Food and Feed Safety and Nutritional Assessment of Insect-Protected Soybean MON 87701 (OECD Unique Identifier MON-877Ø1-2)

Conclusion Based on Data and Information Evaluated According to FDA's Policy on Foods from New Plant Varieties

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Monsanto 09-SY-197F

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CERTIFICATION

Monsanto Company is submitting this food and feed safety and nutritional assessment in compliance with the FDA's 1992 policy statement regarding foods derived from new plant varieties (57 FR 22984). At the agency's request, and where appropriate, this submission also complies with the recommendations contained in the proposed rule for Premarket Biotechnology Notice (PBN) Concerning Bioengineered Foods (66 FR 4706).

Specifically, as recommended in the proposed 21 CFR §192.25(a), the undersigned attests to the following:

1. It is the view of Monsanto Company (hereafter referred to as Monsanto) that: (a) Insect-protected soybean MON 87701 is as safe and nutritious as other commercially available soybean; and (b) the intended uses of the food and feed derived from MON 87701 are in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.
2. Monsanto will make available to FDA, upon request, relevant data or other information not included in this submission, either during the course of FDA's evaluation of the submission, or for cause.
3. Monsanto will make relevant data or other information not included in this submission available to FDA either: (a) by allowing FDA to review and copy these data or information at Monsanto's offices in St. Louis, MO, during customary business hours; or (b) by sending a copy of these data or information to FDA.
4. Monsanto makes no claim of confidentiality regarding either the existence of this submission, or any of the data or other information contained herein. However, Monsanto reserves the right to make a claim of confidentiality regarding any relevant data or other information not included in this submission, but requested by FDA, either in the course of its review of this submission, or for cause. Any such claim of confidentiality will be made at the time such data or information is provided, along with an explanation for the basis of the claim.
5. To the best of Monsanto's knowledge, this submission is representative and balanced, including information, unfavorable as well as favorable, that is pertinent to the evaluation of the safety, nutritional, or other regulatory issues that may be associated with MON 87701.

Signature: Date:

[redacted] 5/28/09

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RELEASE OF INFORMATION

Monsanto is submitting the information in this assessment for review by the FDA as part of the regulatory process. By submitting this information, Monsanto does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., § 552; FDA complies with the provisions of FOIA and FDA's implementation regulations (21 CFR Part 20); and this information is responsive to the specific request. Except in accordance with the Freedom of Information Act, Monsanto does not authorize the release, publication or other distribution of this information (including website posting) without Monsanto's prior notice and consent.

ABBREVIATION AND DEFINITIONS*

~	Approximately
2T-DNA	Plasmid vector containing two separate T-DNA regions each surrounded by left and right borders of the Ti plasmid
7S α'	3' region of the *Sphas1* gene of *Glycine max* encoding the 7sα' seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence
35S	The promoter and leader from the cauliflower mosaic virus (CaMV) 35S RNA
aadA	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7
A5547	The non-transgenic conventional soybean variety used as the recipient for the DNA insertion to create MON 87701 and as the comparator for safety assessment
AA	Amino Acid
ADF	Acid Detergent Fiber
AD8	Allergen, gliadin, and glutenin protein sequence database version 8, compiled by Monsanto Company
AD_2009	Allergen, gliadin, and glutenin protein sequence database compiled and updated by Monsanto Company in 2009
ANOVA	Analysis of Variance
AOAC	Association of Analytical Communities
AOCS	American Oil Chemists Society
APS	Analytical Protein Standard
ASA	American Soybean Association
B	Border
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower Mosaic Virus
CAPS	N-Cyclohexyl-3-aminopropanesulfonic acid
CBI	Confidential Business Information
CEW	Corn earworm [*Helicoverpa zea* (Boddie)]
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
COA	Certificate of Analysis

* Note: Standard abbreviations, e.g., units of measure, are used according to the format described in 'Instructions to Authors' in the *Journal of Biological Chemistry*.

cp4 epsps	Coding sequence (codon-modified) of the *aroA* gene from *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein
CP4 EPSPS	5-Enolylpyruvlshikimate-3-phosphate synthase from *Agrobacterium sp.* strain CP4
Cry	Crystal proteins from *Bacillus thuringiensis*
cry1Ac	Coding sequence for Cry1Ac protein
Cry1Ac	A Cry1 class crystal protein from *Bacillus thuringiensis* subsp. *kurstaki*
CS	Coding Sequence
CSFII	Continuing Survey of Food Intakes by Individuals
CTAB	Cetyltrimethylammonium Bromide
CTP	Chloroplast Transit Peptide
DAP	Days After Planting
DHB	Dihydroxybenzoic acid
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
DEEM-FCID	Dietary Exposure Evaluation Model-Food Commodity Intake Database
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
DW	Dry Weight
DWCF	Dry Weight Conversion Factor
dwt	Dry weight of tissue
E. coli	*Escherichia coli*
E9	3' non-translated sequence from *RbcS2* gene of *Pisum sativum* encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA
EC_{50}	Effective protein concentration to inhibit the growth of the target insect by 50%
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	United States Environmental Protection Agency
EPSPS	5-Enolpyruvylshikimate-3-phosphate Synthase
E-score	Expectation score produced by the FASTA program
EU	European Union
EUP	Experimental Use Permit
EXO	Exonuclease I
FA	Fatty Acid

FAARP	Food Allergy Research and Resource Program Database
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FFDCA	Federal Food, Drug, and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMV	Figwort Mosaic Virus 35S promoter
FW	Fresh Weight
fwt	Fresh weight of tissue
GenBank	A protein database used for bioinformatic searches
GLP	Good Laboratory Practice
GI	Gene Identification number
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
ILDIS	International Legume Database and Information Service
ILSI-CCD	International Life Sciences Institute-Crop Composition Database
IRM	Insect Resistance Management
IUPAC-IUB	International Union of Pure and Applied Chemistry-International Union of Biochemistry
L	Leader
LC_{50}	LC stands for lethal concentration. LC_{50} is the concentration of a substance that causes the death of 50% (one half) of a group of test organisms
Left Border	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA
LOD	Limit of Detection
LOQ	Limit of Quantitation
MAFF	Ministry of Agriculture, Forestry and Fisheries, Japan
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
MH^+	Protonated mass ion
MHLW	Ministry of Health, Labor and Welfare, Japan
MMT	Million Metric Tons
MOE	Margin of Exposure
MON 87701	A Monsanto soybean product candidate, and subject of this application, that produces the insecticidal Cry1Ac protein
MON 810	A Monsanto corn product that produces the insecticidal Cry1Ab protein
MON 531	A Monsanto cotton product that produces the insecticidal Cry1Ac protein
MW	Molecular Weight

MWM	Molecular Weight Marker
N/A	Not Applicable
NCBI	National Convention on Biological Information
NDF	Neutral Detergent Fiber
NFDM	Non-Fat Dried Milk
NMWC	Nominal Molecular Weight Cut-off
NOAEL	No Observed Adverse Effect Level
NOEL	No Observable Effect Level
NTO	Non-Target Organism
OECD	Organization for Economic Co-operation and Development
OR	Origin of replication
ORF	Open Reading Frame
ori-PBR322	Origin of replication from pBR322 for maintenance of plasmid in *E. coli*
ori-V	Origin of replication for *Agrobacterium* derived from the broad host range plasmid RK2
OSL	Overseason Leaf
P	Promoter
PAGE	Polyacrylamide Gel Electrophoresis
PBN	Premarket Biotechnology Notice
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline containing 0.05% (v/v) Tween-20
PCI	Phenol:chloroform: isoamyl alcohol
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PIP	Plant-Incorporated Protectant
PMSF	Phenylmethanesulfonyl Fluoride
PROTEIN	A protein sequence database derived from GenBank release 163
PRT_2009	A protein sequence database derived from GenBank release 169, compiled and updated by Monsanto Company in 2009
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene Difluoride
PVPP	Polyvinylpolypyrrolidone
PV-GMIR9	Plasmid vector used to develop MON 87701
RbcS4	Promoter, leader, and 5' non-translated region of the *Arabidopsis thaliana RbcS4* gene encoding ribulose 1,5-bisphosphate carboxylase small subunit 1A
Right Border	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA

RK2	Broad host range plasmid of Inc-P1 originally isolated in *Klebsiella pneumonia*
rop	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli*
SAS	Statistical Analysis System
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SE	Standard Error
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SOP	Standard Operating Procedure
T	Transcription termination sequence
TBA	Tris-Borate buffer with L-Ascorbic acid
TCA	Trichloroacetic Acid
TDF	Total Dietary Fiber
T-DNA I	Transfer DNA containing the *cry1Ac* expression cassette in plasmid vector PV-GMIR9
T-DNA II	Transfer DNA containing the *cp4 epsps* gene cassette in plasmid vector PV-GMIR9
T-DNA	Transfer DNA
TE	Tris-HCL buffer with EDTA
TFA	Trifluoroacetic Acid
TMB	3,3',5,5'-Tetramethylbenzidene
TOXIN6	Toxin protein sequence database version 6, compiled by Monsanto Company
TOX_2009	Toxin protein sequence database compiled and updated by Monsanto Company in 2009
Tris	Tris (hydroxymethyl) aminomethane
TS	Termination Sequence
TSSP	Tissue-Specific Site Pool
Tween-20	Polyoxyethylenesorbitan monolaurate
USDA-APHIS	United States Department of Agriculture – Animal and Plant Health Inspection Service
USDA-ARS	United State Department of Agriculture – Agricultural Research Service
USDA-ERS	United States Department of Agriculture – Economic Research Service
USDA-GRIN	United State Department of Agriculture – Germplasm Resources Information Network

USDA-NASS	United States Department of Agriculture – National Agricultural Statistics Service
U.S. EPA	United States Environmental Protection Agency
U.S. FDA	United States Federal Drug Administration
v/v	Volume per volume
w/v	Weight per volume

NARRATIVE SUMMARY

Food and Feed Safety and Nutritional Assessment of MON 87701

Monsanto Company has developed biotechnology-derived, insect-protected soybean MON 87701 that produces the Cry1Ac insecticidal crystal (δ-endotoxin) protein[1] derived from *Bacillus thuringiensis* (Bt) subsp. *kurstaki*. Cry1Ac provides protection from feeding damage caused by targeted lepidopteran pests and will reduce or replace current insecticide applications in tropical and subtropical production regions, where these insects cause significant plant damage and yield loss. The *cry1Ac* gene was transferred into the genome of soybean cells using *Agrobacterium tumefaciens*-mediated transformation. The data and information presented in this safety summary demonstrate that the foods and feeds derived from MON 87701 are as safe and nutritious as those derived from commercially-available conventional soybean.

Soybean is the most commonly grown oilseed in the world. In 2007/08, approximately 218.8 million metric tons (MMT) of harvested soybean seed were produced, representing 56% of the world's oilseed production. The impact and severity of insect pest infestations vary greatly across global soybean production regions, primarily due to the different climate and weather conditions, the distribution and environmental tolerance of insect species, and agricultural practices. MON 87701 will be initially commercialized in South America for the control of targeted lepidopteran pests, including the velvetbean caterpillar (*Anticarsia gemmatalis*), soybean looper (*Pseudoplusia includes*), soybean axil borer (*Epinotia aporema*), and sunflower looper (*Rachiplusia nu*). The Cry1Ac protein produced in MON 87701 is expressed at relatively high levels in leaf tissue throughout the entire growing season and provides efficacious control of these target pests.

In the U.S., the insect pressure is greatest on soybean grown in the South, where tropical and subtropical weather favors pest infestation. According to the United States Department of Agriculture (USDA), 16% of the approximately 75 million U.S. soybean acres planted in 2006, mainly those in the Southeastern and Delta states, were treated with insecticides to control the defoliating and pod-feeding insects. Given the limited number of acres that consistently have sufficient lepidopteran insect pressure to require the use of insecticides or other insect control practices, MON 87701 plantings in the U.S will be initially limited to breeding and seed multiplication activities to support the commercialization in South America. To facilitate these activities, Monsanto filed a petition with the United States Department of Agriculture (USDA) in March 2009 requesting that the agency grant non-regulated status for MON 87701. Monsanto will also file an application with the U.S. EPA to request a seed increase registration of the plant-incorporated protectant (PIP) *Bacillus thuringiensis* Cry1Ac protein and the genetic material necessary for its production in soybean. Under the seed increase registration, sale of MON 87701 seed for commercial planting within the U.S. would be prohibited. The EPA has previously established an exemption from the requirement of a tolerance for the PIP *Bacillus thuringiensis* Cry1Ac protein and the genetic material necessary for its

[1] Hereafter referred to as Cry1Ac or Cry1Ac protein.

production in all raw agricultural commodities (40 CFR § 174.510). In the future, if Monsanto decides to commercialize MON 87701 in the U.S., Monsanto will apply to the U.S. EPA for a commercial use registration of the PIP *Bacillus thuringiensis* Cry1Ac and the genetic material (vector PV-GMIR9) necessary for its production in soybean.

MON 87701 was produced by *Agrobacterium*-mediated transformation of soybean with PV-GMIR9, which is a binary vector containing two transfer DNAs (2T-DNAs). The first T-DNA, designated as T-DNA I, contains the *cry1Ac* gene cassette. The second T-DNA, designated as T-DNA II, contains the *cp4 epsps* gene cassette. During transformation, both T-DNAs were inserted into the soybean genome. The *cp4 epsps* gene was used as the selectable marker to select transformed cells and plants. After the transformed cells and subsequent plants were identified, the selectable marker gene was no longer needed. Therefore, a traditional breeding process was deployed to isolate plants that contain only the *cry1Ac* expression cassette (T-DNA I), thereby, producing marker-free MON 87701 plants.

The genetic modification in MON 87701 has been comprehensively characterized. These studies confirm that MON 87701 contains a single insert with the intended sequence, the insert is maintained stably over multiple generations, and the insertion will not result in unintended gene products with similarity to known allergens or toxins. The strategy used to characterize the genetic modification included: 1) Southern blot analyses to assay the entire soybean genome for the presence of DNA derived from the transformation plasmid to confirm that one copy of T-DNA I (*cry1Ac* expression cassette) is present at a single locus in the genome, that sequences from T-DNA II (*cp4 epsps* expression cassette) are absent, and that the insert was stably inherited; 2) DNA sequence analysis to confirm that the sequence of the inserted DNA matched the T-DNA I sequence of the transformation vector; and, 3) segregation analysis to confirm that the inserted DNA is inherited according to Mendelian laws of genetics. Additionally, open reading frame (ORF) bioinformatic analyses of the junction site between the insert and soybean genomic DNA confirm that no relevant similarities exist between any putative polypeptides and known toxins or allergens. The results confirm that MON 87701 contains a single copy of T-DNA I inserted at a single locus of the genome, that the backbone sequences are absent in the insert and multiple generations, and that the integrated DNA is stably inherited and segregates according to the Mendelian laws of genetics.

A detailed characterization and safety evaluation of the newly expressed Cry1Ac protein confirm that it is safe for human and animal consumption. The assessment included: 1) physicochemical and functional characterization; 2) an examination of similarity to known allergens, toxins and other biologically active proteins known to have adverse effects on mammals; 3) an evaluation of digestibility in simulated gastrointestinal fluids; 4) documentation of a history of safe consumption and of structural and functional homologues that lack adverse affects on human or animal health; 5) an investigation of potential mammalian toxicity by an oral gavage assay; and 6) estimation of expression levels for a dietary exposure assessment. All data confirm that Cry1Ac is safe for human and animal consumption.

Because the expression of Cry1Ac in MON 87701 seed is low, it was necessary to produce the quantities needed for Cry1Ac studies in a high-expressing host organism, *E. coli;* tests confirmed that the MON 87701-produced Cry1Ac was physicochemically and functionally equivalent to the *E. coli*-produced Cry1Ac. The MON 87701-produced Cry1Ac contains four additional amino acids at the N-terminus derived from a chloroplast targeting sequence (CTP). Therefore, the Cry1Ac produced by *E. coli* was designed to match the exact amino acid sequence of its counterpart expressed in MON 87701. The Cry1Ac produced in MON 87701 shares an amino acid identity of >99% with the naturally occurring Cry1Ac from Bt subsp. *kurstaki* and shares 100% identity with the Cry1Ac produced in Bollgard® and Bollgard II® cotton, except for the four additional amino acids at the N-terminus.

The safety of the MON 87701-produced Cry1Ac protein was confirmed by both well-established scientific methods and a significant history of safe use. Bioinformatics assessments showed that Cry1Ac does not share amino acid sequence similarities with known allergens, gliadins, glutenins, or protein toxins that have adverse effects on mammals. Digestive fate experiments demonstrated that the full-length Cry1Ac is rapidly digested in simulated gastric fluid (SGF), a characteristic shared among many proteins with a history of safe consumption. A small, transiently stable Cry1Ac fragment from the SGF digestion was very quickly (within 30 sec) degraded during short exposure to simulated intestinal fluid (SIF). Taken together, these data indicate that it is highly unlikely that the Cry1Ac and its fragment will reach absorptive cells of the intestinal mucosa, resulting in low to no allergenic risk.

The safety of Cry1Ac is further confirmed by a history of safe use, low toxicity potential, and no anticipated risk to humans and animals from its presence in the diet. Cry1Ac is a member of the family of Bt Cry proteins that have been used in agriculture as microbial pesticides for over 50 years with no evidence of adverse effects to human or animal health. Since 1996, a number of insect-resistant biotechnology crops expressing Bt Cry proteins have been commercialized. These include Bollgard and Bollgard II cotton that express a Cry1Ac that shares 100% amino acid identity to the MON 87701-produced Cry1Ac (except for the four N-terminal amino acids), and YieldGard® Corn Borer corn that expresses Cry1Ab with an amino acid identity of ~90% with the MON 87701-produced Cry1Ac. Mouse acute oral toxicity tests demonstrate that the Cry1Ac protein is not acutely toxic and does not cause any adverse effect, even at the highest dose levels tested (1290 and 1460 mg/kg body weight for females and males, respectively). A dietary safety assessment based on the expected expression levels of Cry1Ac in the seed of MON 87701, acute toxicity data, and soybean product dietary patterns shows that the margin of exposure (MOE) for the overall U.S. population is $\geq 2.93 \times 10^6$. A dietary assessment for animals shows that the expected consumption of Cry1Ac as part of the daily protein intake in feed rations is 0.0498% for the dairy cow and less than 0.0012% for both the broiler and pig. Taken together, these data indicate that food and feed derived from MON 87701 containing the Cry1Ac protein are safe for consumption as the food and feed derived from conventional soybean.

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MON 87701 is as safe and nutritious as conventional soybean based on a comprehensive compositional and nutritional assessment. The assessment compared the composition of the seed and forage of MON 87701 to a conventional control and commercially available soybean varieties, harvested in 2007 from five field sites located in major U.S. soybean growing regions. Compositional analyses included important nutrients (protein, fat, carbohydrates, fiber, ash, moisture, amino acids, fatty acids, and a vitamin), and anti-nutrients, consistent with OECD guidelines. In each assessment, MON 87701 was compared to an appropriate conventional control, which had a genetic background similar to MON 87701 but did not possess the introduced trait. In addition, the same analytes were assessed in 20 conventional soybean varieties to establish a 99% tolerance interval for each of the analytes for the population of commercial conventional soybean varieties grown concurrently at the same sites. The results show that MON 87701 is nutritionally and compositionally equivalent to, and as safe and nutritious as, conventional soybean.

In a further assessment, the composition data for MON 87701 and the conventional soybean control were statistically compared in a combined-site analysis, followed by individual-site analyses. The combined-site analysis for harvested seed and forage samples showed no significant difference ($p>0.05$) between MON 87701 and the conventional control, for 40 of 55 comparisons. For the majority of analytes where differences were noted ($p<0.05$), the magnitude of differences were generally low (most <5%), the differences were not observed consistently across all sites (individual-site analyses), and the mean values for MON 87701 were within the 99% tolerance interval. Vitamin E levels for MON 87701 were significantly higher than the control in the combined-site analysis (7.69 vs. 6.24 mg/100g DW) and in four of five individual-site analyses, but were within the calculated 99% tolerance interval. It is concluded that the statistical differences represent the natural variability for these soybean analytes and they were not regarded as biologically meaningful. Harvested seed and forage analyte values were comparable to values published in the scientific literature and reported in the International Life Sciences Institute-Crop Composition Database (ILSI-CCD). This further supports the conclusion that harvested seed and forage from MON 87701 are compositionally equivalent to those of conventional soybean.

The data and information presented in this safety summary demonstrate that the food and feed derived from MON 87701 are as safe and nutritious as those derived from commercially-available conventional soybean. The conclusion of food and feed safety of MON 87701 was confirmed based on well established lines of evidence:

1. A detailed molecular characterization of the inserted DNA, where the results confirm the insertion of a single functional *cry1Ac* expression cassette at a single locus within the soybean genome;
2. An extensive set of biochemical evaluations that demonstrate the equivalence of the full-length Cry1Ac protein produced in MON 87701 to the *E. coli*-produced Cry1Ac protein used for safety evaluation;
3. An assessment of the toxic and allergenic potential of Cry1Ac based on a history of safe use, extensive information collected and safety evaluations performed, demonstrates that Cry1Ac is unlikely to be a toxin or allergen; and,

4. The compositional and nutritional assessment confirmed that the seed and forage from MON 87701 are compositionally and nutritionally equivalent to, and as safe as, those of conventional soybean.

All data strongly support the conclusion that food and feed derived from MON 87701 will be as safe and nutritious as food and feed derived from conventional soybean, and that the sale and consumption of MON 87701 soybean seed produced in the U.S. or imported from other production countries would be in compliance with FDA's 1992 "Statement of Policy: Foods Derived from New Plant Varieties" as well as the Federal Food, Drug and Cosmetic Act.

PART II: SYNOPSIS OF CONSULTATION SUMMARY

Section 1. Name and Address of the Submitter

The submitter of this safety and nutritional assessment summary for insect-protected soybean MON 87701 is:

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St. Louis, MO 63167

Communications with regard to this submission should be directed to [redacted] Regulatory Affairs Manager, at the Monsanto address. He can also be contacted by telephone at [redacted] FAX at [redacted] or email at [redacted].

Section 2. The Subject of this Summary and the Plant Species from Which it Was Derived

The subject of this summary is insect-protected soybean MON 87701. The soybean variety used as the recipient for the DNA insertion to create MON 87701 was A5547, a non-transgenic conventional variety developed by Asgrow. A5547 is an elite maturity group V soybean variety, which was developed and selected on the basis of its superior agronomic performance over other soybean lines (Rhodes, 1997). As a soybean variety in maturity group V, A5547 is a determinate variety adapted and most suitable for production in the Mid-South region.

Section 3. Distinctive Designations Given to the Subject of this Summary

The event that is the subject of this summary has been designated MON 87701. In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants," MON 87701 has been assigned the unique identifier MON-877Ø1-2.

Section 4. Identity and Sources of the Genetic Material Introduced into MON 87701

MON 87701 was produced by *Agrobacterium-mediated* transformation of soybean with the plasmid PV-GMIR9 (Figure IV-2), which is a binary vector containing 2T-DNAs. The first T-DNA, designated as T-DNA I, contains the *cry1Ac* gene expression cassette. The second T-DNA, designated as T-DNA II, contains the *cp4 epsps* gene cassette. During transformation, both T-DNAs were inserted into the soybean genome. The *cp4 epsps* gene was used as the selectable marker needed for the selection of the transformed cells and plants. After the transformed cells, and subsequently the plants, were identified, the selectable marker gene no longer was needed. Therefore, a traditional breeding process was deployed to isolate plants that only contain the *cry1Ac* expression cassette (T-DNA I), thereby producing marker-free MON 87701 plants.

Molecular characterization of MON 87701 by Southern blot analyses demonstrated that the DNA inserted into the soybean genome is present at a single locus and contains one functional copy of the *cry1Ac* expression cassette. No T-DNA II (*cp4 epsps* gene expression cassette) genetic elements or backbone sequences from the transformation plasmid were detected in MON 87701. In addition, no partial genetic elements, linked or unlinked to the inserted expression cassette were detected. The stability of the integrated DNA (*cry1Ac* gene) was demonstrated by confirming that the Southern blot fingerprint of MON 87701 was maintained across five breeding generations. The stability was further confirmed by the inheritance of the insect-protection trait in MON 87701 that followed the expected Mendelian segregation pattern.

Section 5. The Intended Technical Effect of MON 87701

MON 87701 encodes for the Cry1Ac insecticidal crystal (Cry) protein (δ-endotoxin) derived from *Bacillus thuringiensis* (Bt) subsp. *kurstaki* that provides protection from feeding damage caused by targeted lepidopteran pests in soybean production regions. The MON 87701 product concept is to reduce or replace current insecticide applications to control lepidopteran pests of soybean in tropical and subtropical production regions where these insects cause significant plant damage and yield loss.

Studies previously have been conducted to evaluate the spectrum of insecticidal activity of Cry1Ac protein produced from Bt var. *kurstaki* HD-73 against a variety of agronomically important insects and one non-insect arthropod taxon (Luttrell et al., 1999; MacIntosh et al., 1990; MacRae et al., 2005). Species tested included seven species of *Lepidoptera*: beet armyworm (*Spodoptera exigua*), black cutworm (*Agrotis ipsilon*), cabbage looper (*Trichoplusia ni*), corn earworm (*Helicoverpa zea*), European corn borer (*Ostrinia nubilalis*), tobacco budworm (*Heliothis virescens*), and tobacco hornworm (*Manduca sexta*); five species of *Coleoptera*: alfalfa weevil (*Hypera postica*), cotton boll weevil (*Anthonomis grandis*), horseradish flea beetle (*Phyllotreta armoraciae*), southern corn rootworm (*Diabrotica undecimpunctata howardi*), and Japanese beetle (*Popillia japonica*); one species of *Diptera*: yellow fever mosquito (*Aedes aegypti*); one species of *Blattodea*: German cockroach (*Blatella germanica*); one species of *Hemiptera*: green peach aphid (*Myzus persicae*); one species of *Isoptera*: termite (*Reticulitermes flavipes*); and one species of mite: two-spotted spider mite (*Tetranychus urticae*). The results showed that Cry1Ac had activity against all seven of the representative lepidopteran insects. However, there was no indication of Cry1Ac activity against any of the ten non-lepidopteran species (Luttrell et al., 1999; MacIntosh et al., 1990; MacRae et al., 2005). The results from these assays suggest that the Cry1Ac protein has insecticidal activity against lepidopteran insect pests but not against the range of non-lepidopteran pests that were tested.

Section 6. The Applications and Uses of MON 87701

Soybean is the most commonly grown oilseed in the world. In 2007/08, approximately 218.8 million metric tons (MMT) of soybean were produced, representing 56% of world oilseed seed production (ASA, 2008; Soyatech, 2009). The MON 87701 product concept is to reduce or replace current insecticide applications to control lepidopteran pests of

soybean in tropical and subtropical production regions, where these insects cause significant plant damage and yield loss. MON 87701 will offer growers in these regions an effective pest management tool and help to maintain soybean yield potential. The major benefits of MON 87701 are:

1. Consistent and reliable control of lepidopteran pests: The Cry1Ac protein is expressed at consistently high levels in insect-protected soybean MON 87701 throughout the entire growing season, providing nearly complete control of the targeted lepidopteran pests for the entire season (MacRae et al., 2005). Given the difficulty in controlling certain soybean lepidopteran pests, MON 87701 should provide protection that is superior to existing chemical and cultural control practices.
2. Reduced production costs and improved farming efficiency: Growers must work diligently to control lepidopteran pests at an early stage to prevent severe crop damage. Just like other Bt crops, insect-protected soybean MON 87701 provides better control of key lepidopteran insect pests than insecticides with less scouting required. MON 87701 reduces the risk of losses due to suboptimal timing of an insecticide application under traditional farm pest management, resulting in the prevention of potential damage to the crop later in the season. In addition, growers will find it more convenient to grow MON 87701 because no special equipment is required, the labor and time required to spray insecticides under traditional insect control practices is reduced or eliminated, and applicator exposure to chemical pesticides is reduced.
3. Control of target insects while maintaining beneficial species: The major lepidopteran pests causing significant soybean defoliation and yield loss across tropical and subtropical regions are the velvetbean caterpillar (*A. gemmatalis*), soybean looper (*P. includens*), soybean borer (*E. aporema*), and sunflower looper (*R. nu*) (Aragon et al., 1997). MON 87701 will provide efficacious control of these insect pests with reduced reliance on the insecticides currently used to control these lepidopteran pests. At the same time, MON 87701 does not impose any adverse impact on beneficial species compared to conventional insecticide-based programs.
4. Yield benefits and insecticide use reduction: In multi-year field tests in Argentina, MON 87701 was found to provide a significant yield increase of up to 4.5% relative to conventional soybean treated with insecticide under mild to moderate lepidopteran insect infestations. In addition to the benefits associated with its specificity for target pests, the reduced use of insecticides against lepidopteran pests will result in cost savings on insecticide and labor.

Section 7. Applications for Which MON 87701 is Not Suitable

Monsanto Company is not aware of food or feed uses of conventional soybean that are not applicable to MON 87701.

PART III: STATUS OF SUBMISSIONS TO OTHER REGULATORY AGENCIES

Section 1. Status of Submission to USDA-APHIS

Monsanto has requested a Determination of Nonregulated Status for MON 87701, including all progenies derived from crosses between MON 87701 and other soybean, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) in March 2009. Under regulations administered by USDA-APHIS (7 CFR 340), MON 87701 is currently considered a "regulated article." Monsanto will continue to conduct all field tests for MON 87701 in strict compliance with USDA field regulations until a Determination of Nonregulated Status is granted for MON 87701. Once MON 87701 is deregulated, it would not require authorization for import, inter-state movement or environmental release.

Section 2. Status of Submission to U.S. EPA

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 Environmental Protection Agency (EPA). Pesticides produced *in planta*, referred to as plant-incorporated protectants (PIPs), are also subject to regulation by the EPA under FIFRA.

Pursuant to § 408(d) of the Federal Food Drug and Cosmetic Act [21 U.S.C. 346 a(d)], DEKALB Genetics Corporation (subsequently acquired by Monsanto) petitioned EPA for an exemption from the requirement of a tolerance for the Cry1Ac protein and the genetic material necessary for its production in or on all raw agricultural commodities in 1997. On April 11, 1997, the EPA established an exemption from the requirement of a tolerance for the PIP *Bacillus thuringiensis* Cry1Ac protein and the genetic material necessary for its production in all raw agricultural commodities (40 CFR § 180.1155). On April 25, 2008, the EPA re-designated the Cry1Ac tolerance exemption as 40 CFR § 174.510 for administrative reasons.

In September 2006, Monsanto filed an experimental use permit (EUP) application for MON 87701 and the genetic material necessary for its production with the U.S. EPA, to allow MON 87701 field-testing and safety evaluations. EUP (524-EUP-1) was granted in September 2007 by the EPA. To support future breeding and seed multiplication activities in the U.S., Monsanto will file an application with the EPA for a Section 3 seed increase registration of the PIP *Bacillus thuringiensis* Cry1Ac protein, and the genetic material (vector PV-GMIR9) necessary for its production, in soybean. Under this type of seed increase registration, commercial sale of MON 87701 seed for planting within the U.S. would be prohibited by law.

In the future, should Monsanto decide to sell MON 87701 seed for commercial planting in the U.S., Monsanto would be required by the EPA to apply for a FIFRA Section 3

commercial use registration for the PIP *Bacillus thuringiensis* Cry1Ac protein, and the genetic material (vector PV-GMIR9) necessary for its production in soybean.

Section 3. Status of Submissions to Foreign Governments

To support commercial introduction of MON 87701 in South America where the soybean pests targeted by MON 87701 are most prevalent, regulatory submissions will be made to the appropriate authorities in countries where the product will be commercialized. All countries that will plant MON 87701 commercially have their own independent and functioning regulatory system to assess the food, feed, and environmental safety for the planting, use and consumption of MON 87701.

Regulatory submissions also will be made to countries that import significant quantities of soybean or its processed fractions from the U.S. or South America and have established regulatory approval processes in place. These will include submissions to a number of foreign government regulatory authorities, including: the Ministry of Agriculture, People's Republic of China; Japan's Ministry of Agriculture, Forestry, and Fisheries (MAFF) and the Ministry of Health, Labor, and Welfare (MHLW); the Canadian Food Inspection Agency (CFIA) and Health Canada; the European Food Safety Authority (EFSA); the Intersectoral Commission for Biosafety of Genetically Modified Organisms (CIBIOGEM), Mexico; and regulatory authorities in other soybean importing countries with functioning regulatory systems. As appropriate, notifications of importation will be made to importing countries that do not have a formal approval process.

PART IV: DEVELOPMENT OF MON 87701

Section 1. Soybean as a Crop

Soybean is the most commonly grown oilseed in the world. In 2007/08, approximately 218.8 MMT of harvested soybean were produced, representing 56% of the world's oilseed production (ASA, 2008; Soyatech, 2009). Soybean is grown as a commercial crop in over 35 countries. The major producers of soybean are the U.S., Brazil, Argentina, China, and India, which accounted for approximately 91% of the global soybean production in 2007 (Table IV-1). Approximately one-third of the 2007 world soybean production occurred in the U.S. (Soyatech, 2009). The soybean produced in China and India are primarily for domestic use, while a significant portion of that produced in U.S., Brazil, and Argentina is traded globally in the form of soybean harvested seed, soybean meal or soybean oil. Globally, the U.S. was the largest soybean export country, while Argentina led the soybean meal and soybean oil export markets in 2007 (ASA, 2008; Soyatech, 2009).

Table IV-1. World Soybean Production in 2007/2008

Country	Production (million metric tons)
U.S.	70.4
Brazil	61.0
Argentina	47.0
China	13.5
Other	8.1
India	9.3
Paraguay	6.8
Canada	2.7
EU	0.7

Source: Soyatech (2009).

Approximately 50% of the world's soybean seed supply was crushed to produce soybean meal and oil in 2007 (ASA, 2008; Soyatech, 2009), and the majority was used to supply the feed industry for livestock use or the food industry for edible vegetable oil and soybean protein isolates. Another 34% of the world soybean seed supply was traded to other geographies, with China, the EU, Japan, and Mexico being the top soybean seed import geographies (ASA, 2008). The remainder of the soybean seed produced was used as certified seed, feed, or stocks.

Soybean is used in various food products, including tofu, soybean sauce, soymilk, energy bars, and meat products. A major food use for soybean is purified oil, for use in margarines, shortenings, cooking, and salad oils. Soybean oil generally has a smaller

contribution to soybean's overall value compared to soybean meal because the oil constitutes just 18 to 19% of the soybean's weight. Nonetheless, soybean oil accounted for approximately 30% of all the vegetable oils consumed globally, and was the second largest source of vegetable oil worldwide, slightly behind palm oil at approximately 32% market share (Soyatech, 2009).

Soybean meal is used as a supplement in feed rations for livestock. Soybean meal is the most valuable component obtained from processing the soybean, accounting for roughly 50-75% of its overall value. By far, soybean meal is the world's most important protein feed, accounting for nearly 69% of world protein meal supplies (ASA, 2008). Industrial uses of soybean range from a carbon/nitrogen source in the production of yeasts via fermentation to the manufacture of soaps, inks, paints, disinfectants, and biodiesel. Industrial uses of soybean have been summarized by Cahoon (2003) and the American Soybean Association (ASA, 2008).

Global soybean plantings reached 90.5 million hectares in 2007/08, an 9.9% increase compared to 82.3 million hectares planted in 2002/03 (Soyatech, 2009). Soybean production has realized, on average, a 6.2% annual growth rate between 1995/96 to 2006/07. Increased planting flexibility, increased yield from narrow-row seeding practices, a higher rate of corn-soybean rotations, and low production costs favored expansion of soybean areas in the mid-1990s, with expansion concentrated in areas where soybean yields were highest.

1.1. Scientific Name and Taxonomic Classification of Soybean

Cultivated soybean, *Glycine max* (L.) Merr., is a diploidized tetraploid (2n=40), which belongs to the family Leguminosae, the subfamily Papilionoideae, the tribe Phaseoleae, the genus *Glycine* Willd., and the subgenus *Soja* (Moench) F.J. Herm.

Family: Leguminosae
Subfamily: Papilionoideae
Tribe: Phaseoleae
Genus: *Glycine*
Subgenus: *Soja* (Moench) F.J. Herm.
Species: *max*

The genus *Glycine* Willd. is of Asian and Australian origin and is divided into two subgenera, *Glycine* and *Soja* (Moench) F.J. Herm. The subgenus *Glycine* consists of 22 wild perennial species, which are indigenous to Australia, west, central and south Pacific Islands, China, Russia, Japan, Indonesia, Korea, Papua New Guinea, the Philippines, and Taiwan (Hymowitz, 2004). The subgenus *Soja* includes the cultivated soybean, *G. max* (L.) Merr. and its wild annual relatives from Asia, *G. soja* Sieb. and Zucc. The list of species in the genus *Glycine* Willd. is presented in Table IV-2.

Table IV-2. List of Species in the Genus Glycine Willd., 2n Chromosome Number, Genome Symbol and Distribution

Genus		2n	Genome[1]	Distribution
Subgenus *Glycine*				
1.	*G. albicans* Tind. & Craven	40	I1	Australia
2.	*G. aphyonota* B. Pfeil	40	--[2]	Australia
3.	*G. arenaria* Tind.	40	HH	Australia
4.	*G. argyrea* Tind.	40	A2A2	Australia
5.	*G. canescens* F.J. Herm.	40	AA	Australia
6.	*G. clandestina* Wendl.	40	A1A1	Australia
7.	*G. curvata* Tind.	40	C1C1	Australia
8.	*G. cyrtoloba* Tind.	40	CC	Australia
9.	*G. dolichocarpa* Tateishi and Ohashi	80	--	(Taiwan)
10.	*G. falcate* Benth.	40	FF	Australia
11.	*G. hirticaulis* Tind. & Craven	40	H1H1	Australia
		80	--	Australia
12.	*G. lactovirens* Tind. & Craven.	40	I1I1	Australia
13.	*G. latifolia* (Benth.) Newell & Hymowitz	40	B1B1	Australia
14.	*G. latrobeana* (meissn.) Benth.	40	A3A3	Australia
15.	*G. microphylla* (Benth.) Tind.	40	BB	Australia
16.	*G. peratosa* B. Pfeil & Tind.	40	--	Australia
17.	*G. pindanica* Tind. & Craven	40	H3H2	Australia
18.	*G. pullenii* B. Pfeil, Tind. & Craven	40	--	Australia
19.	*G. rubiginosa* Tind. & B. Pfeil	40	--	Australia
20.	*G. stenophita* B. Pfeil & Tind.	40	B3B3	Australia
21.	*G. tabacina* (Labill.) Benth.	40	B2B2	Australia
		80	Complex[3]	Australia, West Central and South Pacific Islands
22.	*G. tomentella* Hayata	38	EE	Australia
		40	DD	Australia, Papua New Guinea
		78	Complex[4]	Australia, Papua New Guinea
		80	Complex[5]	Australia, Papua New Guinea, Indonesia, Philippines, Taiwan
Subgenus *Soja* (Moench) F.J. Herm.				
23.	*G. soja* Sieb. & Zucc.	40	GG	China, Russia, Taiwan, Japan, Korea (Wild Soybean)
24.	*G. max* (L.) Merr.	40	GG	Cultigen (Soybean)

[1] Genomically similar species carry the same letter symbols.
[2] Genome designation has not been assigned to the species.
[3] Allopolyploids (A and B genomes) and segmental allopolyploids (B genomes).
[4] Allopolyploids (D and E, A and E, or any other unknown combination).
[5] Allopolyploids (A and D genomes, or any other unknown combination).
Note: Table is adapted from Hymowitz (2004).

Glycine soja grows wild in China, Japan, Korea, the Russian Far East, and Taiwan, and is commonly found in fields, hedgerows, roadsides, and riverbanks (Lu, 2004). The plant is an annual, slender in build with narrow trifoliolate leaves. The purple or very rarely white flowers are inserted on short, slender racemes. The pods are short and tawny with hirsute pubescence, producing oval-oblong seeds (Hermann, 1962).

Glycine max (L.) Merr., the cultivated soybean, is an annual that generally exhibits an erect, sparsely branched, bush-type growth habit with trifoliolate leaves. The leaflets are broadly ovate, and the purple, pink, or white flowers are borne on short axillary racemes or reduced peduncles. The pods are either straight or slightly curved, and one to three ovoid to subspherical seeds are produced per pod.

A third and unofficial species named *G. gracilis* is also described within the context of the *Soja* subgenus in addition to *G. soja* and *G. max*. The *G. gracilis* is only found in Northeast China, is intermediate in morphology between *G. max* and *G. soja*, and is sometimes considered a variant of *G. max*. The three species in the *Soja* subgenus can cross-pollinate, and the hybrid seed can germinate normally and subsequently produce fertile pollen and seed (Singh and Hymowitz, 1989). The taxonomic position of *G. gracilis* has been an area of debate, and neither ILDIS (International Legume Database and Information Service) nor USDA-GRIN (USDA Germplasm Resources Information Network) recognizes *G. gracilis* as a distinct species. The wild and weedy relatives (*G. soja* and *G. gracilis*) of soybean do not occur in the U.S., and, therefore, are not likely to contribute to the potential for outcrossing (USDA-APHIS, 2006).

1.2. Characteristics of the Recipient Soybean

The soybean variety used as the recipient for the DNA insertion to create MON 87701 was A5547, a non-transgenic conventional variety developed by Asgrow Seed Company. A5547 is an elite maturity group V soybean variety developed and selected on the basis of its superior agronomic performance over other soybean lines (Rhodes, 1997). As a soybean variety in maturity group V, A5547 is a determinate variety adapted and most suitable for production in the Mid-South region.

In developing the data to support the safety assessment of insect-protected soybean MON 87701, the conventional soybean variety A5547 was used as the comparator. In general, the genetic background of MON 87701 was similar to that of the control, so the effect of the genetic insertion and the presence of the Cry1Ac protein could be assessed in an unbiased manner. Since MON 87701 was derived from the A5547 conventional variety, it was deemed appropriate to use the non-transformed A5547 as the control variety as its use would minimize the potential bias in subsequent comparative assessments. In addition, commercial conventional and Roundup Ready soybean (40-3-2) varieties were used as reference materials to establish ranges of responses or values representative of commercial soybean varieties. The reference varieties used at each location were selected based on their availability and agronomic fit.

1.3. History of Soybean

Domestication of soybean is thought to have taken place in China during the Shang dynasty (approximately 1500 to 1027 B.C.) or earlier (Hymowitz, 1970). However, historical and geographical evidence could only be traced back to the Zhou dynasty (1027 to 221 B.C.) where the soybean was utilized as a domesticated crop in the northeastern part of China. By the first century A.D., the soybean probably reached central and southern China as well as peninsular Korea. The movement of soybean germplasms was probably associated with the development and consolidation of territories and the degeneration of Chinese dynasties (Ho, 1969; Hymowitz, 1970).

From the first century A.D. to approximately the 15th and 16th centuries, soybean was introduced into several countries, with land races eventually developing in Japan, Indonesia, Philippines, Vietnam, Thailand, Malaysia, Myanmar, Nepal, and Northern India. The movement of soybean throughout this period was due to the establishment of sea and land trade routes, the migration of certain tribes from China, and the rapid acceptance of seeds as a staple food by other cultures (Hymowitz and Newell, 1981; Hymowitz et al., 1990).

Starting in the late 16th century and throughout the 17th century, soybean was used by the Europeans, and in the 17th century, soybean sauce was a common item of trade from the East to the West.

Soybean was introduced into North America in the 18th century. In 1851, soybean was introduced in Illinois and subsequently throughout the Corn Belt. In 1853, soybean seed were deposited at the New York State Agricultural Society, the Massachusetts Horticultural Society, and the Commissioner of Patents. The two societies and the Commissioner of Patents sent soybean seed to dozens of growers throughout the U.S. Soybean has been extensively cultivated and improved through conventional breeding programs following its introduction in the U.S. and has become a key source of nutrients for food and feed use in the U.S. (Hymowitz and Singh, 1987).

Section 2. Characterization of the Vector Used in Transformation

This section describes the vector, the donor genes and the regulatory sequences used in the development of MON 87701, and the deduced amino acid sequence of the Cry1Ac protein produced in MON 87701. In this section, T-DNA refers to DNA that is transferred to the plant during transformation. An expression cassette is comprised of a coding sequence and the regulatory elements necessary for the expression of the coding sequence.

2.1. Method of Transformation

MON 87701 was developed through *Agrobacterium*-mediated transformation of soybean meristem tissue utilizing transformation vector, PV-GMIR9 (Figure IV-2, Part IV). PV-GMIR9 is a binary vector that contains well-characterized DNA segments required for selection and replication of the plasmid vector in bacteria and transfer of the T-DNAs into plant cells. Vector PV-GMIR9 contains two separate T-DNAs (hence the descriptor

"2T-DNAs") that can be effectively used to generate marker-free plants (Komari et al., 1996). The first T-DNA, designated as T-DNA I, contains the gene cassette bearing the gene of interest *cry1Ac*, and the second T-DNA, designated as T-DNA II, contains the gene cassette of selectable marker gene *cp4 epsps*. During the process of *Agrobacterium*-mediated transformation, the distinct T-DNAs containing the *cry1Ac* and *cp4 epsps* genes were integrated into the soybean genome at independent, unlinked loci, and the rest of the backbone of the vector PV-GMIR9 was not inserted into plant cells. A technique used in traditional breeding was then used to isolate plants that only contain T-DNA I (*cry1Ac* expression cassette) but do not contain T-DNA II (*cp4 epsps* expression cassette). This resulted in the production of marker-free, insect-protected soybean MON 87701.

The *Agrobacterium*-mediated soybean transformation to produce MON 87701 was based on the method described by Martinell et al., (2002), which allows the generation of transformed plants without utilization of callus. Briefly, meristem tissues were excised from the embryos of germinated A5547 seed. After co-culturing with the *Agrobacterium* carrying the vector, the meristems were placed on selection medium containing glyphosate to inhibit the growth of untransformed plant cells, and spectinomycin and chloramphenicol to inhibit the growth of excess *Agrobacterium* so that only cells containing T-DNA II and/or T-DNA I and T-DNA II survived. The absence of the *Agrobacterium* used for transformation was confirmed by PCR targeting backbone sequence of plasmid PV-GMIR9. The meristems then were placed in media conducive to shoot and root development. Rooted plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The R_0 plants generated through this process were self-pollinated to produce the R_1 seed. During the selfing of the R_0 plants to produce the R_1 seed, the unlinked insertions of T-DNA I (*cry1Ac* gene expression cassette) and T-DNA II (*cp4 epsps* gene expression cassette) were segregated. A non-lethal dose of glyphosate herbicide was applied to R_1 plants. The resulting plants with minor injury were selected for further analyses, whereas plants showing no injury, i.e., containing T-DNA II (*cp4 epsps* gene expression cassette), were eliminated from subsequent development. Subsequently, plants containing only a single T-DNA I (*cry1Ac* gene cassette) were identified and selected by a combination of analytical techniques, including ELISA and TaqMan PCR analysis. Only R_1 plants that were homozygous for the T-DNA I cassette and not having the T-DNA II cassette were advanced for development. These R_1 plants were self-pollinated to generate a population of R_2 plants which were repeatedly self-pollinated through subsequent generations. These progeny were subjected to further molecular assessments to ensure the plants contained a single, intact insert and phenotypic assessments to ensure the plants met commercial specifications. MON 87701 was selected as the lead event based on its superior phenotypic characteristics and molecular profile. Additional tests on MON 87701 were initiated to further characterize the genetic insertion and the expressed Cry1Ac protein, and to confirm the food, feed, and environmental safety relative to conventional soybean. The major steps involved in the development of MON 87701 are depicted in Figure IV-1.

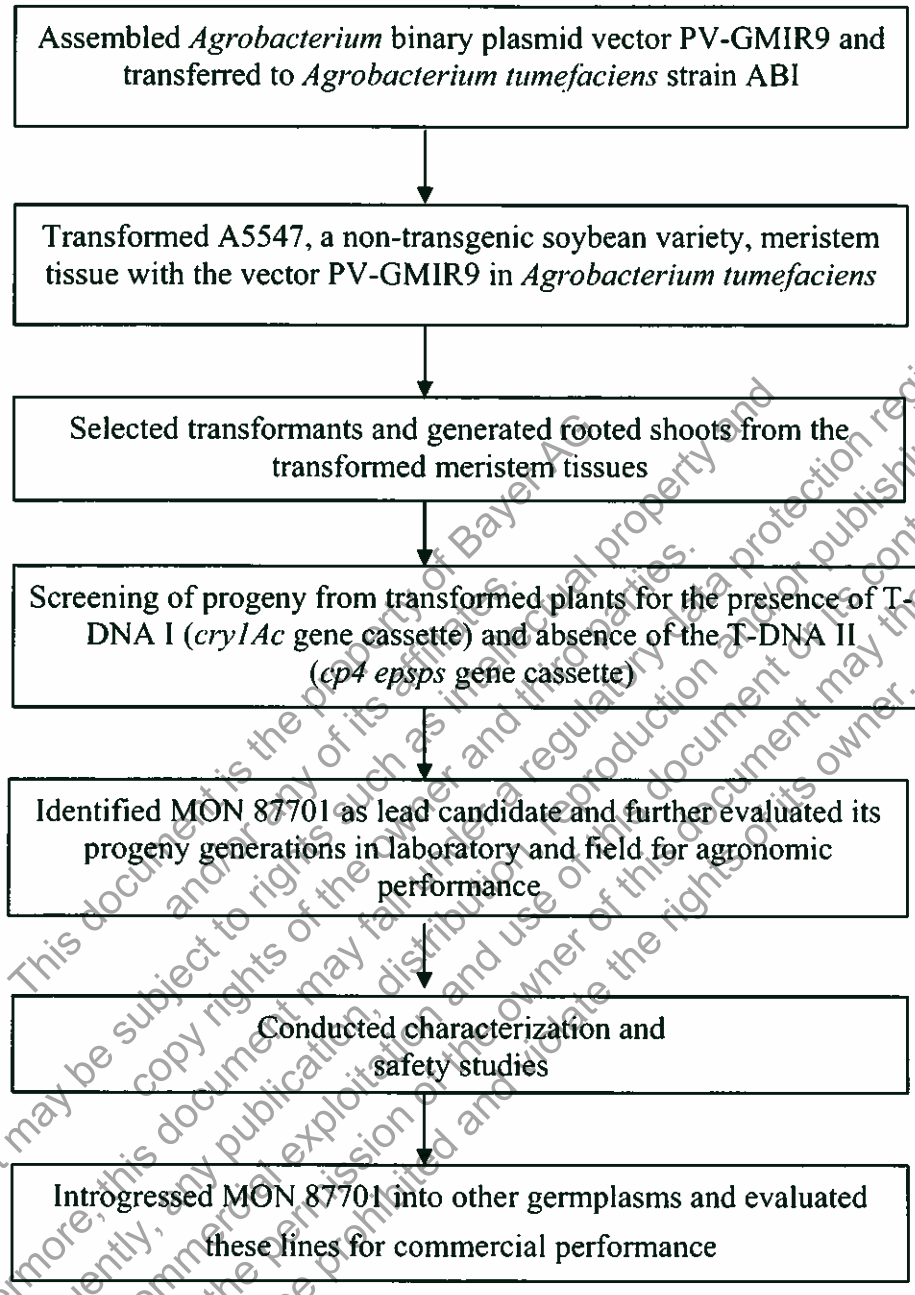

Figure IV-1. Schematic of the Development of MON 87701

2.2. Plasmid PV-GMIR9

The vector PV-GMIR9, used for the transformation of soybean to produce MON 87701, is shown in Figure IV-2 and its genetic elements are described in Table IV-3. This vector is approximately 15.5 kb and contains two T-DNAs delineated by left and right border regions. Each of the two T-DNAs contains a single expression cassette. The first T-DNA (designated as T-DNA I) contains the *cry1Ac* expression cassette, which results in the expression of the Cry1Ac protein. The *cry1Ac* expression cassette contains the *cry1Ac* coding sequence under the regulation of the *RbcS4* promoter and leader, *CTP1* chloroplast targeting sequence, and the *7S α'* 3' non-translated sequence. The second T-DNA (designated as T-DNA II) contains the *cp4 epsps* gene expression cassette. The *cp4 epsps* expression cassette contains the *cp4 epsps* coding sequence under the regulation of the *FMV* promoter, the *shkG* leader, the *CTP2* chloroplast targeting sequence and the *E9* 3' non-translated sequence. Utilizing a vector with two T-DNAs is the basis for an effective approach to generate marker-free plants. It allows for the T-DNA with the trait of interest (e.g., *cry1Ac,* T-DNA I) and the T-DNA encoding the selectable marker (e.g., *cp4 epsps*, T-DNA II) to insert into two independent loci within the genome of the plant. Following selection of the transformants, the inserted T-DNA encoding the selectable marker (e.g., T-DNA II) can be segregated from progeny through the use of traditional breeding techniques and genetic selection processes, while the inserted T-DNA containing the trait(s) of interest is maintained (e.g., T-DNA I). The result is a marker-free soybean containing only the *cry1Ac* expression cassette.

The backbone region outside of the T-DNAs contains two origins of replication for maintenance of plasmid in bacteria (OR-*ori V,* OR-*ori-pBR322*), a bacterial selectable marker gene (*aadA*), and a coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (*rop*). A description of the genetic elements and their prefixes (e.g., P-, L-, I-, TS-, OR-, B-, CS-, and T-) in PV-GMIR9 is provided in Table IV-3.

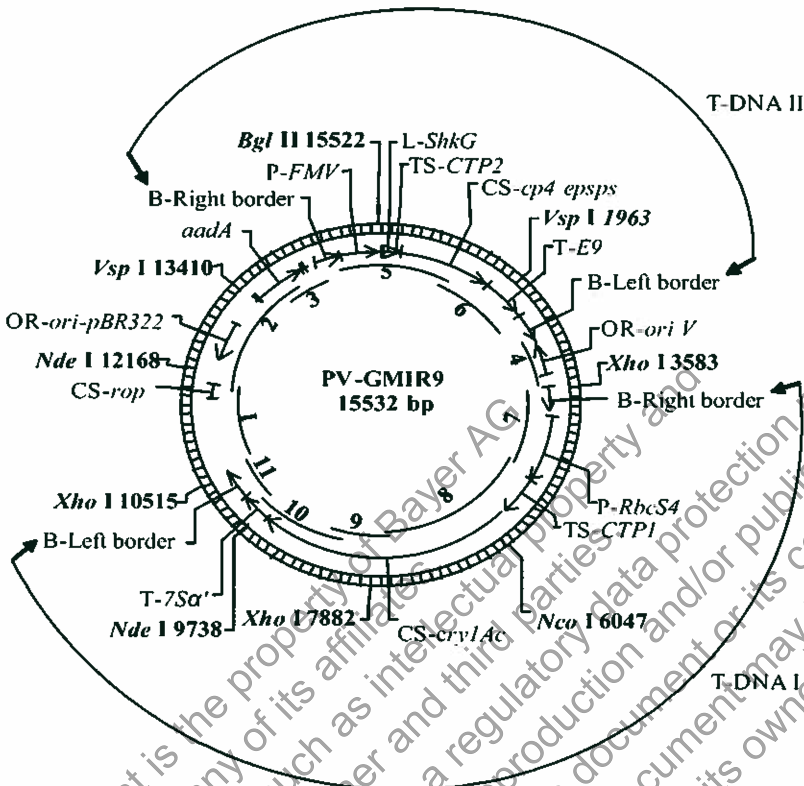

Figure IV-2. Genetic Elements and Restriction Sites of Vector PV-GMIR9 Used in Southern Blot Analysis (Probes 1-11)

Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	Backbone Probe 1	10513	12013	1.5
2	Backbone Probe 2	11813	13640	1.8
3	Backbone Probe 3	13440	14549	1.1
4	Backbone Probe 4	2852	3595	0.74
5	T-DNA II Probe 5	14907	1375	2.0
6	T-DNA II Probe 6	1225	2409	1.2
7	T-DNA I Probe 7	3596	5596	2.0
8	T-DNA I Probe 8	5471	6971	1.5
9	T-DNA I Probe 9	6846	8046	1.2
10	T-DNA I Probe 10	7846	9650	1.8
11	T-DNA I Probe 11	9450	10512*	1.1

A circular map of the plasmid vector PV-GMIR9 used to develop MON 87701 is shown. Genetic elements and restriction sites used in Southern blot analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern blot analyses are shown on the interior of the map. PV-GMIR9 contains two T-DNA regions designated as T-DNA I and T-DNA II. The left and right border regions of T-DNA II share 100% homology with those of T-DNA I and thus were not included in the T-DNA II analysis.

* Nucleotide 10512 is a vector backbone sequence.

Table IV-3. Summary of Genetic Elements in Plasmid Vector PV-GMIR9

Genetic Element	Location in Plasmid	Function (Reference)
	T-DNA II (Continued from bp 15532)	
Intervening Sequence	1-14	Sequences used in DNA cloning
L[1]-*ShkG*	15-81	5' non-translated leader sequence from the *Arabidopsis ShkG* gene encoding EPSPS (Klee et al., 1987) that is involved in regulating gene expression
TS[2]-*CTP2*	82-309	Targeting sequence encoding the chloroplast transit peptide from the *ShkG* gene of *Arabidopsis thaliana* encoding EPSPS (Klee et al., 1987)
CS[3]-*cp4-epsps*	310-1677	Codon modified coding sequence of the *aroA* gene from *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein (Barry et al., 1997; Padgette et al., 1996)
Intervening Sequence	1678-1719	Sequences used in DNA cloning
T[4]-*E9*	1720-2362	3' non-translated sequence from *RbcS2* gene of *Pisum sativum* encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	2363-2409	Sequences used in DNA cloning
B[5]-Left Border	2410-2851	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
	Vector Backbone	
Intervening Sequence	2852-2937	Sequences used in DNA cloning
OR[6]-*ori V*	2938-3334	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	3335-3595	Sequences used in DNA cloning
	T-DNA I	
B-Right Border	3596-3952	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	3953-4061	Sequences used in DNA cloning

Table IV-3 (cont'd). Summary of Genetic Elements in Plasmid Vector PV-GMIR9

Genetic Element	Location in Plasmid	Function (Reference)
P^{7}-*RbcS4*	4062-5784	Promoter, leader, and 5' non-translated region of the *Arabidopsis thaliana RbcS4* gene encoding ribulose 1, 5-bisphosphate carboxylase small subunit 1A, (Krebbers et al., 1988). Promoter expresses in above ground tissues
TS-*CTP1*	5785-6048	Targeting sequence encoding the transit peptide of the *Arabidopsis RbcS4* encoding small subunit 1A transit peptide, from *Arabidopsis thaliana*, present to direct the *cry1Ac* protein to the chloroplast (Krebbers et al., 1988)
CS-*cry1Ac*	6049-9585	Codon-modified coding sequence of the Cry1Ac protein of *Bacillus thuringiensis* (Fischhoff and Perlak, 1995)
Intervening Sequence	9586-9594	Sequences used in DNA cloning
T-*7S α'*	9595-10033	3' region of the *Sphas1* gene of *Glycine max* encoding the 7S α' seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence (Schuler et al., 1982). The element functions to terminate transcription and direct polyadenylation of the mRNA.
Intervening Sequence	10034-10069	Sequences used in DNA cloning
B-Left Border	10070-10511	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)
Vector Backbone (Continued from bp 3595)		
Intervening Sequence	10512-11786	Sequences used in DNA cloning
CS-*rop*	11787-11978	Coding sequence for repressor of primer protein derived from ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	11979-12405	Sequences used in DNA cloning
OR-*ori-pBR322*	12406-12994	Origin of replication from pBR322 for maintenance of plasmid in *Escherichia coli* (Sutcliffe, 1978)
Intervening Sequence	12995-13524	Sequences used in DNA cloning

Table IV-3 (cont'd). Summary of Genetic Elements in Plasmid Vector PV-GMIR9

Genetic Element	Location in Plasmid	Function (Reference)
aadA	13525-14413	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3' (9)-O-nucleotidyltransferase from the transposon Tn7 (Fling et al., 1985) (GenBank accession) that confers spectinomycin and streptomycin resistance
Intervening Sequence	14414-14549	Sequences used in DNA cloning
T-DNA II (Continued from bp 2851)		
B-Right Border	14550-14906	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982)
Intervening Sequence	14907-14939	Sequences used in DNA cloning
P-*FMV*	14940-15503	Promoter for the 35S RNA from figwort mosaic virus (FMV) (Rogers, 2000) that directs transcription in most plant cells
Intervening Sequence	15504-15532	Sequences used in DNA cloning

L[1] -Leader; **TS**[2] - Targeting Sequence; **CS**[3] - Coding Sequence; **T**[4] - Transcription Termination Sequence; **B**[5] - Border; **OR**[6] - Origin of Replication; **P**[7] – Promoter.

2.2.1. T-DNA I

This section describes the genetic elements contained in T-DNA I that were integrated into the soybean genome to produce MON 87701.

2.2.1.1. The *cry1Ac* Coding Sequence and Cry1Ac Protein (T-DNA I)

MON 87701 expresses the insecticidal Cry1Ac protein from Bt subsp. *kurstaki*, which provides resistance to certain lepidopteran pests. Except for four additional amino acids at the N-terminus, the Cry1Ac produced in MON 87701 shares an amino acid identity of >99% with the Cry1Ac from Bt subsp. *kurstaki* and shares 100% identity with the Cry1Ac produced in Bollgard cotton (see Figure VI-1). The four amino acids at the N-terminus are derived from the chloroplast targeting sequence. The deduced full-length amino acid sequence of the Cry1Ac protein is shown in Figure IV-3.

```
   1 MASSMLSSAT MVASPAQATM VAPFNGLKSS AAFPATRKAN NDITSITSNG GRVNCMQVWP
  61 PIGKKKFETL SYLPDLTDSG GRVNCMQAMD NNPNINECIP YNCLSNPEVE VLGGERIETG
 121 YTPIDISLSL TQFLLSEFVP GAGFVLGLVD IIWGIFGPSQ WDAFLVQIEQ LINQRIEEFA
 181 RNQAISRLEG LSNLYQIYAE SFREWEADPT NPALREEMRI QFNDMNSALT TAIPLFAVQN
 241 YQVPLLSVYV QAANLHLSVL RDVSVFGQRW GFDAATINSR YNDLTRLIGN YTDHAVRWYN
 301 TGLERVWGPD SRDWIRYNQF RRELTLTVLD IVSLFPNYDS RTYPIRTVSQ LTREIYTNPV
 361 LENFDGSFRG SAQGIEGSIR SPHLMDILNS ITIYTDAHRG EYYWSGHQIM ASPVGFSGPE
 421 FTFPLYGTMG NAAPQQRIVA QLGQGVYRTL SSTLYRRPFN IGINNQQLSV LDGTEFAYGT
 481 SSNLPSAVYR KSGTVDSLDE IPPQNNNVPP RQGFSHRLSH VSMFRSGFSN SSVSIIRAPM
 541 FSWIHRSAEF NNIIASDSIT QIPAVKGNFL FNGSVISGPG FTGGDLVRLN SSGNNIQNRG
 601 YIEVPIHFPS TSTRYRVRVR YASVTPIHLN VNWGNSSIFS NTVPATATSL DNLQSSDFGY
 661 FESANAFTSS LGNIVGVRNF SGTAGVIIDR FEFIPVTATL EAEYNLERAQ KAVNALFTST
 721 NQLGLKTNVT DYHIDQVSNL VTYLSDEFCL DEKRELSEKV KHAKRLSDER NLLQDSNFKD
 781 INRQPERGWG GSTGITIQGG DDVFKENYVT LSGTFDECYP TYLYQKIDES KLKAFTRYQL
 841 RGYIEDSQDL EIYSIRYNAK HETVNVPGTG SLWPLSAQSP IGKCGEPNRC APHLEWNPDL
 901 DCSCRDGEKC AHHSHHFSLD IDVGCTDLNE DLGVWVIFKI KTQDGHARLG NLEFLEEKPL
 961 VGEALARVKR AEKKWRDKRE KLEWETNIVY KEAKESVDAL FVNSQYDQLQ ADTNIAMIHA
1021 ADKRVHSIRE AYLPELSVIP GVNAAIFEEL EGRIFTAFSL YDARNVIKNG DFNNGLSCWN
1081 VKGHVDVEEQ NNQRSVLVVP EWEAEVSQEV RVCPGRGYIL RVTAYKEGYG EGCVTIHEIE
1141 NNTDELKFSN CVEEEIYPNN TVTCNDYTVN QEEYGGAYTS RNRGYNEAPS VPADYASVYE
1201 EKSYTDGRRE NPCEFNRGYR DYTPLPVGYV TKELEYFPET DKVWIEIGET EGTFIVDSVE
1261 LLLMEE.
```

Figure IV-3. Deduced Amino Acid Sequence of the CTP1 Targeting Sequence and the Full- Length Cry1Ac Protein Produced in MON 87701

The underlined amino acids represent the CTP1 targeting sequence (positions 1-88). The amino acid sequence of the full-length Cry1Ac protein produced in MON 87701 (positions 85-1266) consists of the deduced Cry1Ac amino acid sequence from PV-GMIR9 and the four additional amino acids in bold font (positions 85-88) derived from CTP1.

2.2.1.2. The *cry1Ac* Regulatory Sequences

Each expression cassette contains regulatory sequences involved in the expression of the respective coding sequences. T-DNA I contains the *cry1Ac* expression cassette, which consists of the *cry1Ac* coding sequence under the regulation of the *RbcS4* promoter and leader, *CTP1* targeting sequence, and the *7S α'* 3' nontranslated sequence. The *RbcS4* promoter and leader are from the *Arabidopsis thaliana* ribulose 1,5-bisphosphate carboxylase small subunit 1A gene (Krebbers et al., 1988) and drives transcription of the *cry1Ac* gene in above-ground portions of the plant (see Table VI-4). The *CTP1* targeting sequence is the sequence encoding the transit peptide from the *Arabidopsis thaliana*

small subunit 1A gene (Krebbers et al., 1988) and is present to direct the Cry1Ac protein to the chloroplast. The *7S α' 3'* non-translated region is from the *Glycine max* 7S seed storage protein gene (Schuler et al., 1982) and is present to terminate transcription and direct polyadenylation of the *CTP1- cry1Ac* transcript.

2.2.1.3. T-DNA Borders

Plasmid PV-GMIR9 contains right border and left border regions (Figure IV-2 and Table IV-3) that were derived from *Agrobacterium tumefaciens* plasmids (Depicker et al. 1982; Barker et al. 1983). The border regions each contain a 24-25 bp sequence, called the "nick" site, which is the site of DNA exchange during transformation. The border regions delineate the T-DNA and are involved in their efficient transfer into the soybean genome. Because PV-GMIR9 is a two T-DNA vector, it contains two right border regions and two left border regions, where one set is for T-DNA I and the other set is for T-DNA II (see description above).

2.2.2. T-DNA II

This section describes the genetic elements contained in T-DNA II that were utilized during the plant transformation and selection process but are not present in MON 87701.

2.2.2.1. The *cp4 epsps* Coding Sequence and the CP4 EPSPS Protein (T-DNA II)

The *cp4 epsps* gene expression cassette is not present in MON 87701. The *cp4 epsps* gene expression cassette was used as a selectable marker during the transformation to produce MON 87701, but was segregated away by traditional breeding techniques at the R1 generation. The CP4 EPSPS protein confers tolerance to glyphosate and has been used safely and successfully in many Roundup Ready crops such as canola, corn, cotton, soybean, and sugar beet.

2.2.2.2. The *cp4 epsps* Regulatory Sequences

T-DNA II contains the *cp4 epsps* expression cassette, which consists of the *cp4 epsps* coding sequence under the regulation of the Figwort Mosaic Virus (*FMV*) promoter, the *shk*G leader, the CTP2 targeting sequence and the *E9* 3' non-translated sequence. The *FMV* promoter is from the FMV 35S RNA gene (Rogers, 2000) and drives transcription of *cp4 epsps* in most plant cell types. The *shkG* leader is the 5' untranslated region (UTR) from the *Arabidopsis thaliana shkG* gene (encoding EPSPS) (Klee et al., 1987) and acts to enhance expression. The CTP2 targeting sequence is the sequence encoding the transit peptide from the *ShkG* gene of *Arabidopsis thaliana* (Klee et al., 1987) and is present to direct the CP4 EPSPS protein to the chloroplast. The *E9* non-translated region is the 3' non-translated sequence from the *RbcS2* gene of *Pisum sativum* (Coruzzi et al., 1984) and is present to direct polyadenylation of the *CTP2-cp4 epsps* transcript.

2.2.2.3. T-DNA Borders

The right and left borders are described under Section 2.2.1.3, Part IV.

2.2.3. Genetic Elements outside the T-DNA Borders

Genetic elements that exist outside of the T-DNA borders are those that are essential for the maintenance and selection of the vector PV-GMIR9 in bacteria. The origin of replication OR-*ori V* is required for the maintenance of the plasmid in *Agrobacterium* (Stalker et al., 1981) and is derived from the broad host plasmid RK2. The origin of replication OR-pBR322 is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid pBR322 (Sutcliffe, 1978). CS-*rop* is the coding sequence of the repressor of primer (ROP) protein and is necessary for the maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989). The selectable marker *aadA* is a bacterial promoter and coding sequence for an enzyme from transposon Tn7 that confers spectinomycin and streptomycin resistance (Fling et al., 1985) in *E. coli* and *Agrobacterium* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the soybean genome. The absence of the backbone sequence in MON 87701 has been confirmed by Southern blot analyses (see Section 3.3, Part IV).

Section 3. Characterization of the Introduced Genetic Material

This section details the molecular analyses that characterized the integrated DNA insert in MON 87701. The results confirmed: a) the presence of each genetic element at the insertion site and not at any region outside of the insert, b) the lack of plasmid backbone elements, and c) the insert stability across generations. In addition, DNA sequencing analyses were performed, and results confirmed the expected nucleotide sequence of the insert in MON 87701 as well as the organization of the genetic elements. Furthermore, insert segregation analysis confirmed that the expected and the observed segregation ratios were identical. This result is consistent with the finding of a single chromosomal insertion of the *cry1Ac* gene cassette that segregates according to Mendel's laws of genetics.

3.1. Introduction

Southern blot and DNA sequence analyses were used to characterize the T-DNA insert in MON 87701. Southern analysis was used to assay the entire soybean genome for sequences derived from the transformation vector PV-GMIR9. The sequence analysis was used to determine the composition and intactness of the inserted DNA and to evaluate the region of the genomic DNA directly adjacent to the insert. The analyses were performed on the R5 generation, the same generation used to initiate the integration of MON 87701 into commercial germplasm (Figure IV-11). The Southern blot strategy was designed to ensure sufficient sensitivity while utilizing probes that span the entire transformation vector. A linear map depicting the restriction sites within the insert DNA sequence, as well as within the soybean genomic DNA immediately flanking the insert in MON 87701, is shown in Figure IV-4. A map of plasmid vector PV-GMIR9 annotated with the probes used in the Southern analysis is presented in Figure IV-2. The high level of sensitivity was demonstrated for each blot by including and detecting a 1/10th genome equivalent of the positive control. The Southern blots were performed in a way to maximize the resolution of DNA fragments. Two restriction enzyme sets were

specifically chosen to minimize the possibility that two DNA fragments could comigrate on the gel.

For each digest, there were duplicated samples that consisted of equal amounts of digested DNA. One set of samples was run for a longer period of time (Long Run) than the second set (Short Run). The long run allows for greater resolution of large molecular weight DNA, whereas the short run allows the detection of small molecular weight DNA. For estimating the sizes of bands present in the Long-Run lanes of Southern blots, the molecular weight markers on the left of the figure were used. For estimating the sizes of bands present in the Short-Run lanes, the molecular weight markers on the right of the figure were used.

The DNA sequencing analyses complement the Southern blot analyses. Whereas Southern blots determined that MON 87701 contains T-DNA I-derived sequences at a single insertion site, sequencing of the insert and the flanking genomic DNA determined that T-DNA I inserted as predicted in MON 87701. Each genetic element is intact and the sequence of the insert matches the corresponding sequence in PV-GMIR9. In addition, genomic rearrangements at the insertion site were assessed by comparing the insert and flanking sequences in MON 87701 to the insertion site sequences in conventional soybean.

The stability of the DNA insert across multiple generations also was demonstrated by Southern blot fingerprint analyses. Five generations of MON 87701 were digested with one of the enzyme sets utilized for the copy number analysis and were hybridized with probes that would detect restriction fragments that encompass the entire insert (two hybridization bands). This fingerprint strategy consists of two border fragments that assay not only the stability of the insert, but also the stability of genomic DNA directly adjacent to the insert.

The results of these experiments are summarized in Table IV-5. The insert in MON 87701 matches the T-DNA sequence of PV-GMIR9 starting with the Right Border of T-DNA I and ending at Left Border of T-DNA I. The information and results derived from the molecular analyses were used to construct a linear map of the insert in MON 87701. This linear map, shown in Figure IV-4 depicts restriction sites identified in the insert and the soybean genomic DNA flanking the insert, and provides information on the expected banding patterns and sizes of the DNA fragments after restriction enzyme digestions. Based on the insert linear map and the plasmid map, a table summarizing the expected DNA fragments for Southern blot analyses is presented in Table IV-4. The probes used in the Southern blot analyses, and the map of PV-GMIR9, are presented in Figure IV-2. The materials and methods used in the analyses are presented in Appendix A.

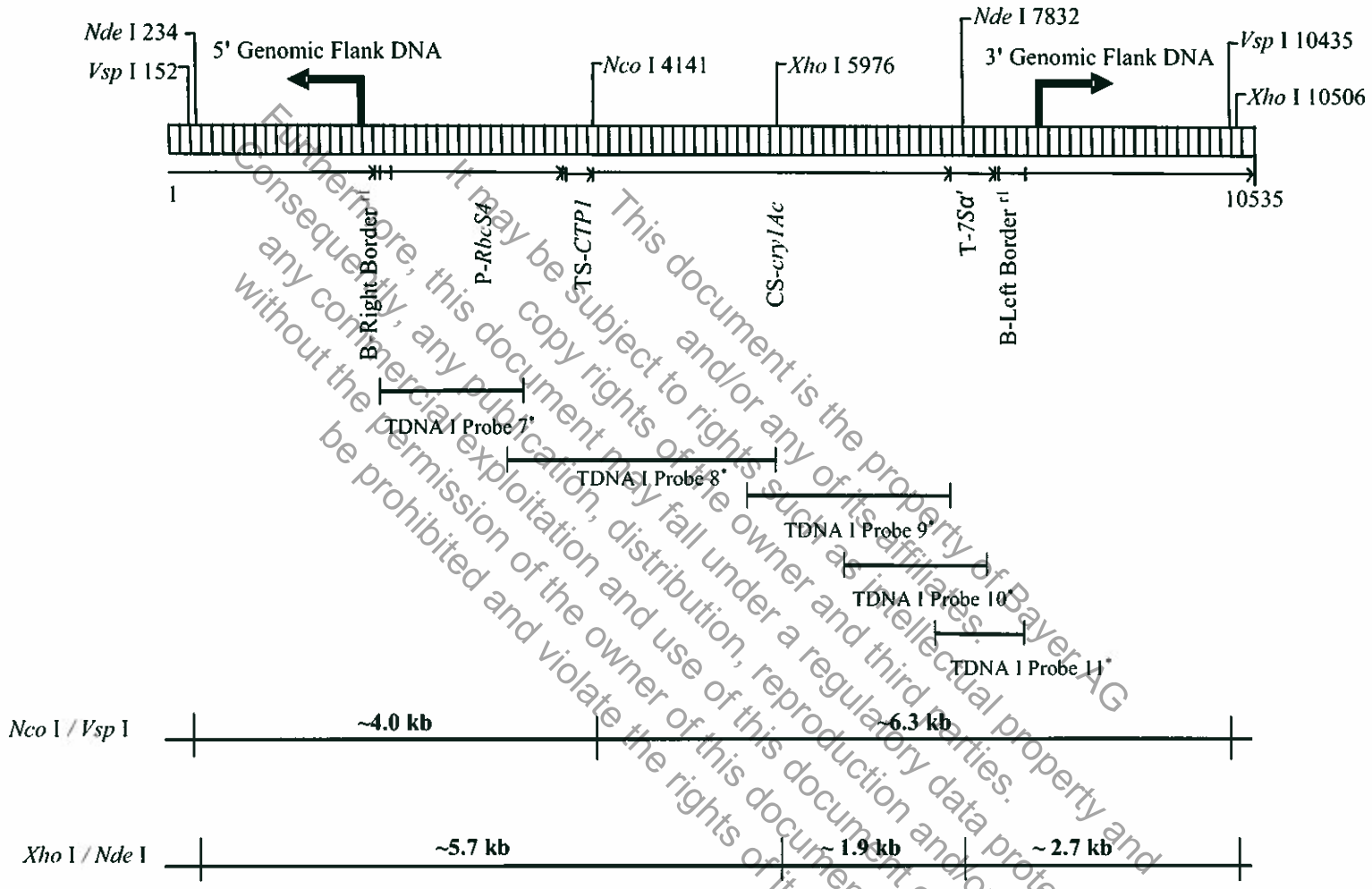

Figure IV-4. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87701

Linear DNA derived from T-DNA I of vector PV-GMIR9 incorporated into MON 87701. Arrows indicate the end of the insert and the beginning of soybean genomic flanking sequence. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analyses.

* These probes are not drawn to scale and are the estimated locations of the T-DNA I probes. Probes are described in Figure IV-2.

Table IV-4. Summary Chart of the Expected DNA Fragments Based on Restriction Enzymes and Probes

Probes Used	7	8, 10	9, 11	1, 2, 3	4	5, 6	8
Southern Blot Figure	IV-5	IV-6	IV-7	IV-8	IV-9	IV-10	IV-12
Plasmid PV-GMIR9							
***Bgl* II / *Nco* I**	~6.0 kb ~9.5 kb	~6.0 kb ~9.5 kb	~6.0 kb ~9.5 kb	~9.5 kb	~6.0 kb	~6.0 kb ~9.5 kb	~6.0 kb ~9.5 kb
Probe Templates [1]	~~[2]	~1.5 kb ~1.8 kb	~1.2 kb ~1.1 kb	~1.8 kb ~1.5 kb ~1.1 kb	~~[2]	~2.0 kb ~1.2 kb	~1.5 kb
MON 87701							
***Nco* I / *Vsp* I**	~4.0 kb	~4.0 kb ~6.3 kb	~6.3 kb	No band	No band	No band	~6.3 kb ~4.0 kb
Xho I / Nde I	~5.7 kb	~5.7 kb ~1.9 kb	~5.7 kb ~2.7 kb	No band	No band	No band	--[3]

[1] probe templates were spiked when multiple probes are used in Southern blot analysis.
[2] '~~' indicates that only the plasmid template was used since the Southern blot was hybridized with one probe.
[3] '--' indicates that the particular restriction enzyme or the combination of enzymes was not used in the analysis.

Table IV-5. Summary of Genetic Elements in MON 87701

Genetic Element	Location in Sequence	Function (Reference)
Sequence flanking 5' end of the insert	1-2000	Soybean genomic DNA
B[1]-Right Border	2001-2045	45 bp DNA region from the right border region remaining after integration (Depicker et al., 1982)
Intervening Sequence	2046-2154	Sequence used in DNA cloning
P[2]- *RbcS4*	2155-3877	Promoter, leader, and 5' non-translated region of the *Arabidopsis thaliana RbcS4* gene encoding ribulose 1,5-bisphosphate carboxylase small subunit 1A (Krebbers et al., 1988)
TS[3]-*CTP1*	3878-4141	Targeting sequence encoding the transit peptide of the *Arabidopsis RbcS4* encoding small subunit 1A transit peptide, from *Arabidopsis thaliana*, present to direct the Cry1Ac protein to the chloroplast, (Krebbers et al., 1988)
CS[4]-*Cry1Ac*	4142-7678	Codon-modified coding sequence of the Cry1Ac protein of *Bacillus thuringiensis* (Fischhoff and Perlak, 1995)
Intervening Sequence	7679-7687	Sequences used in DNA cloning
T[5]-*7S α'*	7688-8126	3' region of the *Sphas1* gene of *Glycine max* encoding the 7S *α'* seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β-conglycinin coding region with the termination codon and the polyadenylation sequence (Schuler et al., 1982). The element functions to terminate transcription and direct polyadenylation of the mRNA.
Intervening Sequence	8127-8162	Sequence used in DNA cloning
B[1]-Left Border	8163-8426	264 bp DNA region from the left border region remaining after integration (Barker et al., 1983)
Sequence flanking 3' end of the insert	8427-10535	Soybean genomic DNA

[1]**B** – Border; [2]**P** – Promoter; [3]**TS** – Targeting Sequence; [4]**CS** – Coding Sequence; [5]**T** –3' non-translated transcriptional termination and polyadenylation signal sequences.

3.2. Insert and Copy Number

The number of copies and insertion sites of T-DNA I sequences in the soybean genome were evaluated by digesting the test and control genomic DNA samples with the two enzyme sets *Nco* I / *Vsp* I and *Xho* I / *Nde* I, which cleave within the insert and known flanking sequences. The enzymes used generate a restriction fragment containing T-DNA I and adjacent plant genomic DNA with a unique banding pattern. If T-DNA I sequences are present at a single integration site in MON 87701, then probing with the sequence from T-DNA I should result in the restriction fragments described in Figure IV-4 and Table IV-4. Any additional integration sites would be detected as additional bands. The blots were hybridized with overlapping T-DNA I probes spanning the entire inserted DNA sequence (Probes 7-11, Figure IV-2). Each Southern blot contained several controls. Genomic DNA isolated from the conventional soybean control, A5547, was used as a negative control to determine if the probes hybridized to any endogenous sequences. Conventional soybean spiked with either plasmid DNA or probe template was used as a positive hybridization control and to demonstrate sensitivity of the Southern blot. The results of these analyses are shown in Figures IV-5 to IV-7.

3.2.1. T-DNA I Probe 7

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure IV-5, lanes 1 and 8) or *Xho* I / *Nde* I (Figure IV-5, lanes 3 and 10) and hybridized with Probe 7 (Figure IV-2) showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA, previously digested with *Nco* I / *Bgl* II, (Figure IV-2, Probe 7) produced the expected bands at ~6.0 kb and ~9.5 kb (Figure IV-5, lane 7). In Figure IV-5, lane 6, the ~0.1 genome equivalent spike produced the expected band at ~6.0 kb, but the ~9.5 kb band is too faint to identify, since only a small portion of probe 7, which spans the Right Border region, has homology to the 9.5 kb portion of the vector. The ability to detect the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I (Figure IV-5, lanes 2 and 9) and hybridized to Probe 7 is expected to produce one band at ~ 4.0 kb. The long run (Figure IV-5, lane 2) produced a single band at ~4.1 kb (at or above the 4.1 kb marker) and the short run (Figure IV-5, lane 9) also produced a single band of the correct size. MON 87701 DNA digested with *Xho* I / *Nde* I (Figure IV-5, lanes 4 and 11) and hybridized with Probe 7 is expected to produce a single band of ~5.7 kb. The long run (Figure IV-5, lane 4) produced a single band at ~6.2 kb (at or above the 6.1 kb marker) and the short run (Figure IV-5, lane 11) produced a single band at ~5.7 kb. The apparent shift in migration of the bands in the long run versus the short run can be attributed to the method used to record the molecular weight markers on the agarose gel and on the autoradiograph and does not alter the conclusion that a single band was detected of the correct size. Thus, there is a single detectable insert containing Probe 7 sequences. The results presented in Figure IV-5 indicate that the sequence covered by Probe 7 resides at a single detectable locus of integration in MON 87701.

3.2.2. T-DNA I Probes 8 and 10

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure IV-6, lanes 1 and 8) or *Xho* I / *Nde* I (Figure IV-6, lanes 3 and 10) and hybridized with Probes 8 and 10 (Figure IV-2) showed no detectable hybridization bands, as expected for the negative control. Pre-digested conventional soybean DNA spiked with probe template (Figure IV-2, Probes 8 and 10) generated from plasmid PV-GMIR9 produced the expected bands at ~1.5 kb, and ~1.8 kb (Figure IV-6, lanes 5 and 6). In lane 6, there is a faint band at ~3.6 kb, which likely represents a minor PCR artifact that was generated during probe template preparation. Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Nco* I / *Bgl* II produced the two expected bands at ~6.0 kb and ~9.5 kb (Figure IV-6, lane 7). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I and hybridized with Probes 8 and 10 (Figure IV-6, lanes 2 and 9) produced two bands. The ~4.0 kb band is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) and the adjacent genomic DNA flanking the 5' end of the insert (Figure IV-4). The ~6.3 kb band represents the 3' border fragment containing the 3' end of the inserted DNA and the adjacent genomic DNA flanking the 3' end of the insert. MON 87701 DNA digested with *Xho* I / *Nde* I (Figure IV-6, lanes 4 and 11) produced two bands. The ~5.7 kb band observed in Figure IV-6 (lanes 4 and 11) is the expected size for the border fragment containing the 5' end of the inserted DNA (T-DNA I) and the adjacent genomic DNA flanking the 5' end of the insert (Figure IV-4). The ~1.9 kb band observed in Figure IV-6 (lanes 4 and 11) represents the 3' border fragment containing the 3' end of the inserted DNA along with the adjacent genomic DNA flanking the 3' end of the insert. The results presented in Figure IV-6 indicate that sequence covered by Probes 8 and 10 resides at a single detectable locus of integration in MON 87701.

3.2.3. T-DNA I Probes 9 and 11

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure IV-7, lanes 2 and 11) or digested with *Xho* I / *Nde* I (Figure IV-7, lanes 4 and 13) and hybridized with Probes 9 and 11 (Figure IV-2) produced several hybridization signals. This was expected because the 7Sα' 3' non-translated region genetic element within T-DNA I originally was isolated from soybean. These hybridization signals result from the probes hybridizing to endogenous targets residing in the soybean genome and are not specific to the inserted DNA. These signals were produced in all lanes, including those containing the conventional soybean DNA material and therefore are considered to be endogenous background hybridization. Pre-digested conventional soybean DNA spiked with probe template (Figure IV-2, Probes 9 and 11) generated from plasmid PV-GMIR9, produced the expected bands at ~ 1.2 kb for probe template 9 (Figure IV-7, lanes 5 and 6) and ~1.1 kb for probe template 11 (Figure IV-7, lanes 7 and 8). Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Nco* I / *Bgl* II produced the two bands at ~6.0 kb and ~9.5 kb (Figure IV-7, lane 9). Detection of the spiked controls indicates that the probes are recognizing their target sequences. There is nonspecific hybridization at the bottom of the blot that spans lanes 5-13. This region of

the blot corresponds to the short run and genomic DNA was not that far in the gel. It is clear that no bands are discernable within this region of the blot.

MON 87701 DNA digested with *Nco* I / *Vsp* I and hybridized with Probes 9 and 11 (Figure IV-7, lanes 1 and 10) produced one unique band in addition to the endogenous background hybridization. The ~6.3 kb band is the expected size for the border fragment containing the 3' end of the inserted DNA (T-DNA I) and the adjacent genomic DNA flanking the 3' end of the insert (Figure IV-4). MON 87701 DNA digested with *Xho* I/ *Nde* I (Figure IV-7, lanes 3 and 12) produced three unique bands, as expected, in addition to the endogenous background hybridization. The expected band at ~5.7 kb migrated together with an endogenous hybridization signal observed in Figure IV-7, lanes 3 and 12. The ~5.7 kb band represents the 5' border fragment containing the 5' end of the inserted DNA along with the adjacent genomic DNA flanking the 5' end of the insert. The ~1.9 kb band represents a portion of the *cry1Ac* expression cassette. The ~2.7 kb band represents the 3' border fragment containing the 3' end of the inserted DNA and the adjacent genomic DNA flanking the 3' end of the insert. The results presented in Figure IV-7 indicate that sequences covered by Probes 9 and 11 reside at a single detectable locus of integration in MON 87701. Taken together, the data presented in Figures IV-5, IV-6, and IV-7 indicate that MON 87701 contains a single copy of T-DNA I at a single insertion site.

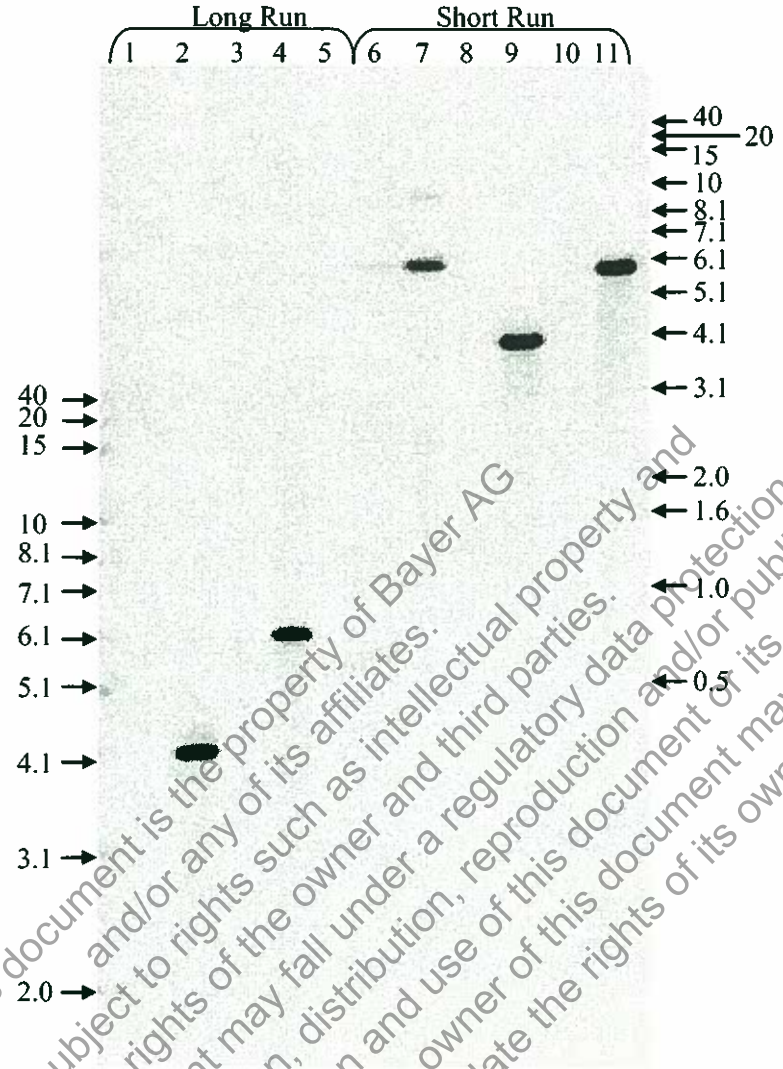

Figure IV-5. Southern Blot Analysis of MON 87701: T-DNA I Copy Number Analysis (Probe 7)

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the T-DNA I sequence (Figure IV-2, Probe 7). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Blank
6: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~0.1 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I)
9: MON 87701 (*Nco* I / *Vsp* I)
10: Conventional soybean (*Xho* I / *Nde* I)
11: MON 87701 (*Xho* I / *Nde* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

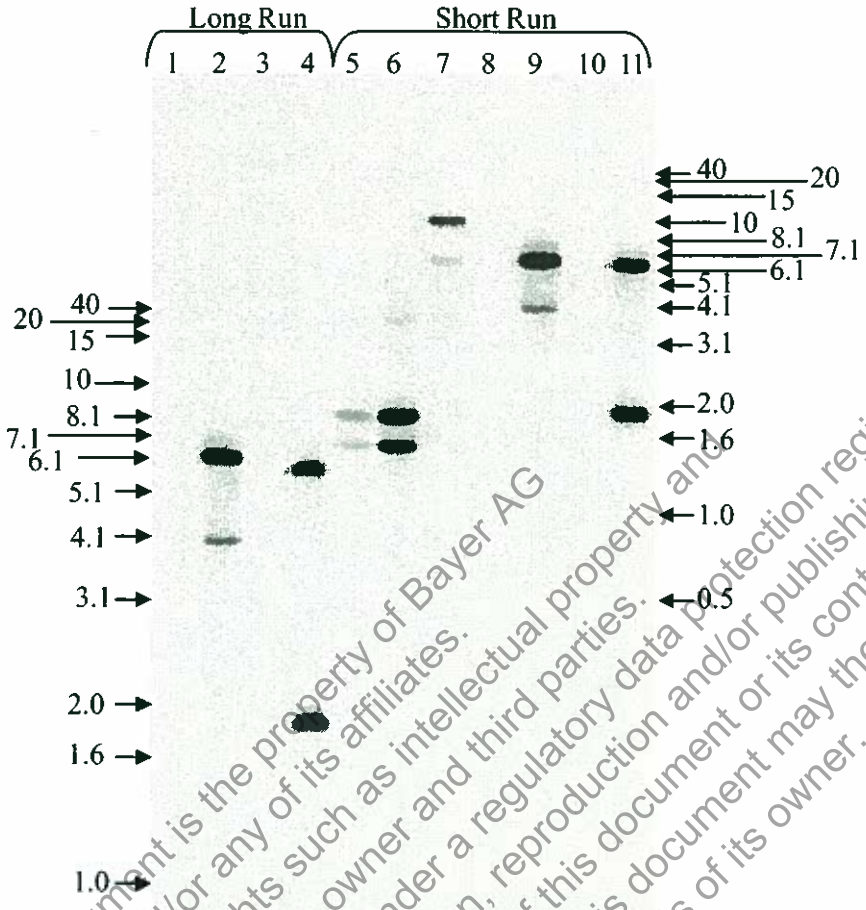

Figure IV-6. Southern Blot Analysis of MON 87701: T-DNA I Copy Number Analysis (Probes 8 and 10)

The blot was hybridized with two overlapping ^{32}P-labeled probes that spanned portions of the T-DNA I sequence (Figure IV-2, Probes 8 and 10). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Conventional soybean spiked with probe templates (*Nco* I / *Vsp* I) [~0.1 genome equivalent]
6: Conventional soybean spiked with probe templates (*Nco* I / *Vsp* I) [~1.0 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I)
9: MON 87701 (*Nco* I / *Vsp* I)
10: Conventional soybean (*Xho* I / *Nde* I)
11: MON 87701 (*Xho* I / *Nde* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

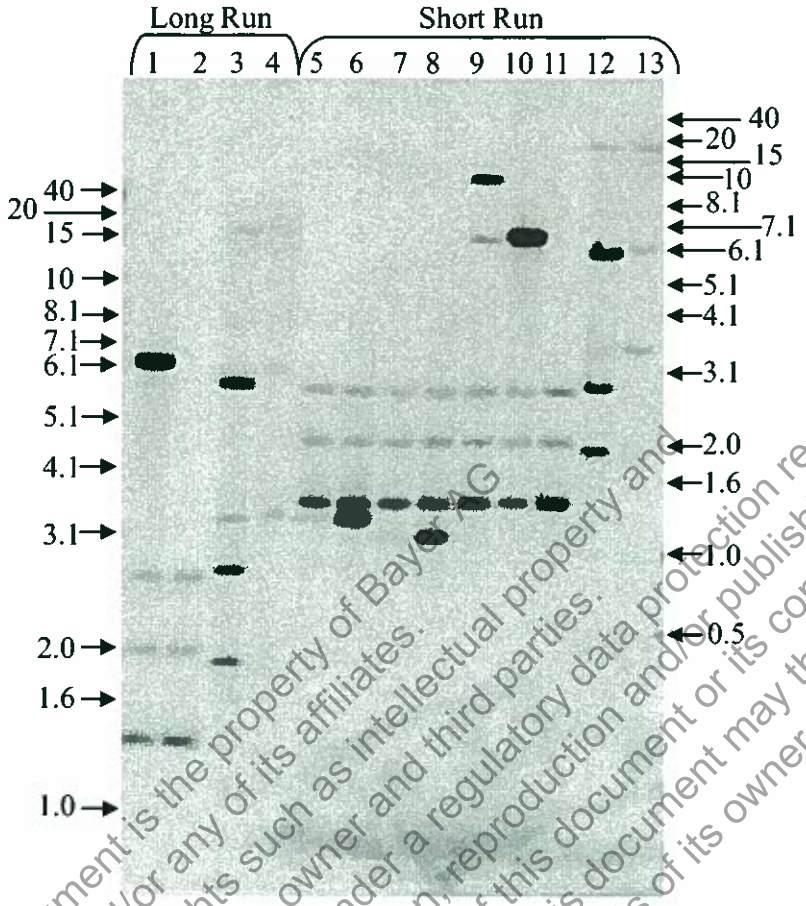

Figure IV-7. Southern Blot Analysis of MON 87701: T-DNA I Copy Number Analysis (Probes 9 and 11)

The blot was hybridized with two overlapping ^{32}P-labeled probes that spanned portions of the T-DNA I sequence (Figure IV-2, Probes 9 and 11). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: MON 87701 (*Nco* I / *Vsp* I)
2: Conventional soybean (*Nco* I / *Vsp* I)
3: MON 87701 (*Xho* I / *Nde* I)
4: Conventional soybean (*Xho* I / *Nde* I)
5: Conventional soybean spiked with probe 9 template (*Nco* I / *Vsp* I) [~0.1 genome equivalent]
6: Conventional soybean spiked with probe 9 template (*Nco* I / *Vsp* I) [~1.0 genome equivalent]
7: Conventional soybean spiked with probe 11 template (*Nco* I / *Vsp* I) [~0.1 genome equivalent]
8: Conventional soybean spiked with probe 11 template (*Nco* I / *Vsp* I) [~1.0 genome equivalent]
9: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl II*) [~1.0 genome equivalent]
10: MON 87701 (*Nco* I / *Vsp* I)
11: Conventional soybean (*Nco* I / *Vsp* I)
12: MON 87701 (*Xho* I / *Nde* I)
13: Conventional soybean (*Xho* I / *Nde* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

3.3. Southern Blot Analysis to Determine the Presence or Absence of Plasmid PV-GMIR9 Backbone

The presence or absence of plasmid backbone sequences in the soybean genome was evaluated by digesting the test and control genomic DNA samples with *Nco* I / *Vsp* I or *Xho* / *Nde* I and hybridizing with overlapping backbone probes spanning the entire backbone sequence of PV-GMIR9 (Figure IV-2, Probes 1, 2, 3, and 4). If backbone sequences are present in MON 87701, then probing with backbone sequences should result in hybridizing bands. The results of this analysis are shown in Figures IV-8 to IV-9. Each Southern blot contains the same controls as described in Section 3.2., Part IV.

3.3.1. Plasmid Backbone Probes 1, 2 and 3

Conventional soybean control DNA was digested with *Nco* I / *Vsp* I (Figure IV-8, lanes 1 and 8) or *Xho* I / *Nde* I (Figure IV-8, lanes 3 and 10) and hybridized simultaneously with overlapping probes spanning most of the vector backbone of PV-GMIR9 (Figure IV-2, Probes 1, 2, and 3) showed no detectable hybridization bands, as expected for the negative control. Pre-digested conventional soybean DNA spiked with probe template (Figure IV-2, Probes 1, 2, and 3) generated from plasmid PV-GMIR9 produced the expected bands at ~1.5 kb, ~1.8 kb, and ~1.1 kb, respectively (Figure IV-8, lanes 5 and 6). Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Bgl* II / *Nco* I produced the expected size band of ~9.5 kb (Figure IV-8, lane 7). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I (Figure IV-8, lanes 2 and 9) or *Xho* I / *Nde* I (Figure IV-8, lanes 4 and 11) and hybridized with Probes 1, 2, and 3 produced no detectable bands. There is a diffuse area of hybridization that overlaps with lane 4. Because this hybridization is not a distinct band, nor is it present in lane 11, which contains the same enzyme set, this area of hybridization is considered non-specific binding. The data indicate that MON 87701 contains no backbone elements from PV-GMIR9 that overlaps Probes 1, 2, and 3.

3.3.2. Plasmid Backbone Probe 4

Conventional soybean control DNA digested with *Nco* I / *Vsp* I (Figure IV-9, lanes 1 and 7) or *Xho* I / *Nde* I (Figure IV-9, lanes 3 and 9) and hybridized with Probe 4 from the vector backbone of PV-GMIR9 (Figure IV-2, Probe 4) showed no detectable hybridization bands as expected for the negative control. Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Bgl* II / *Nco* I produced the expected band at ~6.0 kb (Figure IV-9, lanes 5 and 6). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I / *Vsp* I (Figure IV-9, lanes 2 and 8) or *Xho* I / *Nde* I (Figure IV-9, lanes 4 and 10) and hybridized with Probe 4 produced no detectable hybridization bands, indicating that MON 87701 contains no detectable PV-GMIR9 backbone elements that are contained within Probe 4. These data, in combination with

the data presented in Section.3.3.1, Part IV indicate that MON 87701 contains no detectable PV-GMIR9 backbone elements.

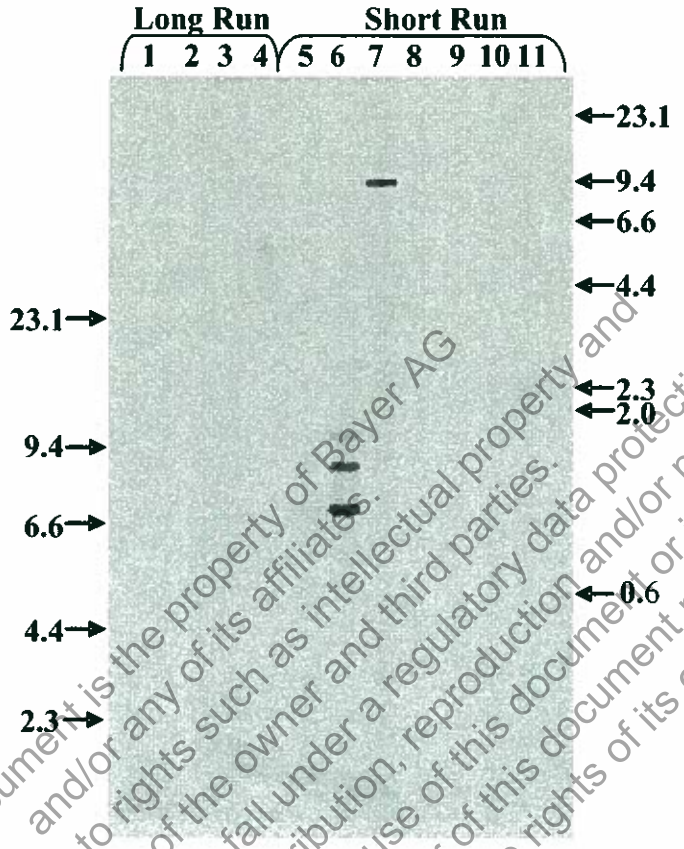

Figure IV-8. Southern Blot Analysis of MON 87701: PV-GMIR9 Backbone Sequence (Probes 1, 2, and 3)

The blot was hybridized with three overlapping ^{32}P-labeled probes that spanned a portion of the PV-GMIR9 backbone sequence (Figure IV-2, Probes 1, 2, and 3). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Conventional soybean spiked with probe templates (*Nco* I / *Vsp* I) [~0.1 genome equivalent]
6: Conventional soybean spiked with probe templates (*Nco* I / *Vsp* I) [~1.0 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I)
9: MON 87701 (*Nco* I / *Vsp* I)
10: Conventional soybean (*Xho* I / *Nde* I)
11: MON 87701 (*Xho* I / *Nde* I)
→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

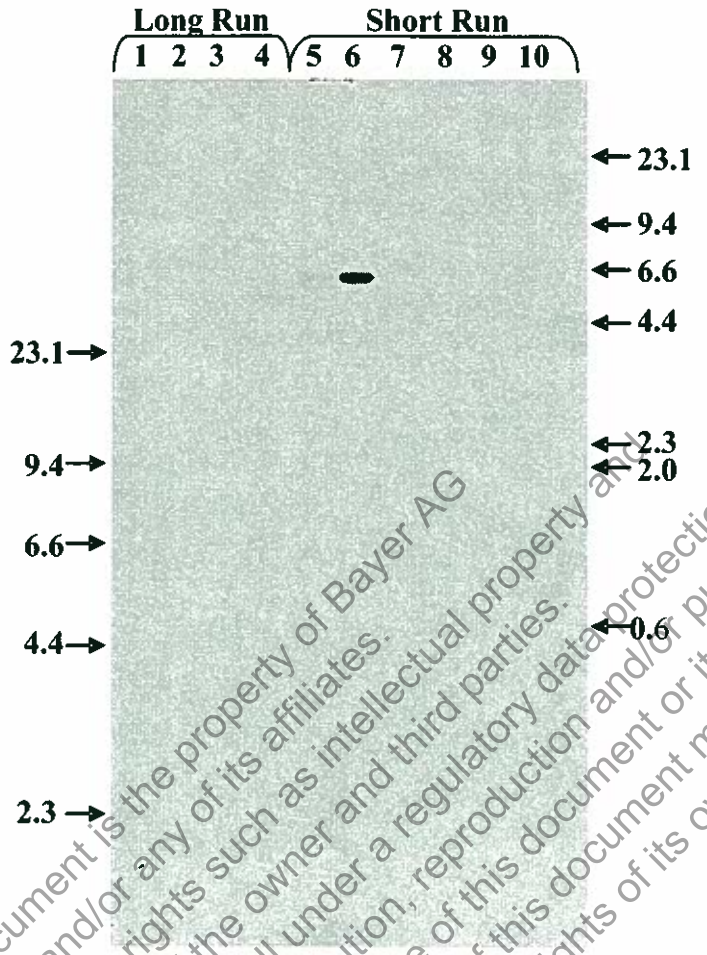

Figure IV-9. Southern Blot Analysis of MON 87701: PV-GMIR9 Backbone Sequence (Probe 4)

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the PV-GMIR9 backbone sequence (Figure IV-2, Probe 4). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~0.1 genome equivalent]
6: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
7: Conventional soybean (*Nco* I / *Vsp* I)
8: MON 87701 (*Nco* I / *Vsp* I)
9: Conventional soybean (*Xho* I / *Nde* I)
10: MON 87701 (*Xho* I / *Nde* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

3.4 Southern Blot Analysis to Determine the Presence or Absence of T-DNA II

The presence or absence of T-DNA II sequences in the soybean genome was evaluated by digesting the test and control genomic DNA samples with the *Nco* I / *Vsp* I or *Xho* I / *Nde* I enzyme sets and hybridizing with overlapping T-DNA II probes spanning the entire T-DNA II sequence of PV-GMIR9 (Figure IV-2, Probes 5 and 6). The left and right border regions of T-DNA II share 100% homology with those of T-DNA I and thus were not included in the T-DNA II analysis. If T-DNA II sequences are present in MON 87701, then probing with the T-DNA II sequences should result in hybridizing bands. The results of this analysis are shown in Figure IV-10. The Southern blot contained the same controls as described in Section 3.2., Part IV.

3.4.1. T-DNA II Probes 5 and 6

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure IV-10, lanes 1 and 9) or *Xho* I / *Nde* I (Figure IV-10, lanes 3 and 11) and hybridized with Probes 5 and 6 (Figure IV-2) showed no detectable hybridization bands, as expected for the negative control. Pre-digested conventional soybean DNA spiked with probe template generated from plasmid PV-GMIR9 produced the expected bands at ~2.0 kb and ~1.2 kb (Figure IV-10, lanes 6 and 7). Conventional soybean DNA spiked with plasmid PV-GMIR9 DNA previously digested with *Bgl* II/ *Nco* I produced the expected size bands of ~6.0 kb and ~9.5 kb (Figure IV-10, lane 8). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

MON 87701 DNA digested with *Nco* I/ *Vsp* I (Figure IV-10, lanes 2 and 10) or *Xho* I / *Nde* I (Figure IV-10, lanes 4 and 12) produced no hybridization bands. These results indicate that MON 87701 contains no detectable T-DNA II elements.

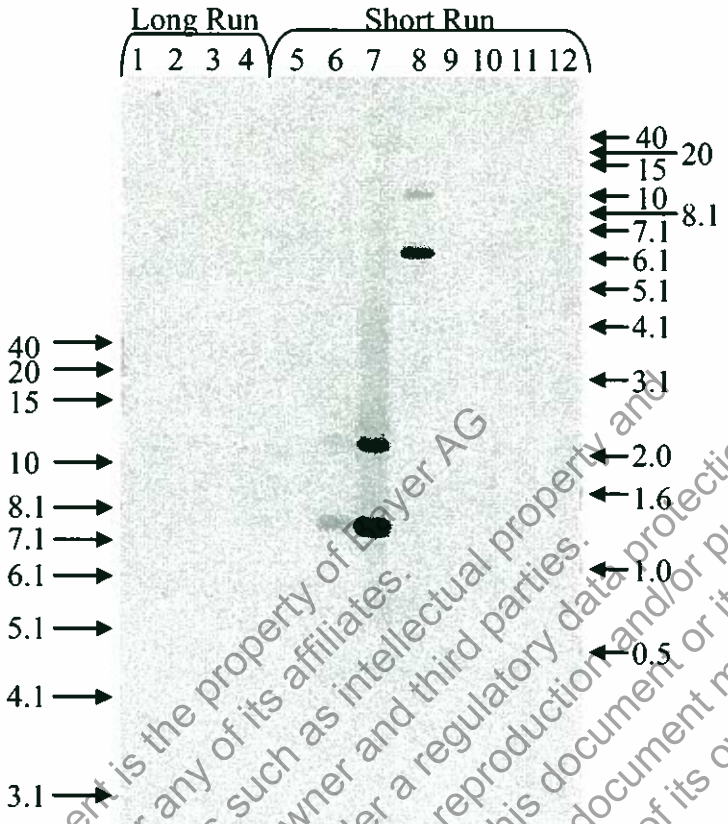

Figure IV-10. Southern Blot Analysis of MON 87701: T-DNA II (Probes 5 and 6)

The blot was hybridized with overlapping ^{32}P-labeled probes that spanned the T-DNA II sequence (Figure IV-2, Probes 5 and 6). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean (*Nco* I / *Vsp* I)
2: MON 87701 (*Nco* I / *Vsp* I)
3: Conventional soybean (*Xho* I / *Nde* I)
4: MON 87701 (*Xho* I / *Nde* I)
5: Blank
6: Conventional soybean spiked with probe templates (*Nco* I / *Vsp* I) [~0.1 genome equivalent]
7: Conventional soybean spiked with probe templates (*Nco* I / *Vsp* I) [~1.0 genome equivalent]
8: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl II*) [~1.0 genome equivalent]
9: Conventional soybean (*Nco* I / *Vsp* I)
10: MON 87701 (*Nco* I / *Vsp* I)
11: Conventional soybean (*Xho* I / *Nde* I)
12: MON 87701 (*Xho* I / *Nde* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

3.5. Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 87701

The organization of the elements within the MON 87701 insert was confirmed by DNA sequence analyses. Several PCR primers were designed with the intent to amplify nine overlapping regions of DNA that span the entire length of the insert (see Appendix A). The amplified DNA fragments were subjected to DNA sequencing analyses. The DNA sequence of the MON 87701 insert is 6427 base pairs long, beginning at base 3908 of PV-GMIR9 located in the right border region and ending at base 10334 in the left border region of PV-GMIR9. A set of primers designed from the 5′ and 3′ DNA flanking sequence of MON 87701 was used to amplify and characterize the insertion site in the conventional soybean control, A5547. A sequence comparison between the PCR product generated from the conventional soybean and the sequence generated from the 5' and 3' flanking sequences of MON 87701 indicates there was a 32 bp deletion of soybean genomic DNA and a 14 bp insertion just 5' to the MON 87701 insertion site. This molecular rearrangement likely occurred in the plant during the *Agrobacterium*-mediated transformation process (Salomon and Puchta, 1998). This analysis confirms that the DNA sequences flanking the 5' and 3' ends of the insert in MON 87701 are native to the soybean genome and that no major unexpected rearrangements occurred during the transformation to produce MON 87701. Results also confirm that the arrangement of the genetic elements in MON 87701 is identical to that in plasmid PV-GMIR9 and is depicted in Figure IV-4 and Table IV-3.

3.6. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87701

In order to demonstrate the stability of MON 87701, Southern blot analyses were performed using DNA obtained from multiple generations of MON 87701. For reference, the breeding history of MON 87701 is presented in Figure IV-11. The specific generations tested are identified in the legend of Figure IV-12. The R_5 generation was used for the molecular characterization analyses shown in Figures IV-5 through IV-10. To analyze stability, four additional generations were evaluated by Southern blot analysis and compared to the R_5 generation. DNA, isolated from each of the selected generations of MON 87701, were digested with the restriction enzymes *Nco* I / *Vsp* I (Figure IV-4) and hybridized with Probe 8 (Figure IV-2). Probe 8 is designed to detect both fragments generated by the *Nco* I / *Vsp* I digest. Any instability associated with the insert would be detected as novel bands within the fingerprint on the Southern blot. The results are shown in Figure IV-12. The Southern blot has the same controls as described in Section 3.2.

Conventional soybean DNA digested with *Nco* I / *Vsp* I (Figure IV-12, lane 4) and hybridized with Probe 8 showed no detectable hybridization bands, as expected for the negative control. Pre-digested conventional soybean DNA spiked with probe template (Figure IV-2, Probe 8) generated from plasmid PV-GMIR9 produced the expected band at ~1.5 kb (Figure IV-12, lanes 1 and 2). Predigested conventional soybean DNA spiked with plasmid PV-GMIR9 DNA, previously digested with *Bgl* II / *Nco* I, produced the expected size bands of ~6.0 kb and ~9.5 kb (Figure IV-12, lane 3). Detection of the spiked controls indicates that the probes are recognizing their target sequences.

DNA extracted from MON 87701 generations R_4, R_5, R_6, R_8, and R_9 digested with *Nco* I / *Vsp* I (Figure IV-12, lanes 5-9) and hybridized with Probe 8 each produced two bands of ~6.3 kb and ~4.0 kb. The ~4.0 kb band is the expected size for the 5' border fragment and the ~6.3 kb band is consistent with the expected size of the 3' border fragment. These bands are consistent with the bands detected in Figure IV-6 (lanes 2 and 9) indicating that the single copy of T-DNA I in MON 87701 is stably maintained across multiple generations.

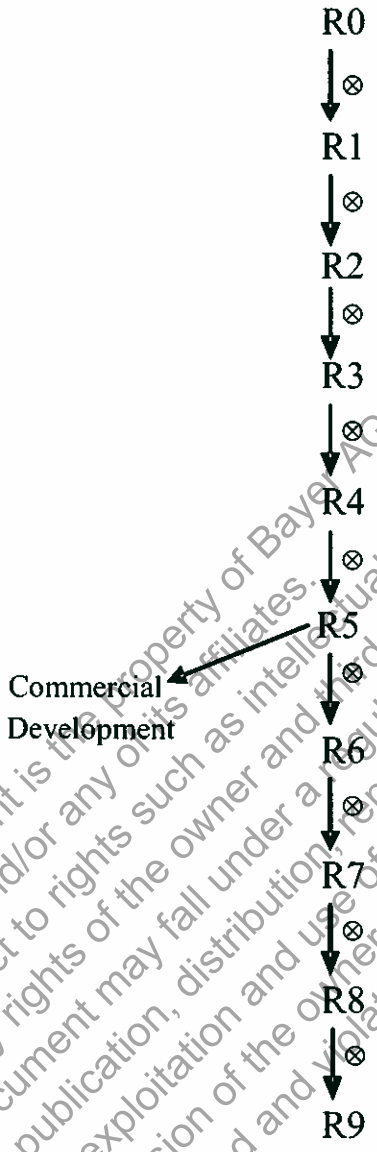

Figure IV-11. MON 87701 Breeding Diagram

All generations were self-pollinated (⊗). The R_5 generation seed material was used for regulatory molecular characterization and commercial development. Seed lots from the R_4, R_5, R_6, R_8, and R_9 generations were used in the molecular generational stability analysis.

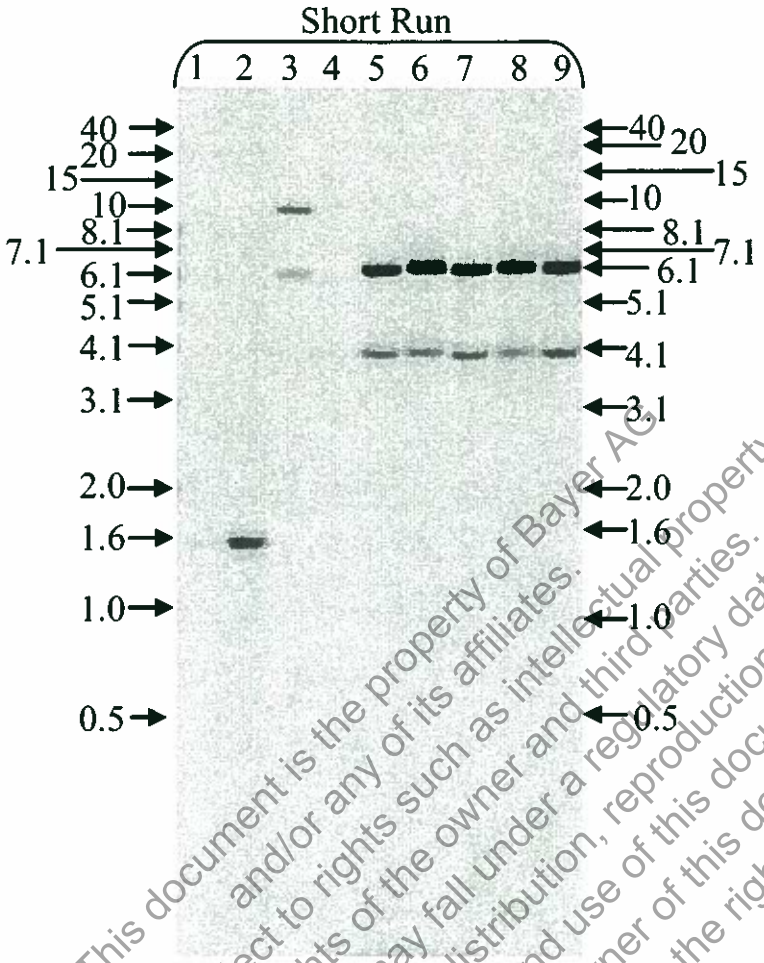

Figure IV-12. Insert Stability of MON 87701: T-DNA I (Probe 8)

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the T-DNA I sequence (Figure IV-2, Probe 8). Each lane contains ~10 µg of digested genomic DNA isolated from soybean leaf tissue. Lane designations are as follows:

Lane 1: Conventional soybean spiked with probe templates (*Nco* I / *Vsp* I) [~0.1 genome equivalent]
2: Conventional soybean spiked with probe templates (*Nco* I / *Vsp* I) [~1.0 genome equivalent]
3: Conventional soybean (*Nco* I / *Vsp* I) spiked with PV-GMIR9 (*Nco* I / *Bgl* II) [~1.0 genome equivalent]
4: Conventional soybean (*Nco* I / *Vsp* I)
5: MON 87701 (R_4) (*Nco* I / *Vsp* I)
6: MON 87701 (R_5) (*Nco* I / *Vsp* I)
7: MON 87701 (R_6) (*Nco* I / *Vsp* I)
8: MON 87701 (R_8) (*Nco* I / *Vsp* I)
9: MON 87701 (R_9) (*Nco* I / *Vsp* I)
→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

3.7. Inheritance of the Genetic Insert in MON 87701

During development of MON 87701, segregation data were recorded to assess the heritability and stability of the *cry1Ac* gene in MON 87701. Chi-square analysis was performed over several generations to confirm the segregation and stability of the *cry1Ac* gene in MON 87701. The Chi-square analysis is based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The breeding path for generating segregation data for MON 87701 is described in Figure IV-13. The transformed R_0 plant was self-pollinated to produce R_1 seed. This seed was planted and the resulting R_1 plants were expected to segregate in a 15:1 ratio of positive to negative individual plants for the insect-protected phenotype. The 15:1 segregation ratio was expected because the *cry1Ac* gene was inserted into the soybean genome (R_0 plant) at two independently segregating loci. An individual plant (#55, designated as MON 87701), homozygous for a single copy of the *cry1Ac* gene, was identified from the R_1 segregating population by TaqMan PCR analysis.

The selected R_1 MON 87701 plant was self-pollinated to give rise to a population of R_2 plants that were repeatedly self-pollinated through the R_5 generation. At each generation, the fixed homozygous plants were tested for the expected segregation pattern of 1:0 (positive : negative) for the *cry1Ac* gene using TaqMan PCR analysis or for the presence of the Cry1Ac protein via ELISA analysis and/or protein specific lateral flow strips.

At the R_5 generation, homozygous MON 87701 soybean plants were bred via traditional breeding (bi-parental cross) with a soybean variety that did not contain the *cry1Ac* gene. The resulting F_1 plants were then self-pollinated to produce F_2 seed. The subsequent F_2 plants were tested for the presence of the MON 87701 insert by TaqMan PCR using an event-specific assay. These plants were predicted to segregate at a 1:2:1 (homozygous positive:hemizygous positive:homozygous negative) ratio according to Mendelian inheritance principles.

The heritability and stability of the *cry1Ac* gene in MON 87701 were further tested in the F_3 generation. Hemizygous positive F_2 plants were selected and self-pollinated to produce F_3 seed. The resulting F_3 plants were tested for the presence of MON 87701 by TaqMan PCR using an event-specific zygosity assay. The F_3 generation was predicted to segregate at a 1:2:1 (homozygous positive:hemizygous positive:homozygous negative) ratio.

A Chi-square (χ^2) analysis was used to compare the observed segregation ratios to the expected ratios according to Mendelian principles. The Chi-square was calculated as:

$$\chi^2 = \sum [(\,|\,o - e\,|\,)^2 / e]$$

where o = observed frequency of the genotype (if PCR used) or phenotype (if ELISA used) and e = expected frequency of the genotype or phenotype. The level of statistical significance was predetermined to be 5% ($p \leq 0.05$).

The results of the χ^2 analysis of the segregating progeny of MON 87701 are presented in Tables IV-6 and IV-7. The χ^2 value in the R_1 generation indicated no statistically significant difference between the observed and expected 15:1 segregation ratio. The insect-protected trait was subsequently fixed in the R_2, R_3, R_4, and R_5 generations, and no further segregation occurred in the generations, as expected. Following the crossing of

the R_5 generation with a soybean variety that did not contain the *cry1Ac* gene, the resulting F_2 and F_3 progeny were assessed for their heritability of the *cry1Ac* gene. The χ^2 values in the F_2 and F_3 generations indicated no statistically significant difference between the observed and expected segregation ratios. These results support the conclusion that the *cry1Ac* gene in MON 87701 resides at a single locus within the soybean genome and is inherited according to Mendelian inheritance principles. These results are also consistent with the molecular characterization data that indicate MON 87701 contains a single, intact copy of the *cry1Ac* expression cassette that was inserted into the soybean genome at a single locus.

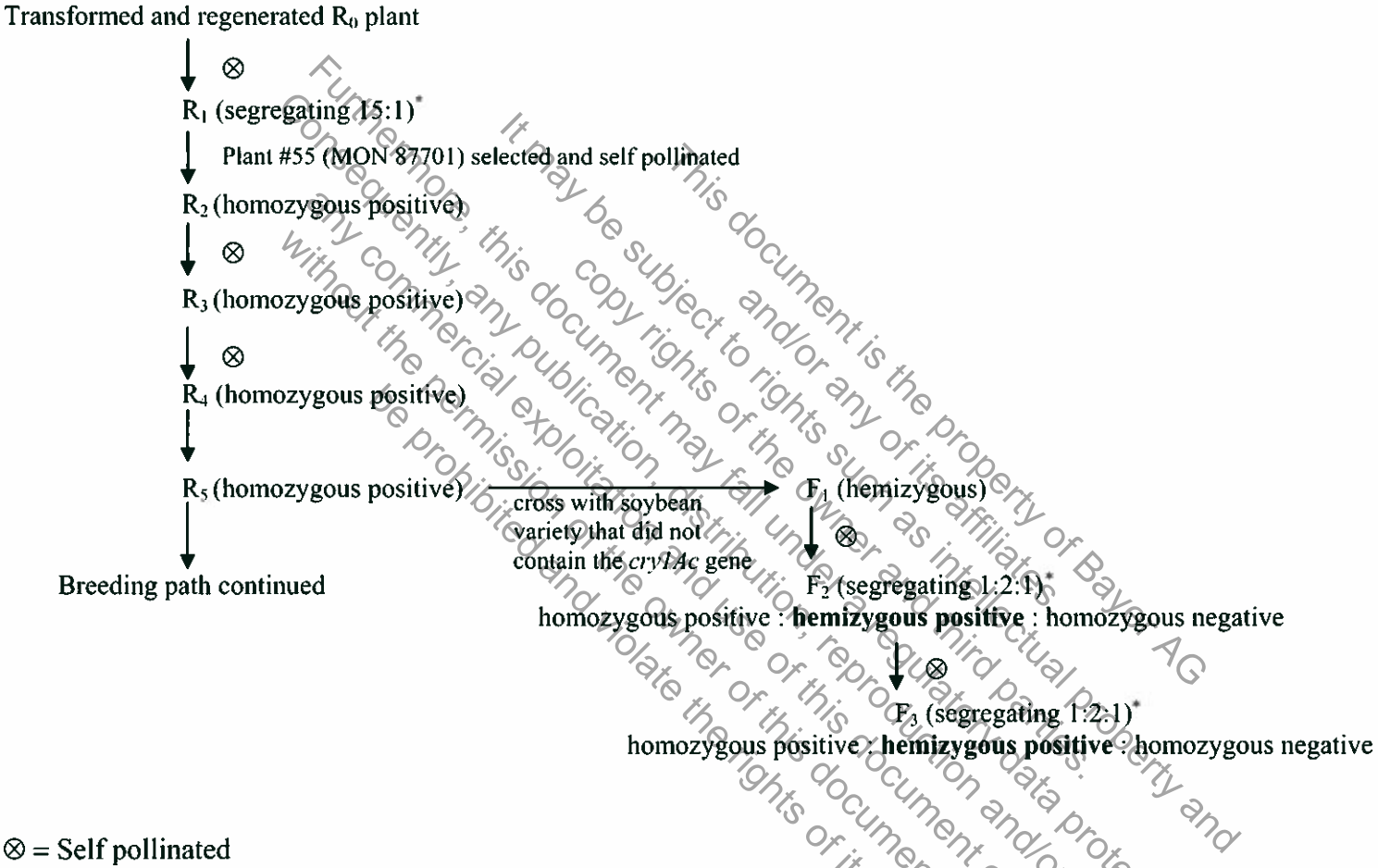

Figure IV-13. Breeding Path for Generating Segregation Data for MON 87701

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* Chi-square analysis conducted on segregation data from the R_1, F_2, and F_3 generations.

Table IV-6. Segregation of the *cry1Ac* Gene during the Development of MON 87701

					Segregation			
Generation	**Expected Ratio**	**Total Plants Tested[1]**	**Observed # Plants Positive**	**Observed # Plants Negative**	**Expected # Plants Positive**	**Expected # Plants Negative**	**χ^2**	**Probability**
R_1	15:1	19	18	1	17.8	1.2	0.03[3]	0.8590
R_2	1:0	80	80	0	80	0	Fixed	N/A
R_3	1:0	48	48	0	48	0	Fixed	N/A
R_4	1:0	598	598	0	598	0	Fixed	N/A
R_5	1:0	629	629	0	629	0	Fixed	N/A

[1] Plants were tested for the presence of the *cry1Ac* gene by protein check strips, ELISA analysis, and/or event-specific Taqman PCR. N/A = Not applicable.

Table IV-7. Segregation of the *cry1Ac* Gene in F_2 and F_3 Progeny from a Cross of MON 87701 with a Soybean Variety that did not Contain the *cry1Ac* Gene

					1:2:1 Segregation				
Generation[1]	**Total Plants Tested[2]**	**Observed # Plants Homozygous Positive**	**Observed # Plants Hemizygous Positive**	**Observed # Plants Homozygous Negative**	**Expected # Plants Homozygous Positive**	**Expected # Plants Hemizygous Positive**	**Expected # Plants Homozygous Negative**	**χ^2**	**Probability**
F_2	297	79	148	70	74.25	148.5	74.25	0.5	0.76
F_3	263	73	121	69	65.75	131.5	65.75	1.8	0.4069

[1] F_2 progeny were from the cross of MON 87701 homozygous positive for the *cry1Ac* gene with a soybean variety that did not contain the *cry1Ac* gene. F_3 progeny were from self-pollinated F_2 plants hemizygous positive for the *cry1Ac* gene.
[2] Plants were tested for the presence of the *cry1Ac* gene by event-specific Taqman PCR.

3.8. Conclusion of Molecular Characterization of MON 87701

Molecular analyses show that one intact copy of the *cry1Ac* expression cassette was integrated at a single chromosomal locus in MON 87701. No additional genetic elements, including backbone sequences from the transformation vector PV-GMIR9, linked or unlinked to the intact DNA insert, were detected in the genome of MON 87701. Generational stability analysis demonstrated that the expected Southern blot fingerprint of MON 87701 has been maintained across five generations of breeding, thereby confirming the stability of the DNA insert over multiple generations. DNA sequence analyses confirmed the sequence identity between the MON 87701 insert and the portion of the T-DNA I from PV-GMIR9 that was integrated into the soybean genome. These results also confirmed the organization of the genetic elements within the *cry1Ac* expression cassette of MON 87701, which was identical to that in plasmid PV-GMIR9. Analysis of the T-DNA insertion site indicates that there is a 32 bp deletion of genomic DNA and 14 bp insertion at the 5' insert-to-plant DNA junction. Results from segregation analyses show heritability and stability of the *cry1Ac* gene occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the DNA insert at a single chromosomal locus.

Section 4. Other Data or Information Regarding the Development of MON 87701

All relevant information regarding development of MON 87701 is described in Parts II-VII of this summary.

PART V: PRESENCE OF GENES THAT ENCODE RESISTANCE TO ANTIBIOTICS

No genes that encode resistance to an antibiotic marker were inserted into the soybean genome during the development of MON 87701. Molecular characterization data presented in Part IV demonstrate the absence of the *aad*A antibiotic resistant marker in MON 87701.

PART VI: CHARACTERIZATION OF THE PROTEIN INTRODUCED INTO MON 87701

The characterization and safety assessment of the Cry1Ac protein produced in MON 87701 was based on the following:

1) Demonstration of its equivalence to the *E. coli*-produced Cry1Ac used in laboratory and regulatory safety evaluations
2) Quantitation of expression levels in key soybean tissues
3) An assessment of allergy potential
4) An evaluation of its potential toxicity, and
5) Dietary exposure assessment for human and animals.

Results indicate that the MON 87701- and *E. coli*-produced Cry1Ac proteins are equivalent. Data also support a conclusion of safe consumption of Cry1Ac based on several lines of evidence. Thus, Cry1Ac has a long history of safe use and has been found to pose negligible risk to human and animal health upon consumption. Cry1Ac lacks structural similarity to known toxins or biologically active proteins known to have adverse effects on mammals. Cry1Ac is present at very low levels in the harvested seed of MON 87701 and will constitute a very small portion of the total protein present in food and feed derived from MON 87701. Cry1Ac is readily digestible in simulated gastrointestinal fluids and shows no oral toxicity in mice. Furthermore, in 1997, the U.S. EPA established an exemption from the requirement of a tolerance for the PIP *Bacillus thuringiensis* (Bt) Cry1Ac protein and the genetic material necessary for its production in or on all raw agricultural commodities (40 CFR § 174.510). These data lead to the conclusion that the Cry1Ac present in the food and feed derived from MON 87701 will be safe for consumption by humans and animals, respectively.

Section 1. Identity and Characterization of the Cry1Ac Protein

The Cry1Ac protein originates from *Bacillus thuringiensis*, a ubiquitous gram-positive soil bacterium that accumulates crystal proteins during sporulation. These crystal (Cry) proteins bind to the specific receptors on the midgut epithelium of targeted lepidopteran insects, and form cation selective pores, which lead to the inhibition of the digestive process and result in the insecticidal activity (Hofmann et al., 1988; Slaney et al., 1992; Van Rie et al., 1990). One valuable feature of this activity is that it is targeted to specific categories of insects, and does not impact broader insect populations or other organisms. For example, Cry1A proteins have insecticidal activity specifically against lepidopteran insects, while Cry3 proteins have insecticidal activity specifically against coleopteran insects (Höfte and Whiteley, 1989). Bt Cry1 proteins are synthesized as ~130 kDa prototoxins consisting of a three-domain toxin portion and a C-terminal extension (De Maagd et al., 2003). Domain I is involved in membrane insertion and pore formation. Domain II is involved in specific receptor recognition and binding. Domain III maintains the structural integrity of the protein molecule and also contributes to specificity (De Maagd et al., 2000 and 2001). The C-terminal portion of the Cry1A protein is thought to

contribute to crystal formation via disulfide bond formation, but is not involved in determining the biological activity or specificity of the toxin toward target insects (Miranda et al., 2001; Rukmini et al., 2000; Schnepf et al., 1998; Diaz-Mendoza et al., 2007).

The Cry1Ac produced in MON 87701 is targeted to chloroplasts due to the addition of a chloroplast transit peptide (CTP) coding sequence at the 5' end of the coding sequence. Following translation and translocation into chloroplasts, the CTP is cleaved. N-terminal sequence analysis of the MON 87701-produced Cry1Ac (Section 1.3, Part VI) indicated the presence of four additional amino acids at the N-terminus compared to Cry1Ac proteins produced in Bt subsp. *kurstaki* and Bollgard cotton (Figure VI-1, Figure VI-4). The additional four amino acids are cysteine (C), methionine (M), glutamine (Q), and alanine (A). While the identities of methionine, glutamine, and alanine were clearly determined by N-terminal sequencing, the identity of the first amino acid, cysteine, was inferred based on the CTP1 coding sequence in MON 87701. The chemistry employed in N-terminal sequencing is known to degrade cysteine (Inglis and Liu, 1970), preventing its clear identification. Except for the CTP-derived four additional amino acids at the N-terminus, the Cry1Ac that accumulates in MON 87701 shares an amino acid identity of >99% with the Cry1Ac from Bt subsp. *kurstaki* and shares 100% identity with the Cry1Ac produced in Bollgard cotton (see Figures VI-1 and VI-4). The presence of these four additional amino acids at the N-terminus of the MON 87701-produced Cry1Ac protein has no impact on protein activity or toxicity due to rapid proteolytic excision of the N-terminus during prototoxin activation.

The sequence encoding for the four additional amino acids derived from the CTP in MON 87701-produced Cry1Ac was included in the N-terminus of the *E. coli*-produced Cry1Ac protein that was used in the safety assessment evaluations for MON 87701. This resulted in the production of a full-length Cry1Ac protein of 1182 amino acids (1178 from Cry1Ac and four from the CTP coding region).

```
Bt Cry1Ac     ----MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGF
Bollgard      ----MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGF
MON87701      CMQAMDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGF

Bt Cry1Ac     VLGLVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFRE
Bollgard      VLGLVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFRE
MON87701      VLGLVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFRE

Bt Cry1Ac     WEADPTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVS
Bollgard      WEADPTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVS
MON87701      WEADPTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVS

Bt Cry1Ac     VFGQRWGFDAATINSRYNDLTRLIGNYTDYAVRWYNTGLERVWGPDSRDWVRYNQFRREL
Bollgard      VFGQRWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRREL
MON87701      VFGQRWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRREL

Bt Cry1Ac     TLTVLDIVALFPNYDSRRYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIERSIRSPHL
Bollgard      TLTVLDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHL
MON87701      TLTVLDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHL

Bt Cry1Ac     MDILNSITIYTDAHRGYYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQ
Bollgard      MDILNSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQ
MON87701      MDILNSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQ

Bt Cry1Ac     GVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQ
Bollgard      GVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQ
MON87701      GVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQ

Bt Cry1Ac     NNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIASDSITQIPA
Bollgard      NNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIASDSITQIPA
MON87701      NNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIASDSITQIPA

Bt Cry1Ac     VKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRVRVRYASV
Bollgard      VKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRVRVRYASV
MON87701      VKGNFLFNGSVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPIHFPSTSTRYRVRVRYASV

Bt Cry1Ac     TPIHLNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESANAFTSSLGNIVGVRNFSGTA
Bollgard      TPIHLNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESANAFTSSLGNIVGVRNFSGTA
MON87701      TPIHLNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESANAFTSSLGNIVGVRNFSGTA

Bt Cry1Ac     GVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSNLVTYL
Bollgard      GVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSNLVTYL
MON87701      GVIIDRFEFIPVTATLEAEYNLERAQKAVNALFTSTNQLGLKTNVTDYHIDQVSNLVTYL

Bt Cry1Ac     SDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQGGDDVF
Bollgard      SDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQGGDDVF
MON87701      SDEFCLDEKRELSEKVKHAKRLSDERNLLQDSNFKDINRQPERGWGGSTGITIQGGDDVF

Bt Cry1Ac     KENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEIYLIRYNAKHETV
Bollgard      KENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEIYSIRYNAKHETV
MON87701      KENYVTLSGTFDECYPTYLYQKIDESKLKAFTRYQLRGYIEDSQDLEIYSIRYNAKHETV

Bt Cry1Ac     NVPGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSHHFSLDIDVG
Bollgard      NVPGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSHHFSLDIDVG
MON87701      NVPGTGSLWPLSAQSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSHHFSLDIDVG

Bt Cry1Ac     CTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKLEW
Bollgard      CTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKLEW
MON87701      CTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKKWRDKREKLEW

Bt Cry1Ac     ETNIVYKEAKESVDALFVNSQYDQLQADTNIAMIHAADKRVHSIREAYLPELSVIPGVNA
Bollgard      ETNIVYKEAKESVDALFVNSQYDQLQADTNIAMIHAADKRVHSIREAYLPELSVIPGVNA
MON87701      ETNIVYKEAKESVDALFVNSQYDQLQADTNIAMIHAADKRVHSIREAYLPELSVIPGVNA
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Bt Cry1Ac     AIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNQRSVLVVPEWEA
Bollgard      AIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNQRSVLVVPEWEA
MON87701      AIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNNQRSVLVVPEWEA

Bt Cry1Ac     EVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEIYPNNTVTC
Bollgard      EVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEIYPNNTVTC
MON87701      EVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNCVEEEIYPNNTVTC

Bt Cry1Ac     NDYTVNQEEYGGAYTSRNRGYNEAPSVPADYASVYEEKSYTDGRRENPCEFNRGYRDYTP
Bollgard      NDYTVNQEEYGGAYTSRNRGYNEAPSVPADYASVYEEKSYTDGRRENPCEFNRGYRDYTP
MON87701      NDYTVNQEEYGGAYTSRNRGYNEAPSVPADYASVYEEKSYTDGRRENPCEFNRGYRDYTP

Bt Cry1Ac     LPVGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE
Bollgard      LPVGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE
MON87701      LPVGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLMEE
```

Figure VI-1. Amino Acid Sequence Alignment for Cry1Ac Proteins

The amino acid sequence alignment for the Cry1Ac proteins produced in *Bacillus thuringiensis* (Cry1Ac, GI-117547*), Bollgard cotton, and MON 87701. Amino acid sequence differences between Cry1Ac from Bt and the two plant-produced proteins are underlined and highlighted in gray in the Bt Cry1Ac sequence. Four amino acids originating from the CTP1 in MON 87701-produced Cry1Ac are in boldface font.

The expression level of the Cry1Ac produced in MON 87701 seed was too low and insufficient for use in the subsequent safety evaluations. Therefore, it was necessary to produce the protein in a high-expressing, recombinant microorganism in order to obtain sufficient quantities of the protein for safety evaluations. A recombinant Cry1Ac was produced in *Escherichia coli*, the sequence of which was engineered to match that of the Cry1Ac produced in MON 87701. The equivalence of the physicochemical characteristics and functional activity between the MON 87701- and *E. coli*-produced Cry1Ac proteins was confirmed by a panel of analytical techniques, including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), glycosylation analysis, and assay of biological activity. The details of the materials, methods, and results are described in Appendix B, while the conclusions are summarized as follows.

The Cry1Ac protein isolated from MON 87701 harvested seed was purified and characterized, and results confirmed the equivalence of MON 87701- and *E. coli*-produced Cry1Ac proteins. SDS-PAGE demonstrated that the MON 87701-produced Cry1Ac comigrated to the same position on the gel as the *E. coli*-produced Cry1Ac protein, indicating the protein from both sources was equivalent in molecular weight. Western blot analysis conducted with a polyclonal antibody against Cry1Ac, demonstrated that the electrophoretic mobility and immunoreactivity of the MON 87701- and *E. coli*-produced Cry1Ac proteins were equivalent. The intactness of the N-terminus of the MON 87701-produced Cry1Ac protein was confirmed with an antibody which is specific to the N-terminal peptide. Tryptic peptide mapping by MALDI-TOF MS yielded

* GenBank gene identification number.

peptide masses consistent with the predicted tryptic peptides generated *in silico* based on the predicted trypsin cleavage sites in the Cry1Ac sequence. In addition, the MON 87701- and *E. coli*-produced Cry1Ac proteins were found to be equivalent based on functional activities against a sensitive lepidopteran species and the lack of glycosylation. Taken together, these data provide a detailed characterization of the Cry1Ac protein isolated from MON 87701 harvested seed and establish its equivalence to the *E. coli*-produced Cry1Ac protein.

The following sections summarize the results and experimental details that are provided in Appendix B.

1.1. Cry1Ac Molecular Weight Equivalence

The equivalence in apparent molecular weights of the full-length purified MON 87701- and *E. coli*-produced Cry1Ac proteins was demonstrated using SDS-PAGE (Figure VI-2). The full-length MON 87701-produced Cry1Ac had an estimated molecular weight of 133.4 kDa (Table VI-1), and migrated to the same position on the SDS-PAGE gel as the *E. coli*-produced Cry1Ac reference standard (Figure VI-2, lane 9). The apparent molecular weight of the full-length *E. coli*-produced Cry1Ac reference protein is 131.7 kDa. The difference in the estimated molecular weights between the MON 87701-produced and *E. coli*-produced Cry1Ac full-length proteins is 1.3% (Table VI-1). Because the experimentally determined difference in apparent molecular weights met the preset acceptance criteria (≤5% difference), the MON 87701- and *E. coli*-produced Cry1Ac proteins are considered equivalent based on their molecular weights.

Table VI-1. Molecular Weight Difference between the Full-Length MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins Based on SDS-PAGE

Molecular Weight of Full-Length MON 87701-Produced Cry1Ac Protein	Molecular Weight of *E. coli*-Produced Cry1Ac Protein	Percent Difference from *E. coli*-Produced Cry1Ac Protein [1]
133.4 kDa	131.7 kDa	1.3 %

[1] Percent difference was calculated as follows: $\frac{133.4 - 131.7}{133.4} \times 100\% = 1.3\%$

1.2. Cry1Ac Immunoreactivity Equivalence

A western blot analysis using goat anti-Cry1Ac serum was conducted to determine the relative immunoreactivity of the MON 87701-produced Cry1Ac protein and the *E. coli*-produced Cry1Ac reference protein. The results demonstrated that the specific anti-Cry1Ac antibody recognized the MON 87701-produced Cry1Ac protein that migrated to a similar position as the *E. coli*-produced Cry1Ac reference protein (Figure VI-3). Furthermore, the immunoreactive signal increased with increasing levels of Cry1Ac

loading. The observed immunoreactivities between the full-length MON 87701- and *E. coli*-produced proteins were similar based on densitometric analysis of the western blot. Faint immunoreactive bands with molecular weights below ~133 kDa represent degradation products of Cry1Ac. Faint immunoreactive bands with molecular weights around 250 kDa also were observed, and most likely represent aggregation of the Cry1Ac protein. Both protein degradation and protein aggregation are commonly observed during protein purification of Cry proteins. Cry proteins naturally aggregate into crystal structures as has been observed for Cry1A proteins (Güereca and Bravo, 1999), while degradation occurs primarily due to the release of endogenous proteases during the purification procedure (Gao et al., 2006).

Densitometric analysis was conducted to compare the immunoreactivity of full-length MON 87701- and *E. coli*-produced Cry1Ac proteins. The relative immunoreactivity of each protein with Cry1Ac-specific antibody was determined by averaging intensity values of six protein bands corresponding to the full-length MON 87701-produced Cry1Ac and six bands corresponding to the full-length *E. coli*-produced Cry1Ac (Table VI-2). The averaged band intensity of the signal from the MON 87701-produced Cry1Ac lanes was 33.3% less than the averaged band intensity of the signal from the *E. coli*-produced Cry1Ac lanes. The observed difference was within the preset acceptance criteria for immunoreactivity (±35% difference). Thus, the immunoblot analysis established identity of the MON 87701-produced Cry1Ac and demonstrated that the MON 87701- and *E. coli*-produced Cry1Ac proteins are equivalent based on their immunoreactivity with Cry1Ac-specific antibody.

Table VI-2. Comparison of Immunoreactive Signals Between Full-Length MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins

Load Amount (ng)	MON 87701-produced Protein Signal Density[1]	*E. coli*-produced Protein Signal Density[1]
10	1.288	1.419
10	1.955	1.798
20	2.908	4.559
20	2.987	3.706
30	4.214	6.547
30	4.140	8.199
Sum	**17.492**	**26.228**
Average Density	**2.915**	**4.371**
Percent difference[2]	**33.3%**	

[1] The density of each band was determined by image analysis of the quantitative western blot shown in Figure VI-3. Values shown for signal density are contour quantity, i.e. average OD × contour area in mm^2.

[2] Percent difference is calculated using the equation:

$$\frac{|Average\ Density\ E.coli - Average\ Density\ Plant\ |}{Average\ Density\ E.coli} \times 100 = Percent\ Difference$$

1.3. N-terminal Sequence Analysis

From N-terminal sequencing analysis of the first 15 amino acids of the MON 87701-produced Cry1Ac, seven definite and two tenuous amino acid assignments were made that matched the predicted N-terminal sequence for a Cry1Ac containing four amino acids derived from the CTP1 (Figure VI-4). As discussed above, the amino acid cysteine is shown in the predicted sequence at position one, based on the CTP1-Cry1Ac coding sequence in MON 87701. Cysteine is unstable during the acid hydrolysis reaction used for N-terminal sequencing, and is usually not explicitly observed (Inglis and Liu, 1970). The clear identification of amino acids in subsequent cycles of the sequencing analysis confirmed that an unidentified amino acid was present at position one. The N-terminal sequence information, therefore, confirms the identity of the Cry1Ac isolated from MON 87701 and the intactness of its N-terminus.

1.4. MALDI-TOF Mass Spectrum Analysis

The identity of the MON 87701-produced Cry1Ac was further confirmed by tryptic peptide mass mapping analysis using MALDI-TOF MS. In general, protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997). Observed tryptic peptides were considered a match to the expected tryptic mass when differences in molecular weight of less than one dalton (Da) were found between the observed and predicted fragment masses. Such matches were made without consideration for potential natural amino acid modifications, such as glycosylation. The protein sample was heat-denatured, chemically reduced, alkylated, and digested with trypsin, and the masses of the tryptic peptides were measured.

There were seventy unique peptide fragments identified that corresponded to the expected masses of the Cry1Ac trypsin-digested peptides (Table D-4, Appendix D). The identified masses were used to assemble a coverage map indicating the matched peptide sequences for the Cry1Ac protein (Figure VI-5), resulting in ~67% (787 out of 1182 total amino acids) coverage of the total protein. This analysis confirmed the identity of the MON 87701-produced Cry1Ac protein.

1.5. Cry1Ac Funtional Activity Equivalence

The functional activities of the MON 87701- and *E. coli*-produced Cry1Ac proteins were compared using an insect growth-inhibition bioassay. Aliquots of MON 87701- and *E. coli*-produced Cry1Ac proteins were tested in an assay using corn earworm (CEW; *Helicoverpa zea)*, an insect species known to be susceptible to Cry1Ac. Dose-response assays were performed for Cry1Ac proteins from both sources in parallel and assays were repeated on three separate days to estimate the mean EC_{50} value, the effective concentration necessary to inhibit CEW growth by 50% relative to a control population of insects not exposed to the insecticidal protein. The mean EC_{50} value determined for the MON 87701-produced Cry1Ac was 0.0039 μg Cry1Ac/ml diet. This EC_{50} value was very similar to the mean EC_{50} value of 0.0036 μg Cry1Ac/ml diet obtained for the *E. coli*-produced Cry1Ac in the same assay (Table VI-3). These results clearly demonstrate that

the Cry1Ac proteins derived from MON 87701 and *E. coli* have equivalent functional activities.

Table VI-3. EC_{50} Values of *E. coli*-Produced and MON 87701-Produced Cry1Ac Proteins in a Corn Earworm Diet-Incorporation Bioassay

		EC_{50} (µg Cry1Ac/ml diet)[1]	
		***E. coli*-produced**	**MON 87701-produced**
Replicate[2]	1	0.0031 ± 0.00035	0.0050 ± 0.00069
	2	0.0026 ± 0.00022	0.0032 ± 0.00021
	3	0.0050 ± 0.00030	0.0034 ± 0.00035
Mean EC_{50}		0.0036	0.0039

[1] EC_{50} represents the Cry1Ac concentration needed to inhibit the growth of the target insect by 50%.
[2] Values shown for each replicate represent EC_{50} estimates ± standard error

1.6. Cry1Ac Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures, simple oligosaccharides or monosaccharides. In contrast, the non-virulent *E. coli* strains used for cloning and expression purposes lack the necessary biochemical synthetic capacity required for protein glycosylation. Therefore, determining whether the MON 87701-produced Cry1Ac is equivalent to the *E. coli*-produced Cry1Ac requires an investigation of its glycosylation status.

To assess whether post-translational glycosylation of the MON 87701-produced Cry1Ac occurred, the purified protein sample was subjected to glycosylation analysis. The *E. coli*-produced Cry1Ac represented a negative control. The positive controls were the transferrin and horseradish peroxidase (HRP) proteins, which are known to have covalently linked carbohydrate modifications. Transferrin and HRP, as well as the purified Cry1Ac proteins isolated from MON 87701 and *E. coli,* were separated on SDS-PAGE, transferred to a PVDF membrane, and glycosylation analysis was performed to detect carbohydrate moieties on the proteins. The results of this analysis are shown in Figure VI-6. The positive controls, transferrin and HRP, were detected at the expected molecular weights of ~75 and ~45 kDa, respectively, in a concentration-dependent manner (Figure VI-6, Panel A, Lanes 2-5). No detectable signal was observed for the MON 87701- and *E. coli*-produced Cry1Ac proteins (Figure VI-6, Panel A, Lanes 6-9). To confirm that sufficient MON 87701- and *E. coli*-produced Cry1Ac proteins were present for carbohydrate detection and glycosylation analysis, the membrane was stained with Coomassie Brilliant Blue R stain to detect proteins (Figure VI-6, Panel B). With this stain, both MON 87701- and *E. coli*-produced Cry1Ac proteins were clearly detected on the membrane (Figure VI-6, Panel B, Lanes 6-9), demonstrating sufficient levels of the proteins were present for carbohydrate detection.

These results indicate that the MON 87701-produced Cry1Ac is not glycosylated and thus is equivalent to the *E. coli*-produced Cry1Ac with respect to the absence of glycosylation.

1.7. Conclusion

The characterization and identity of the MON 87701-produced Cry1Ac was established by demonstrating its equivalence to the *E. coli*-produced Cry1Ac. The molecular weight of the MON 87701- and *E. coli*-produced Cry1Ac proteins was estimated by SDS-PAGE. The SDS-PAGE demonstrated that the proteins migrated at the same molecular weight, indicating that the Cry1Ac proteins from both sources are equivalent in their molecular weight. The electrophoretic mobility and immunoreactive properties of the MON 87701-produced Cry1Ac were shown to be equivalent to those of the *E. coli*-produced Cry1Ac. The N-terminus of the MON 87701-produced Cry1Ac was consistent with the predicted amino acid sequence for this region of the protein. The MALDI-TOF mass spectrometry analysis yielded peptide masses consistent with the expected peptide masses from the translated *cry1Ac* coding sequence. The MON 87701- and *E. coli*-produced Cry1Ac proteins also were found to be equivalent based on their functional activities and the lack of glycosylation.

Taken together, these data provide a detailed characterization of the Cry1Ac isolated from MON 87701 harvested seed and establish its equivalence to the *E. coli*-produced Cry1Ac.

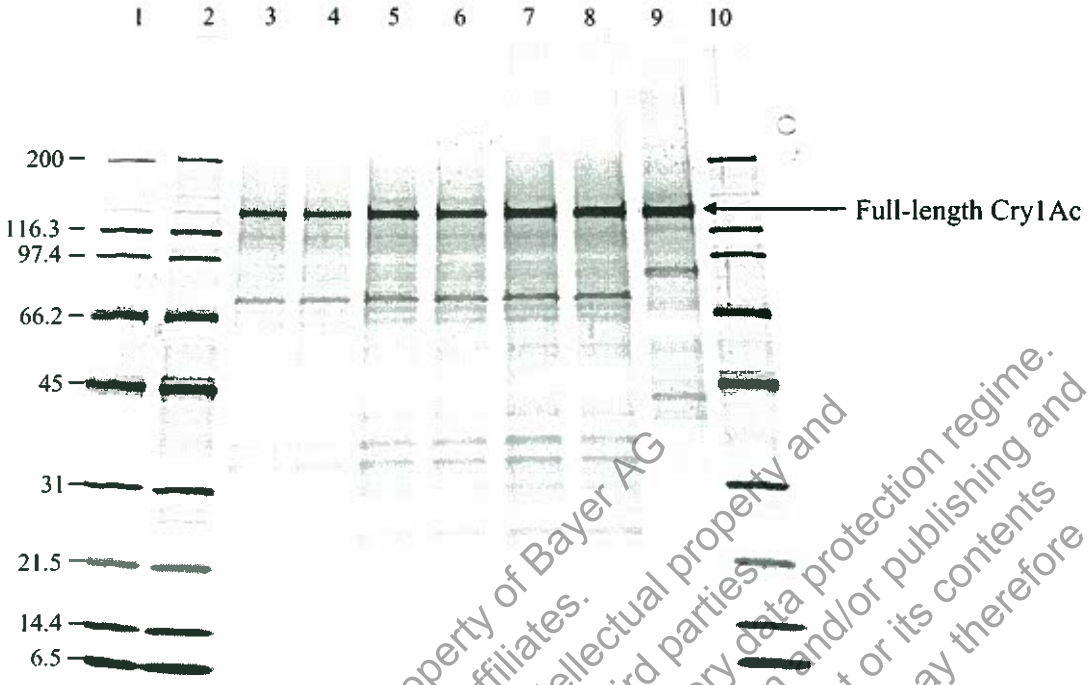

Figure VI-2. SDS-PAGE of MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins

Aliquots of the MON 87701-produced Cry1Ac and the *E. coli*-produced Cry1Ac protein were separated on a Tris-glycine 4-20% polyacrylamide gradient gel and stained with an Owl Silver Staining kit. Approximate molecular weights indicated (in kDa) correspond to the Broad Range Molecular Weight marker (BioRad) loaded in lanes 1, 2, and 10.

Lane	Sample	Amount loaded (ng)
1	Broad Range MW Marker	360
2	Broad Range MW Marker	360
3	MON 87701-produced Cry1Ac	95
4	MON 87701-produced Cry1Ac	95
5	MON 87701-produced Cry1Ac	189
6	MON 87701-produced Cry1Ac	189
7	MON 87701-produced Cry1Ac	284
8	MON 87701-produced Cry1Ac	284
9	*E. coli*-produced Cry1Ac	198
10	Broad Range MW Marker	360

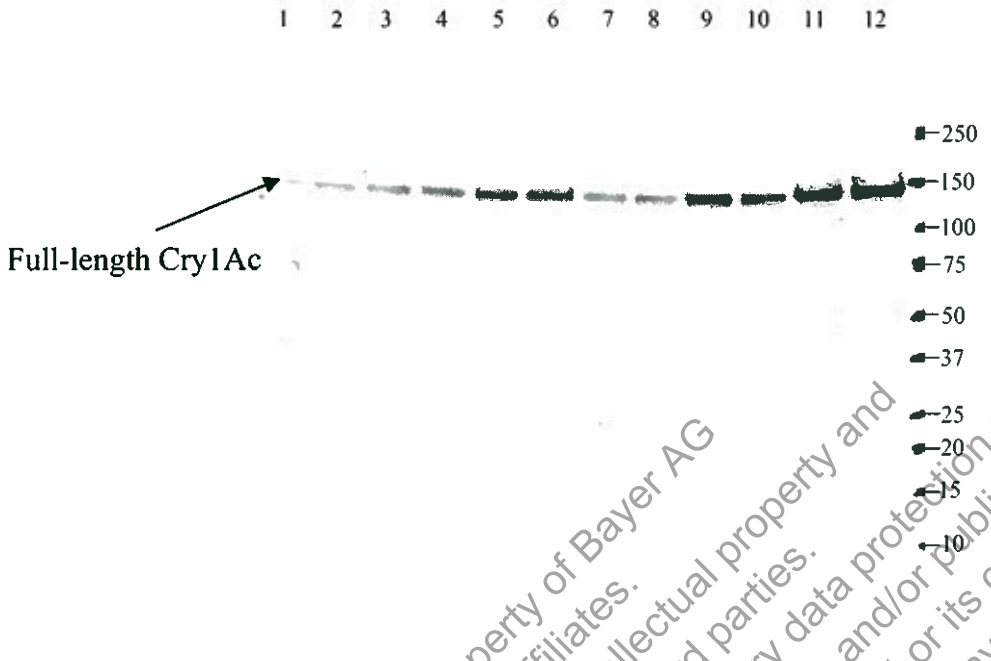

Figure VI-3. Western Blot Analysis of MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins

Aliquots of the purified, MON 87701- and *E. coli*-produced Cry1Ac proteins were separated by SDS-PAGE, and electrotransferred to a PVDF membrane. The membrane was probed with goat anti-Cry1Ac serum and developed using an ECL system (GE Healthcare). Approximate molecular weights (kDa) are shown on the right and correspond to the tick marks indicating the position of molecular weight markers. Amount loaded indicates amount of full-length protein.

Lane	Sample	Amount Loaded (ng)
1	MON 87701-produced Cry1Ac	10
2	MON 87701-produced Cry1Ac	10
3	MON 87701-produced Cry1Ac	20
4	MON 87701-produced Cry1Ac	20
5	MON 87701-produced Cry1Ac	30
6	MON 87701-produced Cry1Ac	30
7	*E. coli*-produced Cry1Ac	10
8	*E. coli*-produced Cry1Ac	10
9	*E. coli*-produced Cry1Ac	20
10	*E. coli*-produced Cry1Ac	20
11	*E. coli*-produced Cry1Ac	30
12	*E. coli*-produced Cry1Ac	30

Amino acid[1] residue # from the N-terminus →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Predicted Cry1Ac Sequence[2] →	C	M	Q	A	M	D	N	N	P	N	I	N	E	C	I
Observed Sequence→	X	M	Q	A	M	D	N	(N)	P	(N)	X	X	X	X	X

[1] The single letter International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB) amino acid code is: A, Alanine; C, Cysteine; D, Aspartic acid; E, Glutamic acid; I, Isoleucine; M, Methionine; N, Asparagine; P, Proline; and Q, Glutamine. X indicates an undesignated call in that cycle of the analysis. Parentheses indicate a tenuous designation.

[2] The predicted Cry1Ac sequence was deduced from the coding region of the full length *cry1Ac* gene present in MON 87701. The observed sequence was obtained by N-terminal sequencing of the MON 87701-produced Cry1Ac.

Figure VI-4. N-terminal Amino Acid Sequence Analysis of the Cry1Ac Protein Purified from Harvested Seed of MON 87701

1	CMQAMDNNPN	INECIPYNCL	SNPEVEVLGG	ERIETGYTPI	DISLSLTQFL
51	LSEFVPGAGF	VLGLVDIIWG	IFGPSQWDAF	LVQIEQLINQ	RIEEFARNQA
101	ISRLEGLSNL	YQIYAESFRE	WEADPTNPAL	REEMRIQFND	MNSALTTAIP
151	LFAVQNYQVP	LLSVYVQAAN	LHLSVLRDVS	VFGQRWGFDA	ATINSRYNDL
201	TRLIGNYTDH	AVRWYNTGLE	RVWGPDSRDW	IRYNQFRREL	TLTVLDIVSL
251	FPNYDSRTYP	IRTVSQLTRE	IYTNPVLENF	DGSFRGSAQG	IEGSIRSPHL
301	MDILNSITIY	TDAHRGEYYW	SGHQIMASPV	GFSGPEFTFP	LYGTMGNAAP
351	QQRIVAQLGQ	GVYRTLSSTL	YRRPFNIGIN	NQQLSVLDGT	EFAYGTSSNL
401	PSAVYRKSGT	VDSLDEIPPQ	NNNVPPRQGF	SHRLSHVSMF	RSGFSNSSVS
451	IIRAPMFSWI	HRSAEFNNII	ASDSITQIPA	VKGNFLFNGS	VISGPGFTGG
501	DLVRLNSSGN	NIQNRGYIEV	PIHFPSTSTR	YRVRVRYASV	TPIHLNVNWG
551	NSSIFSNTVP	ATATSLDNLQ	SSDFGYFESA	NAFTSSLGNI	VGVRNFSGTA
601	GVIIDRFEFI	PVTATLEAEY	NLERAQKAVN	ALFTSTNQLG	LKTNVTDYHI
651	DQVSNLVTYL	SDEFCLDEKR	ELSEKVKHAK	RLSDERNLLQ	DSNFKDINRQ
701	PERGWGGSTG	ITIQGGDDVF	KENYVTLSGT	FDECYPTYLY	QKIDESKLKA
751	FTRYQLRGYI	EDSQDLEIYS	IRYNAKHETV	NVPGTGSLWP	LSAQSPIGKC
801	GEPNRCAPHL	EWNPDLDCSC	RDGEKCAHHS	HHFSLDIDVG	CTDLNEDLGV
851	WVIFKIKTQD	GHARLGNLEF	LEEKPLVGEA	LARVKRAEKK	WRDKREKLEW
901	ETNIVYKEAK	ESVDALFVNS	QYDQLQADTN	IAMIHAADKR	VHSIREAYLP
951	ELSVIPGVNA	AIFEELEGRI	FTAFSLYDAR	NVIKNGDFNN	GLSCWNVKGH
1001	VDVEEQNNQR	SVLVVPEWEA	EVSQEVRVCP	GRGYILRVTA	YKEGYGEGCV
1051	TIHEIENNTD	ELKFSNCVEE	EIYPNNTVTC	NDYTVNQEEY	GGAYTSRNRG
1101	YNEAPSVPAD	YASVYEEKSY	TDGRRENPCE	FNRGYRDYTP	LPVGYVTKEL
1151	EYFPETDKVW	IEIGETEGTF	IVDSVELLLM	EE	

Figure VI-5. MALDI-TOF MS Coverage Map of the Cry1Ac Protein Isolated from MON 87701

The amino acid sequence of the plant-produced Cry1Ac protein was deduced from the coding region of the full-length *cry1Ac* gene present in MON 87701, and from N-terminal sequencing of MON 87701-produced Cry1Ac. Boxed regions indicate amino acids that were identified in tryptic peptides using MALDI-TOF MS. In total, ~67% (787 of 1182 total amino acids) of the expected protein sequence was identified.

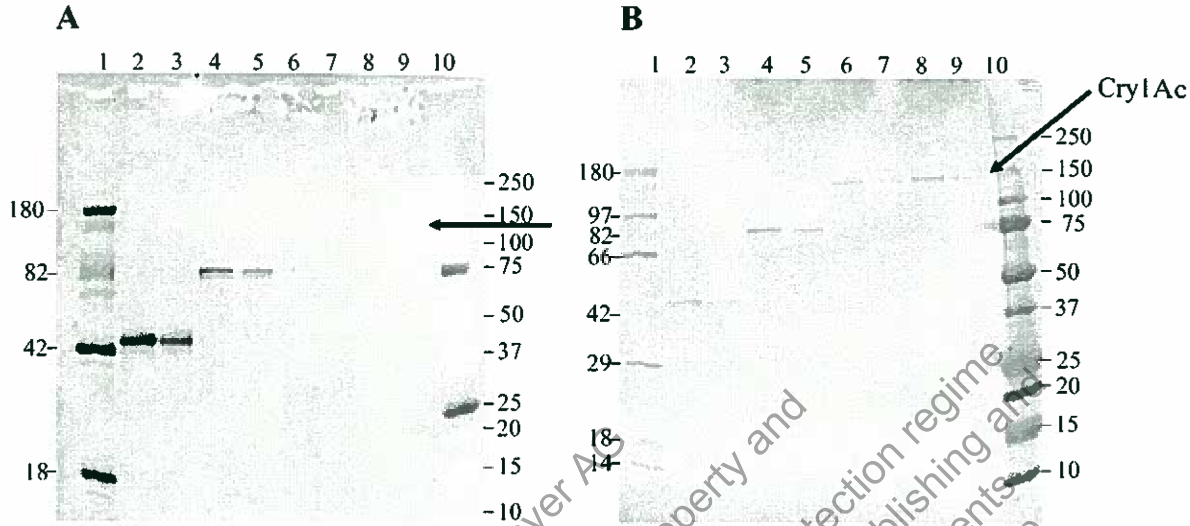

Figure VI-6. Glycosylation Analysis of the MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins

Aliquots of horseradish peroxidase (positive control), transferrin (positive control), the MON 87701-produced Cry1Ac protein, and *E. coli*-produced Cry1Ac protein (negative control), were separated by SDS-PAGE and electrotransferred to a PVDF membrane. For Cry1Ac samples, amount loaded indicates full-length protein amount. Approximate molecular weights indicated (in kDa) correspond to the Precision Plus pre-stained dual color molecular weight marker (BioRad) loaded in Lane 10 and the CandyCane glycosylated marker (Molecular Probes) loaded in Lane 1. (Panel A) Glycosylation Analysis: Where present, periodate-oxidized protein-bound carbohydrate moieties reacted with Pro-Q Emerald 488 glycoprotein stain and emitted a fluorescent signal at 488 nm. The arrow indicates the approximate expected position of Cry1Ac protein on the blot. (Panel B) Total Protein Staining: Following glycosylation analysis, the blot was stained for total protein using Coomassie Brilliant Blue R.

Lane	Sample	Amount Loaded (ng)
1	Candy Cane MW Marker	-
2	Horseradish Peroxidase	100
3	Horseradish Peroxidase	50
4	Transferrin	100
5	Transferrin	50
6	MON 87701-produced Cry1Ac	100
7	MON 87701-produced Cry1Ac	50
8	*E. coli*-produced Cry1Ac	100
9	*E. coli*-produced Cry1Ac	50
10	Precision Plus Dual Color MW marker	-

Section 2. Expression Levels of the Cry1Ac Protein in MON 87701

The levels of the Cry1Ac in various tissues of MON 87701 that are relevant to the risk assessment were assessed by a validated ELISA.[2] The materials and methods for the ELISA analysis, as well as a description of the tissue types, are provided in Appendix C. Tissue samples for analysis were collected from five field trials conducted in the U.S. during 2007. The trial locations were in the states of Alabama, Arkansas, Georgia, Illinois, and North Carolina, which represent relevant soybean-growing regions of the U.S. and provide a range of environmental conditions that would be typical of those encountered in the production of soybean. At each site, three replicated plots of MON 87701 and a conventional soybean control (A5547) were planted using a randomized complete block field design. Over-season leaf (OSL), forage, root, and harvested seed were collected from each replicated plot at all field sites. A description of the tissues collected is provided below.

Tissue	*Soybean development stage*	*Days after planting (DAP)*
OSL-1	V3-V4	23-34
OSL-2	V6-V8	36-45
OSL-3	V10-V12	43-57
OSL-4	V14-V16	52-70
Forage	R6	85-106
Root	R6	85-106
Mature seed	R8	139-156
Pollen/Anther	R2	63

[1]Harvested at or dried to a moisture content of ~10-15%.

Pollen/anther tissue was collected at the R2 growth stage during the 2007 growing season from a field site in Jackson County, IL that was used to generate bulk quantities of MON 87701 and conventional control material. At this site, single plots were established for MON 87701 as well as the conventional soybean control. Four replicate pollen/anther samples were collected from each plot.

Cry1Ac levels were determined in all eight tissue types described above. The results obtained from ELISA analysis are summarized in Table VI-4 for the various tissue types including the tissues collected throughout the growing season. The Cry1Ac levels were determined in over-season leaf (OSL1-4), forage, root, harvested seed, and pollen. The levels of Cry1Ac in tissue samples from the conventional soybean control were below the Cry1Ac assay limit of quantitation (LOQ) or limit of detection (LOD) for each tissue type.

Results showed that mean Cry1Ac levels across the five sites were highest in leaf (340 µg/g dwt in OSL-4), followed by forage (34 µg/g dwt) and mature, harvested seed (4.7

[2] Due to the limited quantity of material available, pollen/anther was evaluated using a non-validated, but optimized ELISA method..

µg/g dwt). If present in root, Cry1Ac levels are less than the ELISA assay LOD of 0.347 µg/g fwt. In over-season leaf tissues harvested throughout the growing season, mean Cry1Ac levels in MON 87701 across all sites ranged from 220 – 340 µg/g dwt. In general, the mean levels of the Cry1Ac protein in leaf remained relatively constant across sampling time points, but levels of the protein were within a broader range as the growing season progressed (Table VI-4).

Table VI-4. Summary of Cry1Ac Protein Levels in Tissues Collected from MON 87701 Produced across Five Sites during the U.S. 2007 Growing Season

Tissue Type	Cry1Ac µg/g fwt (SD)[1,3]	Range[4] (µg/g fwt)	Cry1Ac µg/g dwt (SD)[2]	Range (µg/g dwt)	LOQ/LOD (µg/g fwt)
OSL-1	30 (8.5)	12-40	220 (70)	110-350	2.5/0.74
OSL-2	38 (16)	18-80	260 (100)	130-500	2.5/0.74
OSL-3	34 (17)	14-77	240 (110)	94-480	2.5/0.74
OSL-4	53 (36)	15-110	340 (290)	78-960	2.5/0.74
Root	< LOD	< LOD	NA[5]	NA[5]	0.4/0.347
Forage	9.0 (8.8)	2.5-32	34 (36)	8.2-140	2.0/0.55
Harvested seed	4.2 (0.73)	3.1-5.0	4.7 (0.79)	3.4-5.7	1.0/0.47
Pollen/ anther[6]	2.3 (0.58)	1.8-3.1	NA[7]	NA[7]	ND[8]

1. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
2. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the fwt value by the dry weight conversion factors obtained from moisture analysis data.
3. The mean and standard deviation were calculated across sites (n=15, except OSL-1 where n=13 and pollen/anther where n=4).
4. Minimum and maximum values were determined for each tissue type across sites.
5. Protein levels that were <LOD on a fwt basis were not converted to dwt values.
6. Due to limited quantity, pollen/anther material was evaluated using a non-validated, but optimized ELISA method.
7. Protein level by dry weight was not calculated due to limited quantities of pollen/anther tissue.
8. Due to limited quantities of pollen/anther tissue the LOD and LOQ were not determined.

Section 3. Assessment of Potential Allergenicity of the Cry1Ac Protein

According to guidelines adopted by the Codex Alimentarius Commission (Codex, 2003) for the evaluation of the potential allergenicity of novel proteins, the allergenic potential of a novel protein is assessed by comparing the biochemical characteristics of the novel protein to characteristics of known allergens. A protein is not likely to be associated with allergenicity if: 1) the protein is from a non-allergenic source; 2) the protein represents only a very small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence, and 4) the protein is rapidly digested in mammalian gastrointestinal systems.

The Cry1Ac protein has been assessed for its potential allergenicity according to the recommendations of the Codex Alimentarius Commission (Codex, 2003). The *cry1Ac* coding sequence is derived from *Bacillus thuringiensis*, an organism that is not a source of allergens and has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). Cry1Ac is present at a level of no more than 0.0012% of the total protein in the harvested seed of MON 87701. Bioinformatics analyses demonstrated that Cry1Ac does not share structurally- or immunologically- relevant amino acid sequence similarities with known allergens and, therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes. Digestive fate experiments conducted with the full-length Cry1Ac show that it is rapidly digested in SGF (simulated gastric fluid), although a small, transiently stable fragment is formed. The ~4 kDa Cry1Ac fragment that is transiently stable in SGF was degraded within 30 sec after exposure to simulated intestinal fluid (SIF). Finally, rapid digestion of the full-length Cry1Ac in SGF together with complete degradation of the small, transiently stable fragment in SIF indicates that it is highly unlikely that Cry1Ac or its fragment will reach absorptive cells of the intestinal mucosa. Taken together, these data support the conclusion that the Cry1Ac protein produced in MON 87701 does not pose a significant allergenic risk.

3.1. Source of Cry1Ac

The gene encoding Cry1Ac is derived from Bt var. *kurstaki*, a soil microorganism that is both ubiquitous and abundant in the environment (de Boer and Diderichsen, 1991). Sprays of sporulated Bt have a long history of safe use for pest control in agriculture (Cannon, 1993; EPA, 1988; WHO, 1999). Microbial pesticides containing Bt Cry proteins have been used for more than 50 years and subjected to extensive toxicity testing showing no adverse effects to human health (Betz et al. 2000; EPA 2000; James 2003). During the last decade, a variety of crops containing Cry1 proteins from Bt have been commercialized, thus rendering these plants resistant to several insect pests (De Maagd et al. 1999). For example, corn products that produce Cry1Ab [YieldGard Corn Borer, Bt11] and Cry1F (TC1507) proteins, and cotton products that produce the Cry1Ac protein [Bollgard and Bollard II] are currently registered and sold on the market (Mendelsohn et al., 2003). Moreover, DBT418 corn that produces the tryptic core of Cry1Ac was previously deregulated by USDA (1997). In 1997, the U.S. EPA established an exemption from the requirement of a tolerance for the PIP *Bacillus thuringiensis* Cry1Ac protein and the genetic material necessary for its production in or on all raw

agricultural commodities (40 CFR § 174.510), due to its extremely low mammalian toxicity and lack of similarity with known allergens. There are no known reports of allergies to Bt or its expressed proteins.

3.2. The Cry1Ac Protein as a Proportion of Total Protein

The Cry1Ac protein was detected in all plant tissues, except root, at a number of time points during the growing season. Among these tissues, harvested seed is the most relevant to the assessment of food allergenicity. The mean level of Cry1Ac in harvested seed is 4.7 µg/g dwt. The mean % dry weight of total protein in harvested seed from MON 87701 is 39.27% (or 392,700 µg/g). The percent of Cry1Ac in MON 87701 harvested seed is calculated as follows:

$$(4.7\ \mu g/g \div 392{,}700\ \mu g/g) \times 100\% \approx 0.0012\% \text{ of total soybean protein}$$

Therefore, the Cry1Ac protein represents a very small portion of the total protein in harvested seed of MON 87701.

3.3. Bioinformatic Analysis of Sequence Similarity of the Cry1Ac Protein Produced in MON 87701 to Allergens

In 2003, the Codex Alimentarius Commission published guidelines for the evaluation of the potential allergenicity of novel proteins based on shared amino acid sequence identity (Codex, 2003). The guideline is based on the comparison of amino acid sequences between introduced proteins and allergens, where potential allergenic cross-reactivity may exist if the introduced protein is found to have at least 35% amino acid identity with an allergen over any segment of at least 80 amino acids. The Codex guideline also recommended that a sliding window search with a scientifically justified peptide size could be used to identify immunologically relevant peptides in otherwise unrelated proteins.

The potential for allergenic cross-reactivity between the Cry1Ac produced in MON 87701 and known allergens, gliadins and glutenins was assessed using two search algorithms: 1) a FASTA sequence alignment program was used to determine if any allergens shared at least 35% amino acid identity over at least 80 amino acids with Cry1Ac, and 2) a sliding window search was used to identify any eight-amino acid matches to known allergens (Codex 2003; Thomas et al. 2005). Both bioinformatic search algorithms were used in conjunction with the Food Allergy Research and Resource Program Database allergen database (FARRP)[3] to evaluate if Cry1Ac shared any sequence similarities with known allergens. The AD8 database (release date, January 11, 2008) was assembled from sequences obtained from the FARRP allergen database. The protein sequences in the FARRP allergen database were assembled and evaluated for evidence of allergenicity by an international panel of allergy experts. For in-house searches, redundant sequences present in the FARRP database were removed and obsolete GI sequence numbers were replaced with an up-to-date GI numbers. Due to the removal of obsolete GI numbers and sequence duplicates derived from the same species,

[3] FARRP. 2009. www.allergenonline.com. University of Nebraska.

a total of 1,250 GI sequence numbers were found to be valid and used to assemble a searchable in-house database AD8.

Based on the results of the FASTA comparisons, known allergens were ranked according to their degree of similarity to Cry1Ac. Alignments for full-length search sequences may be considered relevant for similarity to known allergens if the identity is equal to or greater than 35% and equal to or greater than 80 amino acids in aligned sequence length (Codex, 2003). All alignments were inspected visually to determine if an alignment represented biologically relevant sequence similarity. None of the proteins in the AD8 database met or exceeded the threshold of 35% identity over 80 amino acids when compared to the Cry1Ac amino acid sequence. Although none of the obtained alignments satisfied minimum Codex standards, the quality of each alignment was also thoroughly evaluated for their percent identity and *E*-scores (expectation score) as produced from the FASTA bioinformatic program (Pearson, 2000; Pearson and Lipman, 1988). The analysis of the shared percent identity, length of the alignment, as well as the E-score is intended to add additional information to the search for proteins that may have potentially significant homology. The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences require an *E*-score of 1×10^{-5} or smaller to be considered to have sufficient sequence similarity to infer homology. There were no alignments that had an *E*-score below 1, therefore, no alignments between the Cry1Ac sequence and sequences in the AD8 database were observed that were considered relevant from an allergenic assessment perspective.

A sliding eight-amino acid window search was performed to identify whether or not a linearly contiguous match (exact identity matches) of eight amino acids exists between the Cry1Ac amino acid sequence and any amino acid sequences contained within the AD8 allergen database. Results indicate that no alignments of eight contiguous amino acid identities were detected when the Cry1Ac amino acid sequence was compared to known allergen sequences in the AD8 database. Together, these data demonstrate that the Cry1Ac protein does not share any relevant amino acid sequence similarities with known allergens, gliadins, or glutenins.

3.4. Insert Junction Open Reading Frame Analysis

While there was no indication of additional polypeptides produced from the MON 87701 insert other than the Cry1Ac protein, analyses of putative polypeptides encoded by DNA spanning the 5′ and 3′ junctions of the MON 87701 inserted DNA were performed using a bioinformatic comparison strategy. The purpose of the assessment is to evaluate the potential for novel open reading frames (ORFs) that may have concerns for similarity to known allergens and toxins. DNA sequence spanning the 5′ and 3′ junctions of the MON 87701 insertion site was analyzed for translational stop codons (TGA, TAG, TAA), and all ORFs originating or terminating within the MON 87701 insertion site were translated using the standard genetic code from stop codon to stop codon. Five sequences of eight amino acids or greater in length spanning the 5′ junction, and four sequences of eight amino acids or greater in length spanning the 3′ junction were considered as putative polypeptides and used as search sequences for FASTA comparisons against the

AD_2009, TOX_2009 and PRT_2009 databases. In addition, the nine sequences were searched for eight amino sequences that match proteins in the AD_2009 database, using a sliding eight-amino acid window search.

Results of the FASTA sequence alignments demonstrated a lack of structurally relevant similarity between any known allergens, toxins, or bioactive proteins and the nine putative polypeptides. Results from the eight amino acid search demonstrated the lack of immunologically relevant matches between any of the putative polypeptides and the AD_2009 database. Bioinformatic analyses performed using the nine query sequences support the conclusion that even in the highly unlikely event that any of the putative junction polypeptides were translated, they would not share a sufficient degree of sequence similarity with known allergens or toxins. Therefore, there is no evidence for concern regarding health implications of the cross-junction putative polypeptides in MON 87701.

3.5. Assessment of Open Reading Frames Contained in the *cry1Ac* Coding Sequence

Although DNA replication, DNA transcription and mRNA translation are of extremely high fidelity, mutation may in certain rare circumstances lead to the potential translation of mRNA on reading frames other than those defined by the intended translation start codon. In such instances, a novel protein may be produced. Due to the spontaneous nature of mutations, it is not possible to determine when or where in a coding sequence such an event may occur. In order to assess potential risks, bioinformatic analyses were performed to assess the potential of toxicity, allergenicity or biological activity of the putative peptides encoded by translation of reading frames 2 through 6 of the *cry1Ac* coding sequences. The methodology involves translation of two reading frames from the sense strand (frames 2 and 3) and three frames from the reverse complement (anti-sense) strand (frames 4, 5 and 6). Frame 1 corresponds to the *cry1Ac* coding sequence whose bioinformatics assessment was previously described in Section 3.3, Part IV. Frames 2 and 3 are alternate reading frames that correspond to peptides derived from the chloroplast targeting peptide-*cry1Ac* coding sequence, beginning with nucleotide 2 or 3, respectively, through to the final nucleotide. Frames 4, 5 and 6 represent the anti-sense strand of the chloroplast targeting peptide-*cry1Ac* coding sequence that were translated beginning with nucleotide 1, 2 or 3, respectively.

Translated sequences were compared to allergen (AD_2009), toxin (TOX_2009), and public domain (PRT_2009) sequence databases using the FASTA sequence comparison algorithm and significance criteria as described in Section 3.3, Part VI. The allergen, gliadin, and glutenin sequence database (AD_2009) was obtained from FARRP (2009), which was used as provided and contains 1,386 sequences. GenBank protein database, release 169.0 (December 16, 2008), was downloaded from NCBI and formatted for use in these bioinformatic analyses. The resulting database, referred to as the PRT_2009 database, contains 14,717,352 sequences. The toxin database is a subset of sequences derived from the PRT_2009 database that was selected using a keyword search and filtered to remove likely non-toxin proteins. The TOX_2009 database and contains 7,651 sequences.

The results of the search comparisons showed that no relevant alignments were observed against proteins in the AD_2009 database because none of the AD_2009 alignments with frames 2-6 of *cry1Ac* coding sequence met or exceeded the minimum threshold of 35% identity over 80 amino acids (Codex Alimentarius, 2003) or displayed *E*-scores less than 1e-5. Likewise, no FASTA alignments with the TOX_2009 database displayed *E*-scores less than 1e-5. When used as queries for a FASTA search the PRT_2009 database, frames 2 and 5 yielded alignments that displayed *E*-scores less than 1e-5. Inspection of the frame 2 alignments revealed that they were punctuated with numerous stop codons in the query sequence and required numerous gaps to optimize the alignment. As a result, it is unlikely these alignments reflect conserved structure. For those alignments with frame 5 yielding an *E*-score less than 1e-5, all were an identical 46 amino acid overlap displaying greater than 90% identity with patent sequences described as being "Lepidopteran-active *Bacillus thuringiensis* delta-endotoxin polynucleotides." While these alignments likely reflect conserved structure, there is no indication that they reflect the potential for adverse biological activity.

When combined, these data demonstrate the lack of relevant similarities between known allergens, toxins or other biologically active proteins for the five putative peptides derived from the *cry1Ac* coding sequence. As a result, in the event a translation product was derived from possible reading frames 2-6 of *cry1Ac*, these putative polypeptides are not expected to possess functional cross-reactivity with known allergenic proteins or be toxic or display adverse biological activity.

3.6. Stability of the Cry1Ac Protein in Simulated Gastric Fluids

Digestibility of the full length *E. coli*-produced Cry1Ac protein in SGF was assessed by SDS-PAGE and western blot methods. The extent of Cry1Ac digestion was evaluated by visual analysis of stained polyacrylamide gels (Figure VI-7) or by visual analysis of developed X-ray film (Figure VI-8). The LOD of Cry1Ac by Colloidal Brilliant Blue G staining was 0.0025 µg or approximately 0.3% of the protein sample loaded (0.0025 µg divided by 0.8 µg of protein loaded in each lane of the gel; Figure VI-7, panel B). The LOD of Cry1Ac by western blotting was 0.5 ng or approximately 5% of the total protein loaded (0.5 ng divided by 10 ng of the protein loaded in each lane of the gel; Figure VI-8, panel B).

Visual examination of the Colloidal Brilliant Blue G stained gel (Figure VI-7, panel A) showed that the full-length Cry1Ac was digested below the LOD within 30 s of digestion in SGF (Figure VI-7, panel A, Lane 5). Therefore, at least 99.7% (100% - 0.3% = 99.7%) of the full-length Cry1Ac was digested within 30 s of incubation in SGF. No change in the full-length Cry1Ac band intensity was observed in the absence of pepsin in the experimental controls SGF P0 and SGF P7 (Figure VI-7, panel A, lanes 3 and 12) indicating that the digestion of the Cry1Ac was due to the proteolytic activity of pepsin present in SGF and not due to instability of the protein while incubated at pH ~1.2 at ~37 °C for 60 min. A protein fragment of ~4 kDa was observed throughout the digestion (Figure VI-7, panel A, lanes 5-11), but appears to degrade to a smaller ~3.5 kDa fragment visible at the 30 min and 60 min time points on the gel.

Western blot analysis demonstrated that the full-length Cry1Ac was digested below the LOD within 30 s of incubation in SGF (Figure VI-8, panel A, Lane 5). Based on the western blot LOD for Cry1Ac in SGF and the observation that no full-length protein or immunoreactive bands were observed on the western blot at the 30 s digestion time point, it was concluded that at least 95% (100% - 5% = 95%) of the full-length Cry1Ac protein was digested within 30 s of incubation in SGF.

Because the transiently stable Cry1Ac fragment (~4 kDa) observed in the SDS-PAGE analysis was not cross-reactive with Cry1Ac-specific antibodies, the identity of this fragment was established by N-terminal sequencing. Two sequences were identified that match internal Cry1Ac sequences starting at amino acid positions 415 and 882. This indicates that the observed transiently stable fragment was derived from Cry1Ac. As shown in Figure VI-9, the observed sequences fall in a region that is highly conserved between the Cry1Ac and Cry1Ab proteins. The long history of safe use of the Bt Cry1 proteins strongly suggests that the observed transiently stable fragments do not pose any allergenicity concerns.

3.7. Stability of the Cry1Ac protein in SGF Followed by SIF

To better understand the digestive fate of the full-length Cry1Ac protein and the transiently stable fragment produced in SGF, Cry1Ac was exposed to digestion with pancreatin in simulated intestinal fluid (SIF) following the digestion in SGF. After digestion of Cry1Ac in SGF for 2 min, the reaction was quenched and exposed to further digestion in SIF. The digestibility of Cry1Ac in SIF following SGF digestion was evaluated by visual analysis of stained polyacrylamide gels (Figure VI-10). The gel was loaded with ~0.8 μg total protein (based on concentration of the protein prior to the digestion in SGF) for each of the SIF digestion time points. As expected, the full-length Cry1Ac protein was undetectable at the 2 min digestion time point, while the ~ 4 kDa fragment was present (Figure VI-10, lane 3). After exposure to SIF, the ~ 4 kDa fragment was not visible at the SEQ T1 (1 min) digestion time point (Figure VI-10, lane 7). These data clearly indicate that the ~ 4 kDa fragment degrades rapidly (< 1 min) upon exposure to SIF.

3.8. Assessment of Human IgE Binding to MON 87701, Control, and Conventional Soybean Extracts

A study was conducted to quantitatively evaluate the binding levels of IgE antibody in sera from clinically documented, soybean-allergic patients to protein extracts prepared from MON 87701 soybean seed, a conventional soybean control (variety A5547), and 17 commercial soybean reference varieties. The reference soybean varieties were used to establish the range in soybean-specific IgE binding. The reference varieties are commercially available and included high protein, high oil, and food-grade (tofu) soybean that are already on the market and are being used for human consumption.

Sera from 13 clinically documented, soybean-allergic subjects and five non-allergic subjects were used to assess IgE binding to each soybean extract. Only soybean-allergic subjects with a documented case history of soybean allergy and a positive Double-Blind

Placebo Controlled Food Challenge (DBPCFC) were included as soybean positive subjects in this study.

Aqueous extracts were prepared from the ground soybean seeds of MON 87701, the conventional soybean control, and reference varieties. These extracts were then analyzed for soybean-specific IgE antibody binding by a validated ELISA. Each soybean extract was tested in triplicate at a concentration of 10 µg/ml of total soybean protein. Soybean-specific IgE binding was quantified by interpolation against a soybean-specific IgE standard curve and was expressed as ng of IgE/ml of serum. The standard curve was created by loading serial dilutions of human serum PEI 163[4] containing a known amount of soybean-specific IgE, into wells coated with an assay-specific soybean extract from a commercial reference variety, Hensel. The bound soybean-specific IgE was detected using biotin-conjugated antihuman IgE polyclonal antibody and a horseradish peroxidase enzyme-conjugated streptavidin.

The IgE binding values obtained for the 17 reference soybean extracts were used to calculate a 99% tolerance interval for each subject's serum. The 99% tolerance interval represents the range of IgE binding for each subject's serum to the reference soybean extracts. The tolerance interval describes the range that includes 99% of the IgE binding values and that has a statistically predicted 95% confidence level. The IgE binding values obtained for extracts prepared from MON 87701 and the conventional soybean control were compared to the tolerance interval derived for each serum. All of the IgE binding values for MON 87701 and the control, shown in Figure VI-11, are within the reference soybean tolerance limits for each subject's serum. None of the soybean varieties showed IgE binding to sera from non-allergic subjects.

The results of this assessment demonstrate that soybean-specific IgE binding to endogenous allergens in MON 87701 and the conventional soybean control are comparable with the IgE binding to commercially available soybean varieties currently on the market.

3.9. Conclusions on Potential Allergenicity of Cry1Ac

The Cry1Ac protein has been assessed for its potential allergenicity according to the recommendations of the Codex Alimentarius Commission (Codex, 2003). The *cry1Ac* coding sequence is derived from *Bacillus thuringiensis,* an organism that is not a source of allergens and has been used commercially in the U.S. since 1958 to produce microbially derived products with insecticidal activity (EPA, 1988). Cry1Ac represents no more than 0.0012% of the total protein in the harvested seed of MON 87701. Bioinformatics analyses demonstrated that Cry1Ac does not share structurally or immunologically relevant amino acid sequence similarities with known allergens and therefore, is highly unlikely to contain immunologically cross-reactive allergenic epitopes. Digestive fate experiments conducted with the full-length Cry1Ac demonstrated that it is rapidly digested in SGF, a characteristic shared among many

[4] PEI is the abbreviation of the Paul-Ehrlich-Institute. PEI 163 is a single soybean allergic serum that contains a known amount of soybean-specific IgE adequate for constructing the standard curve.

proteins with a history of safe consumption. A ~4 kDa Cry1Ac fragment that is transiently stable in SGF was quickly degraded during a short exposure to SIF. Rapid digestion of the full-length Cry1Ac in SGF, together with rapid degradation of the small transiently stable fragment in SIF, indicates that it is highly unlikely that Cry1Ac or its fragment will reach absorptive cells of the intestinal mucosa. Taken together, these data lead to the conclusion that the Cry1Ac protein produced in MON 87701 does not pose a significant allergenic risk.

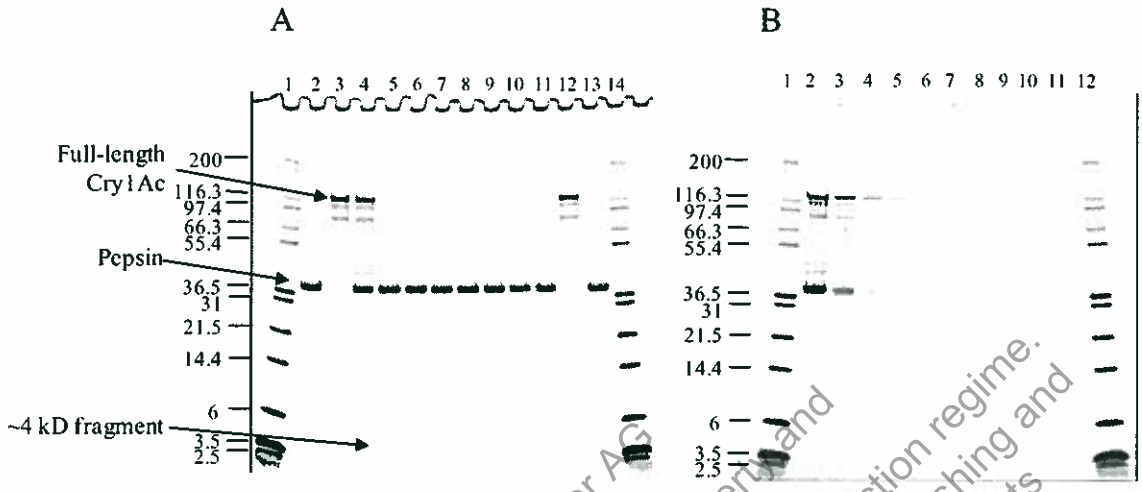

Figure VI-7. Colloidal Brilliant Blue G stained SDS-PAGE Gels of Cry1Ac Protein Digestion in SGF

Panel A corresponds to Cry1Ac protein digestion in SGF. Based on predigestion protein concentrations, 0.8 µg (total Cry1Ac protein) was loaded in lanes containing Cry1Ac protein. The incubation times are indicated. Panel B corresponds to the various amounts of SGF T0 loaded to estimate the LOD of the Cry1Ac protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel.

Lane assignment for Panel A:

Lane	Sample	Incubation Time (min)
1	Molecular weight marker	—
2	SGF N0[1]	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Molecular weight marker	—

Lane assignment for Panel B:

Lane	Sample	Amount (µg)
1	Molecular weight marker	—
2	SGF T0	0.8
3	SGF T0	0.4
4	SGF T0	0.1
5	SGF T0	0.05
6	SGF T0	0.02
7	SGF T0	0.01
8	SGF T0	0.005
9	SGF T0	0.0025
10	SGF T0	0.001
11	SGF T0	0.0005
12	Molecular weight marker	—

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0, N7- negative controls (no Cry1Ac protein); P0, P7- protein control (no pepsin); T0-T7- incubation time point in SGF.

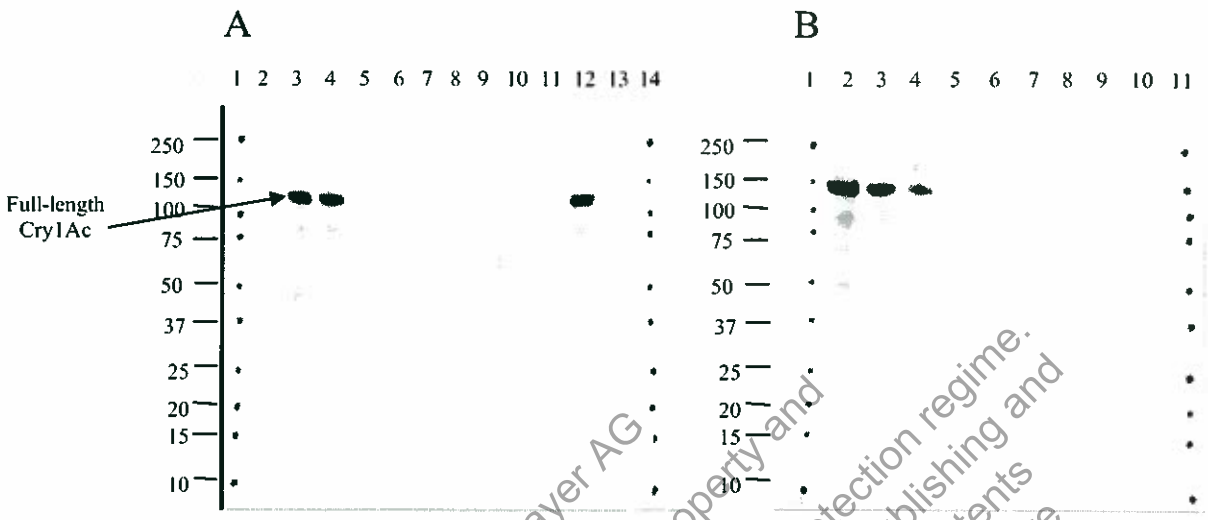

Figure VI-8. Western Blot Analysis of the Digestion of Cry1Ac Protein in SGF

Panel A corresponds to Cry1Ac protein digestion in SGF. Based on pre-digestion protein concentrations, 10 ng (total protein) was loaded in the lanes containing Cry1Ac protein. **Panel B** corresponds to the various amounts of SGF T0 loaded to estimate the LOD of the Cry1Ac protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, Cry1Ac protein migrated to approximately 131.7 kDa. A 5 min exposure is shown. Blank or empty lanes are cropped and lanes renumbered relative to the raw data.

Lane assignment for Panel A:

Lane	Sample	Incubation Time (min)
1	Molecular weight marker	—
2	SGF N0[1]	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Molecular weight marker	—

Lane assignment for Panel B:

Lane	Sample	Amount (ng)
1	Molecular weight marker	—
2	SGF T0	10
3	SGF T0	5
4	SGF T0	2.5
5	SGF T0	1
6	SGF T0	0.5
7	SGF T0	0.2
8	SGF T0	0.1
9	SGF T0	0.05
10	SGF T0	0.025
11	Molecular weight marker	—

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0, N7- negative controls (no test protein), P0, P7- protein control (no pepsin), T0-T7- incubation time point in SGF.

Fragment 1, 415-424

```
cry1Ab          VDSLDEIPPQNNNVPPRQGFSHRLSH
cry1Ac          VDSLDEIPPQNNNVPPRQGFSHRLSH
observed seq        DEIPPQNNNV
```

Fragment 2, 882-894

```
cry1Ab          GEALARVKRAEKKWRDKREKLEWETNIVY
cry1Ac          GEALARVKRAEKKWRDKREKLEWETNIVY
observed seq        ARVKRAEKKWRDK
```

Figure VI-9. Cry1Ac and Cry1Ab Protein Sequences Identified in the ~4 kDa Stable Fragment from SGF

Alignment is shown between the N-terminal sequences from the ~4 kDa stable fragment observed in SGF analysis and the corresponding sequence regions of Cry1Ab (GenBank gene identification number 61221646) and Cry1Ac (GenBank gene identification number 117547).

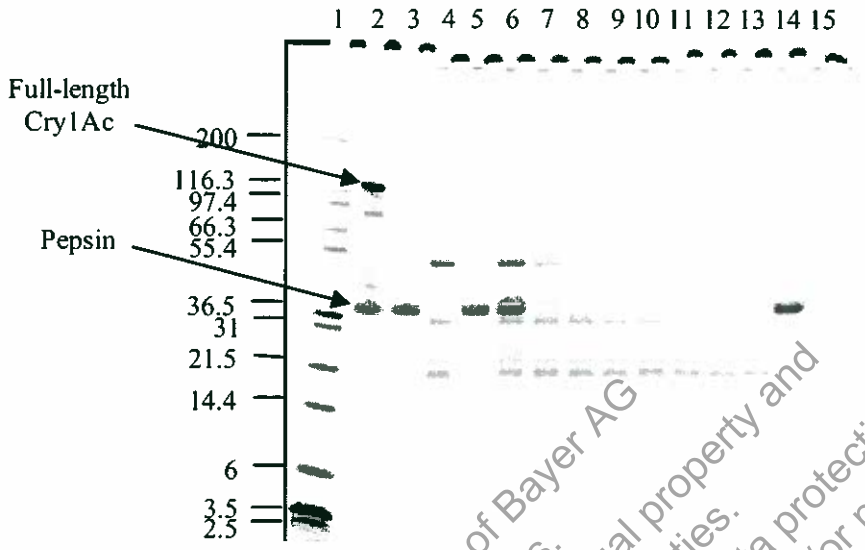

Figure VI-10. Colloidal Brilliant Blue G stained SDS-gel of the Cry1Ac Protein in SGF Followed by SIF

After digestion of the Cry1Ac protein in SGF for 2 min, the reaction was quenched and exposed to further digestion in SIF for the incubation times indicated.
Based on protein concentrations before digestion in SGF, 0.8 µg of total protein was loaded per lane containing Cry1Ac protein.

Lane	Sample	Incubation Time
1	Molecular weight marker	—
2	SEQ 0min	0
3	SEQ 2min	2 min[2]
4	SEQ N0[1]	0
5	SEQ P0	0
6	SEQ T0	0
7	SEQ T1	0.5 min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ P7	2 h
15	SEQ N7	2 h

[1]A numerical code using the numbers 0 through 7 was used to distinguish incubation time points. N0, N7- negative controls (no test protein); P0, P7- protein control (no SIF); T0-T7- incubation time point in sequential digestion assay (SEQ).

[2]Indicates incubation time in SGF.

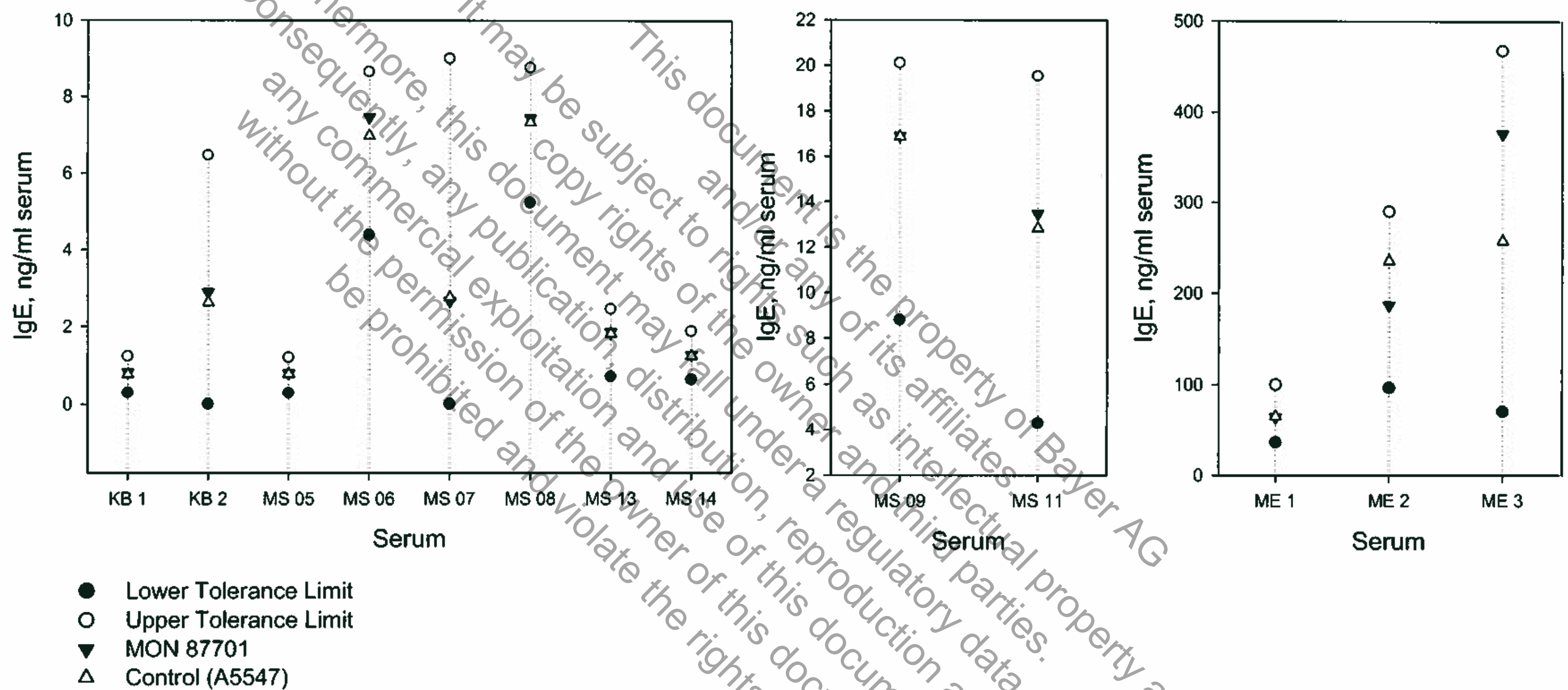

Figure VI-11. Serum IgE Binding Values for MON 87701, Conventional Control (A5547), and the Tolerance Limits for 17 Conventional References.

The lower and upper tolerance limits for 99% tolerance intervals with 95% confidence for each serum are the result of a tolerance interval analysis for 17 commercial soybean varieties. Lower limits of the tolerance intervals that were calculated as less than zero were reported as zero in the analysis. Data are presented in three graphs due to the difference in IgE concentration range between sera.

Section 4. Assessment of the Potential for Toxicity of the Cry1Ac Protein

4.1. Approach to the Assessment of Toxicity

The assessment of the potential toxicity of a newly expressed protein is based on the premise that a protein is not likely to have a toxic effect if:

1. The protein has a demonstrated history of safe use.
2. The protein has no structural similarity to known toxins or other biologically-active proteins that could cause adverse effects in humans or animals.
3. The protein does not exert any acute toxic effects in mammals.

In the following sections, the history of safe use and potential mammalian toxicity of the Cry1Ac protein produced in MON 87701 is discussed. General information regarding the methods used to evaluate the structural similarity to known toxins and acute toxicity is also provided below. The following sections summarize the results of a mammalian toxicity study conducted with Cry1Ac and a comparison of these results to the anticipated levels of human exposure in the U.S. population. Potential health risks were evaluated by calculating a Margin of Exposure (MOE) between the No Observable Adverse Effect Level (NOAEL) from an acute mouse gavage study with Cry1Ac and a conservative estimate of human dietary exposure from MON 87701.

4.2. History of Safe Use of Cry1Ac

4.2.1. Safety of the Donor Organism: *Bacillus thuringiensis*

Bacillus thuringiensis subspecies *kurstaki* is a gram-positive bacterium that is commonly found in soil and has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). Sprays of sporulated Bt have been used for many years in agriculture for pest control. Residual amounts of Bt spores have been detected in several fresh fruits and vegetables, grapes, milk, pasta, and bread (Frederiksen et al., 2006). Despite this exposure, no harm to mammals caused by these bacteria has been identified (Siegel, 2001). In the U.S., an exemption from the requirement of a tolerance for the first microbial Bt product was granted in 1960 by the U. S. FDA after an extensive toxicity and infectivity evaluation program. The testing program consisted of acute, subchronic, and chronic studies, which resembled the testing required for conventional chemical pesticides. Registration was granted by the USDA later the same year. In 1971, the EPA assumed responsibility for all pesticide tolerance exemptions and product registrations. Since then, a variety of naturally occurring and genetically modified microbial Bt products have been registered and included under this tolerance exemption. Microbial pesticides containing Bt Cry1A proteins have been used for more than 50 years and subjected to extensive toxicity testing showing no adverse effects to human health (Baum et al., 1999; EPA, 1988 and 2001; Betz et al., 2000; James, 2003; McClintock et al., 1995; Mendelsohn et al., 2003). The U.S. EPA has also

evaluated the potential impacts of Cry1Ac and Cry1Ab proteins in pesticidal preparations on nontarget organisms, including mammals, birds, fish, beneficial insects, marine animals, and plants, and concluded that they do not pose a risk to these organisms (OECD, 2007).

4.2.2. History of Safe Use of Cry Proteins Produced in Crop Plants

The EPA has established separate tolerance exemptions for various Cry proteins (e.g., Cry1Ab, Cry1Ac, Cry2Ab2, Cry3Aa, and Cry3Bb1) expressed in biotechnology-derived crops (EPA, 2008). The conclusion "of reasonable certainty of no harm" and the resultant tolerance exemptions for the Cry proteins present in food or feed was based on the lack of adverse effects to mammals in numerous toxicological studies.

The Cry1Ac protein has a history of safe use in bacterial preparations used as biopesticides. In addition, Bollgard and Bollgard II cotton, which also produce the Cry1Ac protein, has been used commercially in the U.S. for over a decade (in the case of Bollgard) for the control of lepidopteran pests. Except for the four additional amino acids at the N-terminus, the MON 87701-produced Cry1Ac has 100% amino acid identity with the Bollgard Cry1Ac protein (Figure VI-1). A related protein, Cry1Ab, which has ~90% amino acid identity to the Cry1Ac produced in Bollgard and MON 87701, is expressed in YieldGard Corn Borer corn. The EPA has approved commercial use of the Cry1Ab and Cry1Ac proteins as expressed in corn and cotton (EPA, 2009). An exemption from the requirement for a tolerance was granted in 1996 for Cry1Ab and the genetic material necessary for its production in all plants (40 CFR § 174.511). A tolerance exemption for the Cry1Ac protein and the genetic material necessary for its production in all plants was granted on April 11, 1997 (40 CFR §174.510).

Following the deregulation of Bollgard cotton and YieldGard Corn Borer corn, both products have been grown commercially in the U.S. for more than ten years, and in several other countries. The safety for food and feed consumption of plants expressing Cry1Ac or Cry1Ab proteins has been evaluated by several regulatory agencies around the world, including FDA, and summarized in numerous scientific publications (Betz et al., 2000; Shelton et al., 2002). Detailed human and animal safety assessments and over a decade of safe consumption of these crops confirm their safety. Collectively, this information confirms the safety of the Cry1Ac protein produced in MON 87701.

4.3. Structural Similarity of Cry1Ac to Known Toxins or Other Proteins

The assessment of the potential for protein toxicity includes bioinformatic analysis of the amino acid sequence of the introduced protein. The goal of the bioinformatic analysis is to ensure that the introduced protein does not share homology to known toxins or anti-nutritional proteins associated with adverse health effects.

Potential structural similarities shared between the Cry1Ac protein and sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (i.e., primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (i.e., secondary and tertiary protein structures). Proteins that

share a high degree of similarity throughout the entire sequence are often homologous. Homologous proteins usually have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions.

FASTA bioinformatic alignment searches using the Cry1Ac amino acid sequence were performed with the TOXIN6 database to identify possible homology with proteins that may be harmful to human and animal health. The TOXIN6 database is a subset of 7,176 sequences derived from the protein database (PROTEIN) consisting of publicly available protein sequences from GenBank (GenBank protein database, release 163.0, December 15, 2007). Initially all header lines and the associated protein sequence in PROTEIN database were screened using all possible combinations of upper and lower case characters spelling the words "toxic" and "toxin." The resulting 9,082 header lines and associated sequences then were filtered to exclude the following terms used in combination with "toxic" or "toxin," resulting in 7,176 sequences; these terms were "synthetic," "anti," "putative," "like," "insect," "Cry," "Thuringiensis", and "toxin-reductase."

An *E-score* acceptance criteria of $<1\times10^{-5}$ for any alignment was used to identify proteins from the TOXIN6 database with potential for significant shared structural similarity and function with Cry1Ac. The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences require an *E*-score of 1×10^{-5} or smaller to be considered to have sufficient sequence similarity to infer homology. The FASTA search produced 15 alignments, with the top alignment displaying 98.981% identity in a 1,178 amino acid overlap with a delta-endotoxin. Delta-endotoxin is a term that is synonymous with Bt or Cry proteins and as such, the top alignment does not indicate a potential for human or mammalian toxicity. The 14 additional alignments that were the product of the FASTA search of the TOXIN6 database were with other Cry proteins.

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the Cry1Ac protein and any known toxic or other biologically active proteins that would be harmful to human or animal health.

4.4. Acute Oral Toxicity Study with the Cry1Ac Protein

Most known protein toxins, including the insecticidal Cry proteins, act through acute mechanisms to exert toxicity (Sjoblad et al., 1992; Pariza and Johnson, 2001; Hammond and Fuchs, 1998). The primary exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, which typically require a short-term (two to four week) feeding study to manifest toxicity (Liener, 1994). The amino acid sequence of Cry1Ac produced in MON 87701 is not similar to any of these anti-nutritional proteins or to any other known mammalian protein toxin. In addition, because Cry proteins act through acute mechanisms to control insect pests and have no activity against nontarget organisms such as mammals, the U.S. EPA has determined that a high dose acute test is sufficient to confirm their absence of toxicity towards mammals (McClintock et al.,

1995). Therefore, an acute oral mouse toxicity study was considered appropriate to confirm the lack of mammalian toxicity of Cry1Ac.

An acute toxicology study was conducted with the full-length Cry1Ac that was produced in an *E. coli* expression system. This protein was shown to be physicochemically and functionally equivalent to the Cry1Ac produced in MON 87701. Cry1Ac was administered by oral gavage to 10 male and 10 female CD-1 mice at a total dose of 1290 mg/kg body wt (administered in two doses about four hours apart). Control mice were administered comparable doses of bovine serum albumin (BSA). Following dosing, all mice were subject to detailed clinical observations once daily (twice on day of dosing) for signs of mortality or toxicity. Food consumption was measured on days 0, 7, and 14. Body weights were measured prior to dosing and on study days 0, 7, and 14. All animals were sacrificed on day 14 and subjected to a gross necropsy. There were no treatment-related effects of Cry1Ac on survival, clinical observations, body weight gain, food consumption or gross pathology. A statistically significant reduction in body weight gain was observed in males but not in females dosed with 1,290 mg/kg Cry1Ac relative to BSA-treated controls; however, this result was considered equivocal because at least one male in the study experienced an interruption in water supply. In order to further investigate this possible effect on body weight, an additional group of 10 male CD-1 mice (and BSA controls) was dosed with Cry1Ac by oral gavage at a total dose of 1460 mg/kg body wt (two equal doses four hours apart). There was no effect on body weight in males dosed with 1,460 mg/kg Cry1Ac. Therefore, the effect on body weight in males at 1,290 mg/kg Cry1Ac was not reproduced in the repeat study using a higher dose and was not considered treatment-related. The NOAEL for the Cry1Ac protein was 1,460 mg/kg in males and 1,290 mg/kg in females.

4.5. Dietary Safety Assessment of the Cry1Ac Protein

4.5.1. Estimated Human Exposure to the Cry1Ac Protein from MON 87701

Estimates of acute dietary exposure to the Cry1Ac protein from consumption of foods derived from MON 87701 was determined using the Dietary Exposure Evaluation Model-Food Commodity Intake Database (DEEM-FCID version 2.03, Exponent Inc.) and food consumption data from the 1994-1996 and 1998 USDA Continuing Survey of Food Intakes by Individuals (CSFII). DEEM-FCID differentiates soybean consumption into four fractions: seed, flour, milk, and oil. However, since soybean oil contains negligible amounts of protein (Tattrie and Yaguchi, 1973; Martín-Hernández et al., 2008), it would not be a significant source of dietary exposure to the Cry1Ac from MON 87701 and was thus excluded from this assessment. Estimated human exposure to Cry1Ac from MON 87701 in the U.S. was considered using a reasonable worst case scenario of the 95th percentile estimate of acute soybean consumption estimated on an eater-only[5] basis.

Soybean is a blended commodity that is highly processed before being consumed by humans. Thus, most food products derived from MON 87701 and entering the human food supply likely would be blended with other commercial soybean products before

[5] Includes the population of individuals who consume the food item but not the general population.

being processed and consumed. However, estimating the percentage of consumed soybean products that likely would be derived from MON 87701 is difficult. MON 87701 is intended primarily for use as field or commodity soybean and not for vegetable or garden soybean that are generally used to produce tofu, soybean sprouts, soymilk, edamame or similar food items. Vegetable or food grade soybean generally has a different size, flavor, texture and other characteristics than field soybeans, and is more easily cooked. For the purposes of this dietary risk assessment, the conservative assumption was made that 100% of all soybean products (excluding oil) consumed in the U.S. will be derived from MON 87701, at the time of commercialization.

Since soybean is a blended commodity, the mean level of the Cry1Ac in each of the consumed food fractions (seed, flour, and milk) should be used when estimating total intake of these proteins from consumption of MON 87701. However, specific values for each of these fractions are not available. Thus, the concentration of Cry1Ac in each of these fractions was assumed to be equal to the mean concentration in MON 87701 harvested seed grown in the U.S. in 2007 (4.2 μg/g fresh weight). This is a very conservative assumption since it assumes there is no loss of Cry1Ac during storage, processing, and/or cooking. Soybean contains certain factors, such as trypsin inhibitors, which may act as antinutrients if the soybean is not properly heated during preparation (Rackis, 1974). Thus, virtually all protein-containing soybean fractions are heated during processing prior to consumption by humans and most animals. Cry1Ac is not heat stable and is likely to be denatured during toasting and processing. Thus, the amounts of functionally active Cry1Ac present in consumed soybean products will be substantially lower than the levels assumed for this evaluation.

Based on these assumptions, the 95th percentile acute intake (eater-only) for Cry1Ac was estimated to be 0.00044 mg/kg body weight/day for the overall U.S. population. The 95th percentile estimate of acute intake (eater-only) for non-nursing infants, the most highly exposed subpopulation, was 0.01675 mg/kg body weight/day (Table VI-5).

Table VI-5. Acute (95th Percentile, Eater-Only) Dietary Intake and Margins of Exposure for the Cry1Ac Protein from Consumption of MON 87701 Soybean Meal-Derived Food Products in the U.S.[1]

Population	Protein Intake[2] (mg/kg/day)	Margin of Exposure[3]
General Population	0.00044	2.93×10^6
Non-nursing Infants	0.01675	7.71×10^4

[1] Estimated using DEEM-FCID version 2.03, Exponent Inc., utilizing food consumption data from the 1994-1996 and 1998 USDA-CSFII. Includes soybean seed, flour, and milk. Assumes 100% of soybean products (excluding soybean oil) consumed in the U.S. are derived from MON 87701.

[2] Based on average Cry1Ac levels of 4.2 µg/gram fresh weight in whole seed.

[3] Calculated by dividing the NOAEL from the Cry1Ac acute mouse gavage study by estimated dietary intake of Cry1Ac protein from MON 87701. Although the NOAEL was 1,460 mg/kg in males and 1,290 mg/kg in females, for the purposes of these MOE calculations, the most conservative approach was to consider 1,290 mg/kg to be the NOAEL for Cry1Ac.

4.5.2. Dietary Risk Assessment: Margin of Exposure for the Cry1Ac Protein Derived from MON 87701

A common approach used to assess potential health risks from chemicals or other potentially toxic products is to calculate a Margin of Exposure (MOE) between the lowest NOAEL from an appropriate animal toxicity study and an estimate of human exposure. No adverse health effects were observed when male or female mice were administered a total dose of 1,290 mg/kg of Cry1Ac protein. Therefore, based on an apparent absence of hazard associated with exposure to this protein and the low expression levels of this protein in MON 87701 in harvested seed, a dietary risk assessment for this protein would normally not be considered necessary. Nevertheless, a dietary risk assessment for Cry1Ac was still conducted in order to provide further safety assurance. The dietary risk assessment represents a worst-case scenario as the initial intent is not to commercialize MON 87701 in the U.S.

Potential health risks from the acute dietary intake of Cry1Ac from consumption of food derived from MON 87701 were evaluated by calculating an MOE based on the acute mouse NOAEL for Cry1Ac and the 95th percentile "eater-only" estimates of acute dietary exposure from DEEM-FCID. The MOEs for acute dietary intake of Cry1Ac were estimated to be 2.93×10^6 and 7.71×10^4 for the general population and for non-nursing infants, respectively (Table VI-5). These very large MOEs indicate that there are no meaningful risks to human health from dietary exposure to Cry1Ac derived from MON 87701.

4.5.3. Estimated Animal Exposure to the Cry1Ac Protein from MON 87701

In the United States over 93% of the soybeans grown are either crushed domestically or internationally with less than 7% of the soybean seeds used as feed, seed or residual (USDA-ERS, 2007). During the crushing process soybean meal (SBM) is produced (approximately 0.74 kg of dehulled soybean meal from each kg of soybeans). Of the soybean meal produced in the U.S., approximately 98% is consumed by the livestock industry (Soyatech, 2009). Poultry consumes 50%, swine 27%, cattle 17%, companion animals 3%, and the remainder by others (ASA, 2008). Full-fat soybeans contain a trypsin inhibitor that affects protein digestion in monogastrics and thus it must be heated-treated to deactivate the inhibitor before it can be fed to poultry and swine (Harris, 1990). Heat treatment also enhances the level of ruminal undegradeable protein that is beneficial to the ruminant so long as it is digestible in the lower GI tract. Typically, the feeding level of whole soybeans to dairy cattle is 2.7 (2.3 -3.1) kg/cow/day (Harris, 1990; Hutjens, 1999) and is limited to a maximum of 20% of the swine diet due to its high oil content (Yacentiuk, 2007). Soybean forage can be fed to cattle and is limited to 50% of the total ration dry matter (Brown, 1999). Dehulled soybean meal consumption in different animal species is as follows:

1) the four week old broiler is 27.0 g dry matter/kg body wt/day (30.2% SBM in the diet, 161 g intake/day, diet 89% dry matter, 1.6 kg body wt) (Popescue and Criste, 2003);
2) the young pig is 10.9 g dry matter/kg body wt/day (24.3% SBM in the diet, 2.02 kg intake/day, diet 89% dry matter, 40 kg body wt) (Cromwell et al., 2002);
3) the older pig is 3.8 g dry matter/kg body wt/day (14% SBM in the diet, 3.04 kg of intake/day, diet 89% dry matter, 100 kg body wt) (Cromwell et al., 2002); and,
4) the lactating dairy cow produces 37.4 kg of fat-corrected milk is 7.8 g/kg body wt /day (18.6% SBM in the diet, 27.4 kg of dry matter intake/day, 655 kg body weight) (Bal et al. 2000). The soybean forage intake in lactating dairy cows may be up to 20.9 g/kg body weight/day [50% soybean forage in the diet dry matter (Brown, 1999), 27.4 kg of dry matter intake/day, 655 kg body weight (Bal et al., 2000)].

The exposure of poultry and livestock to MON 87701 may primarily result from feeding soybean meal, with some animals being fed the heat treated full-fat soybeans. For the following exposure calculations, the intake of the soybean meal will be used since the diets contain a higher inclusion level of the protein from soybean meal than from the full-fat soybeans. For example, if we assume that in the crushing process full-fat soybeans yield 74% dehulled soybean meal, then we can multiply the full-fat soybean inclusion levels mentioned above by 74%. The resulting dehulled soybean meal equivalent is equal to or less than the dehulled soybean meal inclusion levels used in this analysis. Soybean meal bought for animal feed would be expected to have gone through a series of commingling steps with non-MON 87701 soybean meal as it makes its way through commerce. MON 87701 soybeans heat-treated and fed as full-fat soybeans may not have been commingled with non-MON 87701 soybeans; therefore, livestock could be exposed to the highest anticipated levels of Cry1Ac protein. For this assessment it will be assumed that MON 87701 soybean meal is the only source of soybean meal in the diet and has not been commingled with non-MON 87701 sources. The second assumption is

that lactating dairy cows will be consuming both soybean forage and soybean meal derived from MON 87701.

4.5.4. Animal Dietary Intake of Cry1Ac

Animals will be exposed to the Cry1Ac protein through dietary intake of feed derived from MON 87701 soybeans and soybean forage in case of the lactating dairy cow. The quantity of soybean meal consumed on a daily basis by poultry and livestock, as well as the levels of Cry1Ac in MON 87701 soybean meal and forage, are necessary to derive an estimate of daily dietary intake (DDI). DDI is computed as follows:

DDI = Daily soybean meal consumption (g) × Cry1Ac protein concentration (µg/g) + daily soybean forage consumption in the case of dairy cattle (g) × Cry1Ac protein concentration (µg/g)

The intake calculations make the conservative assumption that there is no loss of the Cry1Ac protein during the processing of soybeans into soybean meal. It also assumes that 100% of the soybean meal in animal feed is derived from the MON 87701; however in reality, crushing plants that produce large quantities of soybean meal would be commingling soybean meal derived from MON 87701 with soybean meal from non-MON 87701 soybeans.

The potential dietary intake of Cry1Ac from the consumption of MON 87701 soybean meal or soybean forage can be estimated by multiplying the consumption of each commodity by the levels of the protein in that feedstuff.

For the purpose of this dietary intake calculation, which is to characterize a worst case scenario exposure of animals to Cry1Ac protein expressed in soybean meal derived from MON 87701 soybeans and soybean forage allowing for some variability in expression levels, the highest protein levels of the Cry1Ac protein reported for MON 87701 soybeans and soybean forage were used. The mean and high-end range values of the Cry1Ac protein levels in soybeans and soybean forage used in this assessment were from soybean varieties containing MON 87701 grown in the U.S. in 2007.

The mean level of Cry1Ac protein in MON 87701 soybeans is 4.7 µg/g dwt of soybeans (range 3.4 – 5.7 µg/g dwt) (Niemeyer and Silvanovich, 2008). Assuming a crushing yield of 74% (1 kg of soybeans yielding 0.74 kg of dehulled soybean meal), the mean level of Cry1Ac protein in soybean meal derived from MON 87701 soybeans is 6.35 µg/g dwt of soybean meal (range 4.59 – 7.70 µg/g dwt). The mean level of Cry1Ac protein in MON 87701 soybeans forage is 34 µg/g dwt of soybeans (range 8.2 – 140 µg/g dwt).

The estimated mean and maximum daily intake of the Cry1Ac protein by poultry and livestock are shown in Table VI-6.

Table VI-6. Mean and Maximum Daily Intakes of the Cry1Ac Protein in Poultry and Livestock

Species	Total Consumption of SBM (g DM/kg of body weight/day)	Cry1Ac Protein Intake (g/kg of body weight/day dwt)	
		Mean	Highest Level
Chicken broiler[1]	27.0	0.000172	0.000208
Young pig[1]	10.9	0.000069	0.000084
Finishing pig[1]	3.8	0.000024	0.000029
Lactating dairy cow[2]	7.8 SBM + 20.9 Soy forage	0.000760	0.002986

[1] Soybean meal consumed x concentration of Cry1Ac protein in the soybean meal.

[2] Soybean meal consumed x concentration of Cry1Ac protein in the soybean meal + soybean forage consumed x concentration of Cry1Ac protein in the soybean forage.

The broiler chicken, young pig, finishing pig, and lactating dairy typically would consume 18 g dietary protein/kg bw (NRC, 1994), 14 g dietary protein/kg bw (NRC 1998), 4 g dietary protein/kg bw (NRC, 1998), and 6 g dietary protein/kg bw (NRC, 2001), respectively. The highest percentage of Cry1Ac protein (g/kg bw) per total protein (0.0498%)[6] was consumed by the dairy cow due to the much higher levels of Cry1Ac in the soybean forage than in the seed. The percentages of the Cry1Ac protein consumed as part of the daily protein intake for the broiler and pig were less than 0.0012%.

4.6. Conclusion on Potential Toxicity of Cry1Ac Protein Produced in MON 87701

The Cry1Ac protein has been assessed for its potential toxicity according to the recommendations of the Codex Alimentarius Commission. Cry1Ac has a long history of safe use and has been found to pose negligible risk to human and animal health upon consumption. Furthermore, it lacks structural similarity to known toxins or biologically active proteins known to have adverse effects on mammals. Cry1Ac is present at a very low level in the harvested seed of MON 87701 and therefore will constitute a very small portion of the total protein present in food and feed derived from MON 87701. Cry1Ac was readily digestible in simulated gastric and simulated intestinal fluids and showed no oral toxicity in mice. Based on the above information, the consumption of Cry1Ac protein from MON 87701 is safe for humans and animals.

[6] 0.002986 g Cry1Ac /kg bw/day divided by 6 g dietary protein, which is the total dietary protein intake for the dairy cow × 100.

PART VII: FOOD / FEED SAFETY AND NUTRITIONAL ASSESSMENT OF MON 87701

Section 1. Soybean Variety as the Comparable Food and Feed

Soybean is used widely for food and feed purposes, and it is intended that MON 87701 will be utilized in the same manner and for the same uses as conventional soybean. To assess whether the insect-protection trait in MON 87701 caused any unintended effects, compositional analyses were conducted on MON 87701 soybean seed and forage collected from five replicated U.S. locations during the 2007 growing season. Levels of the various analytes assessed from MON 87701 were compared to that of A5547, a conventional soybean variety that has a genetic background similar to that of MON 87701 but does not contain the *cry1Ac* gene cassette or produce the Cry1Ac protein. Additionally, harvested seed and forage from 20 conventional soybean varieties produced in the same field trials alongside MON 87701 and A5547 were also subjected to compositional analyses. Values derived from these conventional varieties were used as references to generate a 99% tolerance interval for each of the analytes for conventional soybean. MON 87701 was found to be compositionally equivalent to conventional soybeans and thus is as safe as conventional soybeans for uses in food and feed applications.

Section 2. Use of Soybean

Soybean is the main source of plant protein consumed by humans and animals. It is the second leading source of vegetable oil of all crops produced in the world, exceeded only by palm oil (ASA, 2008). Utilization of soybean and its derived products is not limited to food and feed, as it has been used for the manufacture of multiple industrial products, including soaps, inks, paints, disinfectants, and biodiesel (Cahoon, 2003). Approximately 5% of the world soybean oil production is used for industrial purposes (Soyatech, 2009). Consequently, food and feed uses of soybean and its processed products remain the predominant use of soybeans produced globally.

2.1. History of Soybean Utilization

In addition to the description below regarding the history and uses of soybean as food and feed, the Organization for Economic Co-operation and Development consensus document on soybean compositional considerations (OECD, 2001) provides an overview of the uses of whole and processed soybean fractions in food applications.

The history and development of soybean has been discussed in Section 1.3, Part IV, including information on the origination of soybean in Northern China. During the course of soybean domestication, the Chinese began using soybean for the preparation of various types of soyfoods, including soymilk, tofu, soy sauce, soy paste, and had started to consume soybean sprouts (Liu, 2004a). As soybean was introduced to neighboring countries through migrations and through sea and land trade, the soyfood products and the methods of product preparation were spread into Korea, Japan, and other regions in

Asia (Hymowitz, 1970). As a result, additional soyfood products, such as tempeh and natto, were developed outside of China (Nout and Kiers, 2004).

The types of traditional soyfoods mentioned above all were made from whole soybean for human consumption. However, human and animal consumption of soybean is primarily in the form of processed fractions such as soybean oil and protein ingredients.

2.2. Soybean as a Food Source

Soybean has the remarkable ability to produce more edible protein per acre of land than any other known crop (Liu, 2004a). On average, dry soybean contains roughly 40% protein and 20% oil. It has the highest protein content among cereals and other legume species, and has the second-highest oil content among all food legumes (Liu, 2004a). Soybean is highly versatile and can be processed into a wide variety of food products. In general, soyfoods can be roughly classified into four major categories (Liu, 2004b):

1. Traditional soyfoods: As discussed above, traditional soyfoods are primarily made from whole soybean. The nonfermented traditional soyfoods include soymilk, tofu, and soybean sprouts, whereas the fermented soyfoods include soybean paste (miso), soy sauce, natto, and tempeh.
2. Soybean oil: Soybean oil constitutes approximately 71% of global consumption of edible fats and oil (ASA, 2008), and is the second largest source of vegetable oil worldwide (Soyatech, 2009). Refined, bleached, and deodorized soybean can be further processed to produce cooking oils, shortening, margarine, mayonnaise, salad dressings, and a wide variety of products that are either based entirely on fats and oils or contain fat or oil as a principal ingredient.
3. Soybean protein products: Soybean protein products are made from defatted soybean flakes, and include soybean flour, soybean protein concentrate, and soybean protein isolate. Soybean flour has a protein content of approximately 50% and is used mainly as an ingredient in the bakery industry. Soybean protein concentrate has a protein content of approximately 70% and is used widely in the meat industry as a key ingredient of meat alternative products such as soybean burgers and meatless "meatballs." Soybean protein isolate has a protein content of 90%, and possesses many functional properties such as gelation and emulsification. As a result, it can be used in a wide range of food applications, including soups, sauce bases, energy bars, nutritional beverages, infant formula, and dairy replacements.
4. Dietary supplements: Soybean is a rich source of certain phytochemicals used as dietary supplements, which include isoflavones and tocopherols. Isoflavones have been shown to inhibit the growth of cancer cells, lower cholesterol levels, and inhibit bone resorption (Messina, 1999). Tocopherols have long been recognized as a classic free radical scavenging antioxidant whose deficiency impairs mammalian fertility. In addition, new biological activities have been reported for the desmethyl tocopherols, such as γ-tocopherol, to possess anti-inflammatory, antineoplastic, and natriuretic functions (Schafer et al., 2003; Hensley et al., 2004; IFIC, 2005). Detailed reviews of soybean as functional foods have been published (IFIC, 2005; Liu, 2004a).

2.3. Soybean as a Feed Source

Soybean meal is the most valuable component obtained from processing the soybean, accounting for roughly 50-75% of its overall value (USDA-ERS, 2005). Soybean meal is produced by solvent (typically hexane) extraction of dehulled soybean flakes. The spent flakes (soybean flakes with the oil removed) are conveyed to a desolventizer-toaster for removal of hexane. The process involves heating the spent flakes to evaporate the hexane and utilizing steam to carry away hexane vapors. This process also provides toasting of the meal to inactivate proteins that may reduce the digestibility and nutritional value of the meal, such as urease, trypsin inhibitors, and lectins. The meal is subsequently dried to about 13 to 14 percent moisture, and is screened and ground to produce a uniform particle size prior to shipment to the end user. The finished meal from dehulled soybean will contain less than 1.5% (w/w) crude fat and approximately 48% (w/w) protein, and is referred to as high protein meal (SMIC, 2006).

Soybean meal is a premier supplemental protein source in livestock and poultry rations due to its nutrient composition, availability, and price. Typically, soybean meal is used to meet the animal's requirement for limiting amino acids, because it is a cost-effective source of amino acids. Soybean meal is also one of the best protein sources for complementing the limiting amino acid profile of corn protein (Kerley and Allee, 2003). Due to its high value and versatility, approximately two-thirds of the global protein meal used as animal feed is derived from soybean, with the remainder divided between rapeseed, cottonseed, sunflower, peanut, and other meals (ASA, 2008). Poultry and swine account for most of the soybean meal utilized in the U.S., with poultry consuming 50%, swine 27%, cattle 17%, and 3% for companion animals and the reminder to other feed uses (ASA, 2008).

Dairy and livestock producers need an inexpensive, readily available, on-farm source of high-quality, high-protein forage adapted to growth during the summer months when other forage legume species typically are restricted in growth. Soybean forage can provide livestock and dairy producers with a source of high-protein feed for their livestock (USDA-ARS, 2006). Harvested forage can be used as hay or to produce silage (MAFRI, 2004).

Section 3. Comparison of the Composition and Nutritional Components of MON 87701 to Conventional Soybean

Compositional comparisons between biotechnology-derived and conventional crops represent an integral part of a nutritional and safety assessment. Compositional assessments are performed using the principles and analytes outlined in the OECD consensus documents for soybean composition (OECD, 2001). These principles are accepted globally and have been employed previously in assessments of soybean products derived through biotechnology.

Compositional equivalence between biotechnology-derived and conventional crops provides an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 1998). The OECD consensus documents emphasize quantitative measurements of essential nutrients, and known antinutrients and toxicants.

This is predicated on the premise that such comprehensive and detailed analyses will most effectively discern any compositional changes that imply potential safety and anti-nutritional concerns. Levels of the components in seed and forage of the biotechnology-derived crop product are compared to: 1) corresponding levels in a non-modified comparator, typically the near isogenic parental line grown under identical conditions, and 2) natural ranges generated from an in-study evaluation of commercial varieties or from data published in the scientific literature. Together with the FDA's 1992 Policy on Food Derived from New Plant Varieties (FDA, 1992), these OECD guidelines framed the strategy for the compositional assessment of MON 87701.

Compositional analyses were conducted to assess whether the nutrient and antinutrient levels in the seed and forage derived from MON 87701 are comparable to those in the conventional soybean control, A5547. In addition, twenty commercial conventional soybean varieties were included in the analysis as references to establish a range of natural variability for each analyte, defined by a 99% tolerance interval. Compositional analysis included the significant nutrients and antinutrients, consistent with OECD guidelines (OECD, 2001). Results of the comparisons indicate that MON 87701 is compositionally and nutritionally equivalent to current conventional commercial soybean varieties that have a history of safe human and animal consumption. Further details of this assessment are provided below.

3.1. Composition Assessment Methodology

Compositional analyses were conducted on the forage and seed collected from MON 87701, the conventional soybean control (A5547), and twenty unique commercial conventional soybean varieties grown at five replicated trial sites (AL, AR, GA, IL, and NC) in a 2007 U.S. field production. Seeds were planted in a randomized complete block design with three replicates per block for MON 87701, the control, and reference soybean varieties. Samples from all three replicates of MON 87701 and control plots at each site were analyzed, whereas one replicate of the twenty unique commercial conventional soybean varieties planted across sites was analyzed. Analysis of a single replicate from each unique reference variety was sufficient to establish a 99% tolerance interval for each analyte, as described below. All samples were collected from plants grown under normal agronomic field conditions for their respective geographic regions.

A total of 64 compositional analytes (seven in forage and 57 in seed) were evaluated. Compositional analyses of the forage samples included proximates (ash, fat, moisture, and protein), carbohydrates by calculation, acid detergent fiber (ADF), and neutral detergent fiber (NDF). Seed samples were analyzed for proximates (ash, fat, moisture, and protein), carbohydrates by calculation, ADF, NDF, amino acids, fatty acids (C8-C22), trypsin inhibitors, phytic acid, lectin, isoflavones (daidzein, glycitein, and genistein), vitamin E, raffinose, and stachyose. Materials and methods used for compositional analysis of the seed and forage of MON 87701, the conventional soybean control, and commercial conventional soybean varieties collected from the 2007 U.S. field production are presented in Appendix F.

In a further assessment, the composition analysis data were statistically compared to that of the conventional soybean control. Of the evaluated components, nine fatty acids in

harvested seed had more than 50% of the observations below the assay limit of quantitation (LOQ) and, as a result, were excluded from the statistical analysis. Therefore, the remaining 55 compositional analytes (seven in forage and 48 in seed) were statistically analyzed. A summary of the statistical results included the calculation of least square means, standard errors, and the range of observed values for MON 87701 and the conventional soybean control. A 99% tolerance interval was also calculated for each analyte that represented, with 95% confidence, 99% of the values contained in the population of commercial conventional soybean varieties. The compositional analysis data across sites (combined-site) are presented in Table VII-1 for forage and in Tables VII-2 (nutrients) and VII-3 (antinutrients) for harvested seed. The compositional analysis data within each site (individual-site) are presented in Appendix G.

Each analyte for MON 87701 was statistically compared to that of the conventional soybean control in a combined-site analysis and five individual-site analyses using SAS, version 9.1, software. The combined-site analysis used a mixed model analysis with both site and site by analyte designated as random effects. Site was treated as a random effect since it is assumed that the sites are a representative sample from a population of sites (Littell et al., 2006). The overall data set was examined for evidence of biologically relevant changes by first examining combined-site differences, followed by the individual-site assessments. Additionally, each mean test value that differed ($p<0.05$) from the control was compared to the 99% tolerance interval generated from the commercial conventional soybean varieties. A summary of the significant differences ($p<0.05$) between MON 87701 and the conventional soybean control for forage and harvested seed are presented in Table VII-4. Finally, this comparative evaluation also considered the natural ranges in soybean component levels published in the scientific literature and the International Life Sciences Institute - Crop Composition Database (ILSI-CCD)[7]. Reported literature and ILSI-CCD ranges for the analytical components present in forage and harvested seed are shown in Table VII-5.

3.2. Overall Assessment of the Composition of MON 87701 Compared to the Conventional Soybean Control

MON 87701 is as safe and nutritious as conventional soybean based on a comprehensive compositional and nutritional assessment as described in Section 3.1, Part VII. The combined-site analysis for seed and forage samples showed no significant difference ($p>0.05$) between MON 87701 and the conventional control for 40 of 55 comparisons. Statistically significant differences were detected for 15 analytes: alanine, 22:0 behenic acid, carbohydrates, daidzein, glycine, histidine, isoleucine, leucine, lysine, protein, serine, threonine, trypsin inhibitor, valine, and vitamin E. However, the magnitude of these differences were generally small (most <5%), were not observed consistently across all sites (individual-site analyses), and the mean values for MON 87701 were within the calculated 99% tolerance interval for the population of commercial conventional soybean varieties grown concurrently at the same field sites. Therefore, it is concluded that since the measured values fall within the range of natural variability for these soybean analytes,

[7] International Life Science Institute Crop Composition Database. Version 3.0. 2006. Available at http://www.cropcomposition.org/.

they were not regarded as biologically relevant. Soybean seed and forage analyte values were also comparable to values published in the scientific literature and reported in ILSI-CCD. This further supports the conclusion that the soybean seed and forage from MON 87701 are compositionally equivalent to those of conventional soybean.

The following sections provide a detailed assessment of the biological relevance of the differences observed between MON 87701 and the conventional soybean control based on the magnitude of the difference, reproducibility across sites, and comparison of mean analyte values to the 99% tolerance interval.

3.3. Levels of Nutrients in Soybean Forage

The levels of nutrients from the combined-site analysis for forage are summarized in Table VII-1. No significant differences ($p \geq 0.05$) were detected in the seven comparisons made between MON 87701 and the conventional soybean control in the combined-site analysis. Forage analyte values were within the 99% tolerance for conventional soybean varieties and comparable to values published in the scientific literature and reported in ILSI-CCD (2006). This supports the conclusion that the forage from MON 87701 is compositionally equivalent to that of conventional soybean.

3.4. Levels of Nutrients in Soybean Seed

The levels of nutrients from the combined-site analysis for soybean seed are summarized in Table VII-2. No significant differences ($p \geq 0.05$) were detected in 27 of 40 nutrient analyte comparisons made between MON 87701 and the conventional soybean control in the combined-site analysis. Significant differences ($p < 0.05$) were detected for 13 nutrient analytes: protein, nine amino acids (alanine, glycine, histidine, isoleucine, leucine, lysine, serine, threonine, and valine), 22:0 behenic acid, carbohydrates and vitamin E (Table VII-4). The magnitude of these differences were generally small (most <5%), except in the case of vitamin E described below.

Biological relevance of these differences was assessed based on the magnitude of the difference, reproducibility across sites, and comparison of mean analyte values to the 99% tolerance interval for the population of commercial conventional soybean varieties grown concurrently at the same field sites. The protein content in MON 87701 was slightly increased, but at a small order of magnitude (3.87%) compared to the conventional soybean control. Similarly, the levels of all nine amino acids are slightly increased (2.15-4.63%) when compared to the conventional soybean control. Based on the relatively high total protein content (35-45%) in commercially available conventional soybean (Table VII-5), and the relatedness of amino acid content to total protein content, it is not unexpected that several of the amino acids in MON 87701 also had elevated levels that are significantly different from the conventional soybean control, and at a magnitude similar to that of protein. Examination of the reproducibility across sites for protein and the nine amino acids found to be different in the combined-site analysis, shows that only one analyte (histidine) was significantly different at more than one site (Table VII-4). Histidine content in MON 87701 was significantly higher than the conventional soybean control at two sites but the magnitudes of the differences were

small (<6%). The differences for protein and nine amino acids are not considered to be biologically relevant changes in composition because the increase in levels were generally small, only one analyte (histidine) showed differences at more than one site, and the mean analyte levels were within the 99% tolerance interval established by commercial conventional soybean varieties grown concurrently at the same field sites. Furthermore, analyte values were comparable to values reported in ILSI-CCD.

The magnitude of differences between MON 87701 and the conventional soybean control for carbohydrates and 22:0 behenic acid were also considered to be relatively small (6.10% and 4.33%, respectively). Examination of the reproducibility across sites shows that only 22:0 behenic acid was significantly different at more than one site. Behenic acid content in MON 87701 was significantly higher than the conventional soybean control at two sites and the magnitudes of the differences were small (<8.3%). The differences for 22:0 behenic acid and carbohydrate are not considered to be biologically relevant changes in composition because the increase in levels were generally small, the differences were not reproducible at more than two sites, and the mean levels were within the 99% tolerance interval established by commercial conventional soybean varieties grown concurrently at the same field sites. Furthermore, analyte values were comparable to those reported in ILSI-CCD.

Vitamin E levels were significantly higher (23.26%) in MON 87701 compared to the conventional soybean control. Examination of the reproducibility across sites shows that vitamin E was significantly higher than the conventional soybean control in four of five individual-site analyses, with the magnitude of differences ranging between 17-37% (Table VII-4). The differences are not biologically relevant changes in composition given that the mean levels of Vitamin E in MON 87701 from the combined-site and individual-site analyses were all well within the 99% tolerance interval established by commercial conventional soybean varieties grown concurrently at the same field sites. From a nutritional perspective, vitamin E is not listed as a key nutrient in soybean by OECD (2001) for food and feed uses, though soybean oil is recognized as a source of vitamin E in the diet (Eitenmiller, 1997). No dietary impact is expected as the Vitamin E levels are also comparable to the values reported in ILSI-CCD.

Based on the data and information presented above, it is concluded that the seed from MON 87701 is compositionally equivalent to conventional soybean with regard to the levels of nutrients. The differences in nutrients were limited in number, not consistently observed across all sites, and reflect the natural variation of conventional soybean, which further supports the compositional equivalence of MON 87701 to conventional soybean.

3.5. Levels of Antinutrients in Soybean Seed

Soybean seed contains several well-described antinutritional factors (OECD, 2001) that include: trypsin inhibitors, lectins, isoflavones (daidzein, genistein and glycitein), stachyose, raffinose, and phytic acid. The levels of these components in the seed of MON 87701, the conventional soybean control (A5547), and 20 commercial conventional soybean varieties were evaluated. The levels of antinutrients from the

combined-site analysis for soybean seed are summarized in Table VII-3. No significant differences ($p \geq 0.05$) were detected in six of eight antinutrient analyte comparisons made between MON 87701 and the conventional soybean control in the combined-site analysis. Statistically significant differences were detected for trypsin inhibitor and daidzen (Table VII-4); however, the magnitude of these differences were small (<11%). Biological relevance of these differences was assessed based on the magnitude of the difference, reproducibility across sites, and comparison of mean analyte values to the 99% tolerance interval.

Trypsin inhibitors are heat-labile antinutrients that interfere with the digestion of proteins and result in decreased animal growth (Liener, 1994). Lectins are also heat labile, and can inhibit growth and cause death in animals if raw soybean is consumed (Liener, 1994). Both trypsin inhibitors and lectins are inactivated during processing of soybean protein products or soybean meal and, when processed appropriately, the final edible soybean fractions should contain minimal levels of these antinutrients. There were no significant differences ($p \geq 0.05$) in lectin levels between MON 87701 and conventional soybean control seed. The level of trypsin inhibitor was significantly lower (-8.79%) in MON 87701 than the conventional control in the combined-site analysis (Table VII-3). Examination of the reproducibility across sites shows that the trypsin inhibitor levels were significantly lower (-20.48%) than the conventional soybean control in only one of five sites (Table VII-4). The difference for trypsin inhibitor is not considered to be a biologically relevant change in composition because the increase in level was small, the difference was not reproducible at more than one site, and the mean levels were within the 99% tolerance interval established by commercial conventional soybean varieties grown concurrently at the same field sites. Furthermore, the trypsin inhibitor values in MON 87701 were comparable to those reported in ILSI-CCD.

There are three principle isoflavones in soybean seed, namely, daidzein, genistein, and glycitein. Although they have been reported to possess biochemical activities including estrogenic and anti-estrogenic effects, it is not universally accepted that the isoflavones are antinutrients as they have also been reported to have beneficial antioxidant, anticarcinogenic and heart-healthy hypocholesterolemic effects (OECD, 2001). It is well documented that isoflavone levels in soybean seed are highly variable and are greatly influenced by many factors (OECD, 2001; Messina, 2001; Nelson et al., 2001).

There were no significant differences ($p \geq 0.05$) in glycitein levels between MON 87701 and conventional soybean control seed. The level of daidzein was significantly higher in MON 87701 (10.36%) than the conventional control in the combined-site analysis (Table VII-3). Examination of the reproducibility across sites shows that the daidzein levels were significantly higher in MON 87701 than the conventional soybean control in two of five individual-site analyses (<16.7%). These results are not unexpected because, as described above, soybean isoflavone levels are greatly influenced by many factors, ranging from environmental conditions, variety, and agronomic practices. The difference for daidzein is not considered to be biologically relevant because the increase in level was small, the difference was not reproducible at more than one site, and the mean levels were within the 99% tolerance interval established by commercial conventional soybean varieties grown concurrently at the same field sites. Furthermore, the daidzein values in MON 87701 were comparable to the values reported in ILSI-CCD.

Antinutrients that showed no differences in the combined-site analysis are not expected to raise biological, nutritional or toxicological concerns. However, further consideration and background is provided to complete the discussion for the group of antinutrients.

As discussed earlier, genistein is one of three principle isoflavones. A difference ($P < 0.05$) in genistein levels between the seed of MON 87701 and the conventional control was detected in one of the five sites (Table VII-4). Stachyose and raffinose are low molecular weight carbohydrates present in soybean seed that are considered to be antinutrients due to their consumption causing flatulence. Significant differences ($P < 0.05$) were detected in stachyose levels in seed from MON 87701 and the conventional soybean control at two sites (Table VII-4). The differences for genistein and stachyose were not consistently observed across all sites and no trends were observed. Furthermore, the mean levels of these components in MON 87701 seed were within the 99% tolerance interval for commercial conventional soybean varieties. Therefore, these differences are not considered biologically relevant from a food or feed safety or nutritional perspective.

Phytic acid present in soybean seed chelates mineral nutrients, including calcium, magnesium, potassium, iron and zinc, rendering them biologically unavailable to monogastric animals consuming the seed (Liener, 2000). Unlike trypsin inhibitors, phytic acid is not heat labile, and remains stable through most soybean processing steps. No significant differences were detected in combined-site or individual-site analyses of phytic acid levels in seed from MON 87701 and its conventional soybean control (A5547).

Based on the data and information presented above, it is concluded that the seed from MON 87701 is compositionally equivalent to conventional soybean with regard to the levels of antinutrients. The differences in antinutrients were limited in number, not consistently observed across all sites, and reflect the natural variation of conventional soybean, which further supports the compositional equivalence of MON 87701 to conventional soybean.

3.6. Conclusion

Compositional data were generated and statistical analyses performed on the forage and harvested seed from MON 87701, the conventional soybean control, and 20 commercial conventional soybean varieties. The overall dataset was evaluated for evidence of biologically relevant changes from a food and feed safety and nutritional perspective. Overall, statistical analyses of both forage and seed showed no significant difference ($p \geq 0.05$) between MON 87701 and the conventional soybean control for 40 of 55 comparisons from the combined-site analyses and 243 of 275 comparisons from the individual-site analyses. For the analytes where differences were noted ($p<0.05$), the magnitude of differences were generally low (most <5%), were not observed consistently across all sites (individual-site analyses), and the mean values for MON 87701 were within the calculated 99% tolerance interval for the population of commercial conventional soybean varieties grown concurrently at the same field sites. Vitamin E

levels were significantly higher than the control in the combined-site analysis and in four of five individual-site analyses, but were within the calculated 99% tolerance interval and, therefore, are not regarded to be biologically relevant. Forage and seed analytical component values were also comparable to published scientific literature and the ILSI-CCD database, further supporting the conclusion that soybean forage and seed produced from MON 87701 are compositionally equivalent to those of conventional soybean.

3.7. Any Intended Changes to the Composition of Food and Feed

The only intended change for MON 87701 is the introduction of the *cry1Ac* coding sequence from Bt var. *kurstaki* to produce a Cry1Ac insecticidal protein that provides protection from feeding damage caused by targeted lepidopteran pests. There have been no intended changes to the composition (including nutrients and antinutrients) of food or feed derived from MON 87701 compared to other conventional soybean varieties. The analyses of soybean seed and forage composition (total of 64 components, seven in forage and 57 in seed) have shown no differences that are meaningful from a food or feed safety or nutritional perspective, either between MON 87701 and the control (A5547) or commercial conventional soybean varieties. Given this extensive compositional characterization, it is concluded that MON 87701 is compositionally and nutritionally equivalent to conventional soybean varieties already on the market.

Table VII-1. Statistical Summary of Combined Site Soybean Forage Fiber and Proximate Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (%DW)						
Acid Detergent Fiber	37.17 (1.72) [30.04 - 58.25]	36.53 (1.72) [27.42 - 42.06]	0.65 (2.01) [-12.02 - 10.46]	-3.42, 4.71	0.749	(27.99 - 47.33) [14.93, 56.87]
Neutral Detergent Fiber	47.16 (2.00) [37.02 - 55.99]	45.57 (2.00) [34.23 - 64.19]	1.59 (2.48) [-18.07 - 18.76]	-3.50, 6.68	0.526	(30.96 - 54.55) [21.51, 66.01]
Proximate (%DW, unless noted)						
Ash	5.84 (0.30) [5.05 - 7.46]	6.32 (0.30) [5.10 - 8.13]	-0.48 (0.33) [-1.72 - 0.92]	-1.25, 0.29	0.190	(4.77 - 8.54) [2.46, 10.14]
Carbohydrates	71.43 (1.12) [68.29 - 76.73]	70.97 (1.12) [63.68 - 74.26]	0.47 (0.61) [-2.28 - 4.62]	-0.79, 1.73	0.452	(60.61 - 77.26) [56.93, 85.88]
Moisture (% FW)	72.86 (1.19) [70.10 - 76.80]	73.41 (1.19) [69.40 - 78.10]	-0.55 (0.49) [-2.30 - 1.70]	-1.67, 0.58	0.296	(66.50 - 80.20) [57.84, 88.56]
Protein	17.39 (1.07) [13.56 - 20.03]	17.07 (1.07) [14.20 - 23.29]	0.32 (0.60) [-3.57 - 2.22]	-0.90, 1.54	0.591	(12.68 - 22.92) [7.05, 27.27]
Total Fat	5.30 (0.34) [3.60 - 6.82]	5.65 (0.34) [4.23 - 7.23]	-0.35 (0.26) [-2.76 - 0.70]	-0.89, 0.19	0.195	(3.48 - 7.88) [1.11, 9.11]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table VII-2. Statistical Summary of Combined-Site Soybean Seed Proximate, Amino Acid, Fatty Acid, Fiber and Vitamin Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Proximate (% DW, unless noted)						
Ash	5.20 (0.18) [4.70 - 5.90]	5.14 (0.18) [4.70 - 5.88]	0.054 (0.043) [-0.14 - 0.21]	-0.046, 0.15	0.246	(4.32 - 5.62) [3.74, 6.45]
Carbohydrates	34.22 (1.50) [21.58 - 39.61]	36.44 (1.50) [29.88 - 43.48]	-2.22 (1.02) [-15.00 - 2.56]	-4.31, -0.14	0.037	(31.97 - 38.00) [28.17, 40.99]
Moisture (% FW)	7.52 (0.38) [5.86 - 10.70]	6.84 (0.38) [5.44 - 8.74]	0.68 (0.47) [-0.96 - 4.70]	-0.28, 1.64	0.159	(5.48 - 11.70) [1.45, 12.81]
Protein	39.27 (0.86) [36.49 - 42.23]	37.80 (0.86) [32.29 - 41.87]	1.46 (0.54) [-0.73 - 6.49]	0.24, 2.68	0.023	(38.14 - 42.66) [35.30, 45.38]
Total Fat	20.29 (0.78) [17.33 - 23.08]	20.12 (0.77) [17.24 - 22.55]	0.17 (0.39) [-1.82 - 1.98]	-0.71, 1.05	0.670	(17.90 - 23.56) [14.74, 25.18]
Amino Acid (% DW)						
Alanine	1.72 (0.029) [1.66 - 1.84]	1.69 (0.029) [1.59 - 1.82]	0.036 (0.016) [-0.034 - 0.099]	0.0044, 0.068	0.027	(1.66 - 1.93) [1.49, 2.02]
Arginine	2.68 (0.069) [2.36 - 3.00]	2.58 (0.069) [2.37 - 2.89]	0.096 (0.058) [-0.16 - 0.31]	-0.039, 0.23	0.138	(2.54 - 2.99) [2.22, 3.25]
Aspartic Acid	4.90 (0.10) [4.61 - 5.26]	4.85 (0.10) [4.46 - 5.34]	0.053 (0.055) [-0.23 - 0.31]	-0.059, 0.17	0.339	(4.74 - 5.50) [4.22, 5.96]
Cystine	0.62 (0.014) [0.57 - 0.67]	0.61 (0.014) [0.56 - 0.69]	0.0051 (0.014) [-0.11 - 0.066]	-0.024, 0.034	0.718	(0.53 - 0.68) [0.45, 0.77]
Glutamic Acid	7.65 (0.15) [7.25 - 8.21]	7.53 (0.15) [6.89 - 8.26]	0.12 (0.084) [-0.29 - 0.46]	-0.056, 0.29	0.177	(7.53 - 8.72) [6.60, 9.37]

Table VII-2. Statistical Summary of Combined-Site Soybean Seed Proximate, Amino Acid, Fatty Acid, Fiber and Vitamin Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Glycine	1.75 (0.026) [1.63 - 1.89]	1.70 (0.026) [1.64 - 1.85]	0.049 (0.017) [-0.0052 - 0.12]	0.014, 0.083	0.007	(1.67 - 1.99) [1.49, 2.09]
Histidine	1.12 (0.015) [1.05 - 1.18]	1.08 (0.015) [1.03 - 1.15]	0.043 (0.011) [-0.00077 - 0.090]	0.021, 0.064	<0.001	(1.04 - 1.24) [0.94, 1.31]
Isoleucine	1.81 (0.037) [1.68 - 1.99]	1.76 (0.037) [1.64 - 1.96]	0.052 (0.020) [-0.044 - 0.12]	0.0061, 0.098	0.031	(1.73 - 2.02) [1.54, 2.14]
Leucine	3.04 (0.066) [2.82 - 3.36]	2.94 (0.066) [2.73 - 3.29]	0.095 (0.040) [-0.044 - 0.23]	0.0018, 0.19	0.046	(2.93 - 3.32) [2.64, 3.52]
Lysine	2.74 (0.060) [2.48 - 2.99]	2.62 (0.060) [2.42 - 2.91]	0.12 (0.046) [-0.12 - 0.39]	0.028, 0.21	0.012	(2.35 - 3.15) [2.05, 3.47]
Methionine	0.53 (0.012) [0.48 - 0.58]	0.53 (0.012) [0.47 - 0.59]	0.0043 (0.014) [-0.094 - 0.080]	-0.023, 0.032	0.754	(0.49 - 0.62) [0.42, 0.68]
Phenylalanine	2.15 (0.056) [1.91 - 2.48]	2.04 (0.056) [1.91 - 2.38]	0.11 (0.052) [-0.036 - 0.41]	-0.013, 0.23	0.073	(1.97 - 2.44) [1.66, 2.64]
Proline	2.01 (0.035) [1.86 - 2.16]	1.96 (0.035) [1.85 - 2.12]	0.042 (0.021) [-0.058 - 0.11]	-0.0069, 0.091	0.082	(1.92 - 2.25) [1.73, 2.35]
Serine	2.03 (0.032) [1.90 - 2.19]	1.96 (0.032) [1.87 - 2.13]	0.060 (0.019) [0.010 - 0.14]	0.020, 0.10	0.004	(1.96 - 2.30) [1.75, 2.38]
Threonine	1.60 (0.020) [1.50 - 1.72]	1.55 (0.020) [1.49 - 1.68]	0.046 (0.016) [-0.016 - 0.13]	0.0078, 0.084	0.024	(1.54 - 1.74) [1.40, 1.83]

Table VII-2. Statistical Summary of Combined-Site Soybean Seed Proximate, Amino Acid, Fatty Acid, Fiber and Vitamin Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

			Difference (Test minus Control)			
Component (Units)[1]	**MON 87701 Mean (S.E.) [Range]**	**A5547 Mean (S.E.) [Range]**	**Mean (S.E.) [Range]**	**95% CI (Lower, Upper)**	**p-Value**	**Commercial (Range) [99% Tolerance Interval**[2]**]**
Amino Acid (% DW)						
Tryptophan	0.51 (0.0068) [0.47 - 0.54]	0.50 (0.0068) [0.46 - 0.53]	0.011 (0.0067) [-0.039 - 0.075]	-0.0024, 0.025	0.102	(0.47 - 0.55) [0.43, 0.59]
Tyrosine	1.13 (0.034) [0.96 - 1.33]	1.10 (0.034) [0.98 - 1.22]	0.039 (0.029) [-0.11 - 0.25]	-0.028, 0.11	0.213	(1.04 - 1.31) [0.85, 1.48]
Valine	1.92 (0.032) [1.80 - 2.07]	1.86 (0.032) [1.76 - 2.04]	0.053 (0.022) [-0.033 - 0.12]	0.0029, 0.10	0.040	(1.83 - 2.13) [1.64, 2.22]
Fatty Acid (% Total FA)						
10:0 Capric Acid	0.20 (0.014) [0.14 - 0.25]	0.21 (0.014) [0.16 - 0.26]	-0.010 (0.020) [-0.11 - 0.048]	-0.053, 0.032	0.607	(0.15 - 0.27) [0.065, 0.34]
14:0 Myristic Acid	0.093 (0.0031) [0.082 - 0.10]	0.094 (0.0031) [0.083 - 0.11]	-0.00056 (0.0019) [-0.0085 - 0.0025]	-0.0048, 0.0037	0.769	(0.064 - 0.097) [0.052, 0.12]
16:0 Palmitic Acid	11.80 (0.12) [11.32 - 12.30]	11.88 (0.12) [11.50 - 12.13]	-0.079 (0.081) [-0.72 - 0.40]	-0.27, 0.11	0.359	(9.80 - 12.38) [8.88, 13.53]
16:1 Palmitoleic Acid	0.092 (0.0033) [0.073 - 0.11]	0.095 (0.0033) [0.078 - 0.11]	-0.0028 (0.0029) [-0.018 - 0.015]	-0.0097, 0.0041	0.372	(0.073 - 0.14) [0.037, 0.15]
17:0 Heptadecanoic Acid	0.094 (0.0021) [0.084 - 0.10]	0.093 (0.0021) [0.082 - 0.099]	0.0011 (0.0018) [-0.0064 - 0.0074]	-0.0030, 0.0052	0.553	(0.076 - 0.10) [0.066, 0.11]
17:1 Heptadecenoic Acid	0.041 (0.0032) [0.023 - 0.048]	0.041 (0.0032) [0.019 - 0.047]	-0.00009 (0.0040) [-0.020 - 0.022]	-0.0092, 0.0090	0.981	(0.020 - 0.064) [0.0058, 0.083]
18:0 Stearic Acid	4.59 (0.22) [3.97 - 5.36]	4.70 (0.22) [4.03 - 5.36]	-0.12 (0.11) [-0.57 - 0.29]	-0.38, 0.14	0.328	(3.21 - 5.24) [1.88, 6.25]

Table VII-2. Statistical Summary of Combined-Site Soybean Seed Proximate, Amino Acid, Fatty Acid, Fiber and Vitamin Content for MON 87701 vs. the Conventional Control (A5547)(cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
18:1 Oleic Acid	22.35 (1.28) [19.21 - 26.64]	22.71 (1.28) [20.34 - 28.78]	-0.36 (0.49) [-3.16 - 2.04]	-1.51, 0.79	0.486	(16.69 - 35.16) [5.01, 42.01]
18:2 Linoleic Acid	52.16 (0.95) [49.32 - 54.63]	51.76 (0.95) [47.18 - 54.07]	0.40 (0.38) [-1.35 - 2.68]	-0.48, 1.29	0.320	(44.17 - 57.72) [38.57, 66.94]
18:3 Linolenic Acid	7.24 (0.45) [5.55 - 8.41]	7.11 (0.45) [5.34 - 8.26]	0.13 (0.12) [-0.40 - 0.68]	-0.13, 0.40	0.276	(4.27 - 8.81) [2.69, 10.81]
20:0 Arachidic Acid	0.51 (0.025) [0.41 - 0.58]	0.51 (0.025) [0.41 - 0.57]	-0.0027 (0.013) [-0.044 - 0.047]	-0.032, 0.026	0.836	(0.36 - 0.55) [0.23, 0.64]
20:1 Eicosenoic Acid	0.24 (0.012) [0.19 - 0.28]	0.23 (0.012) [0.18 - 0.28]	0.0044 (0.010) [-0.065 - 0.046]	-0.020, 0.029	0.683	(0.21 - 0.30) [0.16, 0.33]
20:2 Eicosadienoic Acid	0.040 (0.0030) [0.020 - 0.054]	0.042 (0.0030) [0.020 - 0.047]	-0.0024 (0.0042) [-0.024 - 0.011]	-0.012, 0.0068	0.585	(0.016 - 0.054) [0.0029, 0.083]
22:0 Behenic Acid	0.56 (0.028) [0.46 - 0.65]	0.54 (0.028) [0.45 - 0.65]	0.023 (0.0084) [-0.00071 - 0.078]	0.0041, 0.042	0.022	(0.38 - 0.59) [0.30, 0.67]
Fiber (% DW)						
Acid Detergent Fiber	15.58 (0.49) [13.53 - 17.05]	15.62 (0.49) [14.00 - 19.02]	-0.042 (0.58) [-2.84 - 1.88]	-1.37, 1.28	0.943	(12.79 - 17.98) [11.13, 20.21]
Neutral Detergent Fiber	17.33 (0.70) [15.06 - 21.80]	17.28 (0.70) [15.02 - 22.45]	0.057 (0.74) [-6.43 - 4.47]	-1.67, 1.78	0.940	(13.32 - 23.57) [7.24, 28.70]
Vitamin (mg/100g DW)						
Vitamin E	7.69 (0.52) [6.36 - 9.62]	6.24 (0.52) [4.88 - 7.94]	1.45 (0.27) [0.57 - 2.25]	0.81, 2.09	<0.001	(1.65 - 8.08) [0, 11.09]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of soybean varieties. Negative limits were set to zero.

Table VII-3. Statistical Summary of Combined-Site Soybean Seed Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg FW)	0.96 (0.19) [0.062 - 2.01]	0.72 (0.19) [0.28 - 1.28]	0.24 (0.25) [-0.88 - 1.42]	-0.27, 0.74	0.354	(0.090 - 2.47) [0, 3.40]
Phytic Acid	1.85 (0.12) [1.39 - 2.29]	1.97 (0.12) [1.31 - 2.66]	-0.11 (0.097) [-0.53 - 0.31]	-0.34, 0.11	0.276	(1.10 - 2.32) [0.54, 3.05]
Raffinose	1.33 (0.19) [0.49 - 1.70]	1.34 (0.19) [0.43 - 1.85]	-0.0086 (0.074) [-0.32 - 0.19]	-0.18, 0.16	0.910	(0.52 - 1.62) [0.038, 2.24]
Stachyose	4.59 (0.63) [1.83 - 6.42]	4.93 (0.63) [2.27 - 6.65]	-0.34 (0.22) [-1.00 - 0.73]	-0.83, 0.16	0.156	(1.97 - 5.55) [0.99, 7.93]
Trypsin Inhibitor (TIU/mg DW)	26.06 (1.24) [21.65 - 32.53]	28.57 (1.24) [22.49 - 34.20]	-2.51 (0.96) [-7.75 - 6.33]	-4.48, -0.54	0.014	(20.84 - 37.24) [13.58, 46.02]
Isoflavone (mg/kg DW)						
Daidzein	667.54 (108.30) [188.96 - 983.26]	604.88 (108.30) [198.95 - 830.65]	62.65 (25.68) [-27.87 - 178.54]	3.56, 121.74	0.040	(213.98 - 1273.94) [0, 1585.14]
Genistein	655.57 (88.52) [214.73 - 863.84]	594.58 (88.52) [244.95 - 760.87]	60.99 (36.27) [-30.22 - 178.22]	-23.10, 145.09	0.132	(148.06 - 1024.50) [0, 1352.86]
Glycitein	164.87 (21.23) [61.08 - 228.79]	156.93 (21.23) [61.28 - 227.25]	7.94 (13.22) [-49.56 - 88.71]	-22.57, 38.44	0.564	(32.42 - 208.45) [0, 272.12]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of soybean varieties. Negative limits were set to zero.

Table VII-4. Summary of Differences (p<0.05) for the Comparison of Soybean Component Levels for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean	A5547 Mean	Mean Difference (Test minus Control)		MON 87701 Range	Commercial Tolerance Interval[2]
			Mean Difference (% of A5547)	Significance (p-Value)		
Statistical Differences Observed in *Combined Site* Analysis						
Seed Proximate (% DW)						
Protein	39.27	37.80	3.87	0.023	[36.49 – 42.23]	[35.30, 45.38]
Carbohydrates	34.22	36.44	-6.10	0.037	[21.58 - 39.61]	[28.17, 40.99]
Seed Amino Acid (% DW)						
Alanine	1.72	1.69	2.15	0.027	[1.66 - 1.84]	[1.49, 2.02]
Glycine	1.75	1.70	2.88	0.007	[1.63 - 1.89]	[1.49, 2.09]
Histidine	1.12	1.08	3.94	<0.001	[1.05 - 1.18]	[0.94, 1.31]
Isoleucine	1.81	1.76	2.94	0.031	[1.68 - 1.99]	[1.54, 2.14]
Leucine	3.04	2.94	3.23	0.046	[2.82 - 3.36]	[2.64, 3.52]
Lysine	2.74	2.62	4.63	0.012	[2.48 - 2.99]	[2.05, 3.47]
Serine	2.03	1.96	3.08	0.004	[1.90 - 2.19]	[1.75, 2.38]
Threonine	1.60	1.55	2.95	0.024	[1.50 - 1.72]	[1.40, 1.83]
Valine	1.92	1.86	2.85	0.040	[1.80 - 2.07]	[1.64, 2.22]
Seed Fatty Acid (% Total FA)						
22:0 Behenic Acid	0.56	0.54	4.33	0.022	[0.46 - 0.65]	[0.30, 0.67]

Table VII-4. Summary of Differences (p<0.05) for the Comparison of Soybean Component Levels for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean	A5547 Mean	Mean Difference (Test minus Control)		Test Range	Commercial Tolerance Interval[2]
			Mean Difference (% of A5547)	Significance (p-Value)		
Statistical Differences Observed in *Combined Site* Analysis						
Seed Vitamin (mg/100g DW)						
Vitamin E	7.69	6.24	23.26	<0.001	[6.36 - 9.62]	[0, 11.09]
Seed Antinutrient (TIU/mg DW)						
Trypsin Inhibitor	26.06	28.57	-8.79	0.014	[21.65 - 32.53]	[13.58, 46.02]
Seed Isoflavone (mg/kg DW)						
Daidzein	667.54	604.88	10.36	0.040	[188.96 - 983.26]	[0, 1585.14]
Statistical Differences Observed in *More than One Individual Site*						
Seed Amino Acid (% DW)						
Arginine Site GA	2.80	2.57	8.75	0.011	[2.72 - 2.91]	[2.22, 3.25]
Arginine Site IL	2.61	2.44	6.88	0.045	[2.49 - 2.70]	
Histidine Site GA	1.15	1.09	5.17	0.019	[1.13 - 1.16]	[0.94, 1.31]
Histidine Site IL	1.11	1.05	4.90	0.036	[1.09 - 1.13]	
Tyrosine Site AL	1.32	1.20	9.95	0.034	[1.28 - 1.33]	[0.85, 1.48]
Tyrosine Site IL	1.10	1.01	9.14	0.003	[1.07 - 1.13]	

Table VII-4. Summary of Differences (p<0.05) for the Comparison of Soybean Component Levels for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean	A5547 Mean	Mean Difference (Test minus Control)		Test Range	Commercial Tolerance Interval[2]
			Mean Difference (% of A5547)	Significance (p-Value)		
Statistical Differences Observed in *More than One Individual Site*						
Seed Fatty Acid (% Total FA)						
22:0 Behenic Acid Site AR	0.47	0.46	3.08	0.037	[0.46 - 0.48]	[0.30, 0.67]
22:0 Behenic Acid Site GA	0.60	0.55	8.24	0.029	[0.58 - 0.62]	
Seed Vitamin (mg/100g DW)						
Vitamin E Site AR	6.88	5.03	36.69	<0.001	[6.77 - 7.08]	[0, 11.09]
Vitamin E Site GA	9.16	7.77	17.81	0.011	[8.51 - 9.62]	
Vitamin E Site IL	6.72	5.31	26.56	<0.001	[6.36 - 7.27]	
Vitamin E Site NC	7.83	6.14	27.55	0.017	[7.59 - 8.19]	
Seed Antinutrient (%DW)						
Stachyose Site AL	1.84	2.37	-22.36	0.024	[1.83 - 1.89]	[0.99, 7.93]
Stachyose Site NC	4.56	5.50	-17.12	0.006	[4.32 - 4.72]	
Seed Isoflavone (mg/kg DW)						
Daidzein Site AR	767.90	658.21	16.67	0.031	[747.32 – 793.95]	[0, 1585.14]
Daidzein Site IL	890.96	803.42	10.90	0.042	[834.82 – 983.26]	

Table VII-4. Summary of Differences (p<0.05) for the Comparison of Soybean Component Levels for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean	A5547 Mean	Mean Difference (Test minus Control)		Test Range	Commercial Tolerance Interval[2]
			Mean Difference (% of A5547)	Significance (p-Value)		
Statistical Differences Observed in *One Individual Site*						
Forage Fiber (% DW)						
Neutral Detergent Fiber Site AR	49.83	38.62	29.02	0.023	[46.69 - 55.99]	[21.51, 66.01]
Seed Proximate (% DW)						
Ash Site IL	5.42	5.29	2.42	0.039	[5.20 – 5.55]	[3.74, 6.45]
Carbohydrates Site IL	36.65	39.17	-6.45	0.024	[35.60 – 37.72]	[28.17, 40.99]
Seed Amino Acid (% DW)						
Isoleucine Site GA	1.81	1.74	4.23	0.029	[1.77 - 1.84]	[1.54, 2.14]
Leucine Site GA	3.04	2.91	4.59	0.014	[2.98 - 3.09]	[2.64, 3.52]
Proline Site GA	2.00	1.94	3.56	0.025	[1.99 - 2.02]	[1.73, 2.35]
Tryptophan Site NC	0.49	0.47	4.75	0.006	[0.47 - 0.51]	[0.43, 0.59]
Valine Site GA	1.91	1.84	3.96	0.035	[1.88 - 1.94]	[1.64, 2.22]
Seed Fatty Acid (% Total FA)						
16:0 Palmitic Acid Site IL	11.53	11.71	-1.48	0.025	[11.39 - 11.63]	[8.88, 13.53]
16:1 Palmitoleic Acid Site NC	0.09	0.10	-13.81	0.012	[0.084 - 0.089]	[0.04, 0.15]

Table VII-4. Summary of Differences (p<0.05) for the Comparison of Soybean Component Levels for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean	A5547 Mean	Mean Difference (Test minus Control)		Test Range	Commercial Tolerance Interval[2]
			Mean Difference (% of A5547)	Significance (p-Value)		
Statistical Differences Observed in *One Individual Site*						
Seed Fatty Acid (% Total FA)						
18:0 Stearic Acid Site NC	4.42	4.79	-7.62	0.038	[4.34 - 4.49]	[1.88, 6.25]
18:1 Oleic Acid Site NC	19.78	21.60	-8.42	0.047	[19.21 - 20.21]	[5.01, 42.01]
18:2 Linoleic Acid Site NC	54.21	52.62	3.03	0.046	[53.89 - 54.61]	[38.57, 66.94]
20:1 Eicosenoic Acid Site GA	0.24	0.22	5.27	0.035	[0.23 - 0.24]	[0.16, 0.33]
Seed Antinutrient (TIU/mg DW)						
Trypsin Inhibitor Site GA	23.28	29.27	-20.48	0.005	[21.65 - 25.24]	[13.58, 46.02]
Seed Isoflavone (mg/kg DW)						
Genistein Site AR	807.35	680.07	18.72	0.007	[771.77 - 840.99]	[0, 1352.86]

[1]DW=dry weight; FA=fatty acid; TIU= trypsin inhibitor units
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table VII-5. Literature and ILSI Ranges for Components in Soybean Forage and Seed

Forage Tissue/Component[1]	Literature Range[2]	ILSI Range[3]
Proximate (% dw)		
Ash	5.36 – 8.91	6.72 – 10.78
Carbohydrates	62.25 – 72.28	59.8 – 74.7
Moisture (% fw)	68.50 – 78.40	73.5 – 81.6
Protein	16.48 – 24.29	14.38 – 24.71
Total Fat	2.65 – 9.87	1.30 – 5.13
Fiber (% dw)		
Acid Detergent Fiber (ADF)	23.86 – 50.69	not available
Neutral Detergent Fiber (NDF)	19.61 – 43.70	not available
Seed Tissue Component[1]	**Literature Range[2]**	**ILSI Range[3]**
Proximates (% dw)		
Ash	4.61 – 6.32	3.89 – 6.99
Carbohydrates	32.75 – 40.98	29.6 – 50.2
Moisture (% fw)	6.24 – 11.10	4.7 – 34.4
Protein	34.78 – 43.35	33.19 – 45.48
Total Fat	14.62 – 20.68	8.10 – 23.56
Fiber (% dw)		
Acid Detergent Fiber (ADF)	9.22 – 26.26	7.81 – 18.61
Neutral Detergent Fiber (NDF)	10.79 – 23.90	8.53 – 21.25
Amino Acids (% dw)		
Alanine	1.62 – 1.89	1.51-2.10
Arginine	2.57 – 3.27	2.29-3.40
Aspartic acid	4.16 – 5.02	3.81-5.12
Cystine/Cysteine	0.52 – 0.69	0.37-0.81
Glutamic acid	6.52 – 8.19	5.84-8.20
Glycine	1.59 – 1.90	1.46-2.00
Histidine	0.96 – 1.13	0.88-1.18
Isoleucine	1.59 – 2.00	1.54-2.08
Leucine	2.79 – 3.42	2.59-3.62
Lysine	2.36 – 2.77	2.29-2.84
Methionine	0.45 – 0.63	0.43-0.68
Phenylalanine	1.82 – 2.29	1.63-2.35
Proline	1.83 – 2.23	1.69-2.28
Serine	1.95 – 2.42	1.11-2.48
Threonine	1.44 – 1.73	1.14-1.86
Tryptophan	0.30 – 0.48	0.36-0.50
Tyrosine	1.27 – 1.53	1.02-1.61
Valine	1.68 – 2.09	1.60-2.20

Table VII-5. Literature and ILSI Ranges for Components in Soybean Forage and Seed (cont'd.)

Forage Tissue/Component[1]	Literature Range[2]	ILSI Range[3]
Fatty Acids	**(% dw)**	**(% total)**
8:0 Caprylic	not available	0.148 – 0.148
10:0 Capric	not available	not available
12:0 Lauric	not available	0.082 – 0.132
14:0 Myristic	not available	0.071 – 0.238
14:1 Myristoleic	not available	0.121 – 0.125
15:0 Pentadecanoic	not available	not available
15:1 Pentadecenoic	not available	not available
16:0 Palmitic	1.44 – 2.35	9.55 – 15.77
16:1 Palmitoleic	not available	0.086 – 0.194
17:0 Heptadecanoic	not available	0.085 – 0.146
17:1 Heptadecenoic	not available	0.073 – 0.087
18:0 Stearic	0.54 – 1.12	2.70 – 5.88
18:1 Oleic	2.87 – 8.82	14.3 – 32.2
18:2 Linoleic	6.48 – 11.6	42.3 – 58.8
18:3 Gamma Linolenic	not available	not available
18:3 Linolenic	0.72 – 2.16	3.00 – 12.52
20:0 Arachidic	0.04 – 0.7	0.163 – 0.482
20:1 Eicosenoic	0.026 – 0.057	0.140 – 0.350
20:2 Eicosadienoic	not available	0.077 – 0.245
20:3 Eicosatrienoic	not available	not available
20:4 Arachidonic	not available	not available
22:0 Behenic	0.044 – 0.073	0.277 – 0.595
22:1 Erucic	not available	not available
Vitamins (mg/100g dw)		
Vitamin E	1.29 – 4.80	0.19-6.17
Antinutrients		
Lectin (H.U./mg fw)	0.45 – 9.95	0.09 – 8.46
Trypsin Inhibitor (TIU/mg dw)	20.79 – 59.03	19.59 – 118.68
Phytic Acid (% dw)	0.41 – 1.92	0.63 – 1.96
Raffinose (% dw)	0.26 – 0.84	0.21 – 0.66
Stachyose (% dw)	1.53 – 2.98	1.21 – 3.50
Isoflavones		
Daidzein	224.03 – 1485.52	60.0 – 2453.5
Genistein	338.24 – 1488.89	144.3 – 2837.2
Glycitein	52.72 – 298.57	15.3 – 310.4

[1]fw=fresh weight; dw=dry weight

[2]Lundry et al. (2008).

[3]ILSI-CCD (2006). Available at http://www.cropcomposition.org/. Conversions: % dw x 10^4 = µg/g dw; mg/g dw x 10^3 = mg/kg dw; mg/100g dw x 10 = mg/kg dw; g/100g dw x 10 = mg/g dw.

Section 4. Other Information Relevant to the Safety and Nutritional Assessment of MON 87701

Having demonstrated the compositional equivalence of harvested soybean seed and forage from MON 87701 to the harvested seed and forage from conventional soybean varieties already on the market, and considering the history of safe use of the host organism, no additional information was considered necessary to support the safety and nutritional assessment of MON 87701.

Section 5. Substantial Equivalence of MON 87701 to A5547 and Conventional Soybean Varieties

A detailed compositional assessment of soybean seed and forage was presented (Section 3, Part VII) where the levels of key nutrients, antinutrients and other components in MON 87701 were examined and compared to that of the conventional soybean control, A5547, a variety with background genetics similar to that of MON 87701. Additionally, tolerance intervals representing 99% of the values of each analyte for a commercial conventional soybean population were established. Results demonstrate that the levels of key nutrients, antinutrients and other components of MON 87701 are comparable to those of conventional soybean.

The assessment compared the composition of seed and forage from MON 87701 to a conventional control and commercially available soybean varieties collected during 2007 from five field sites in U.S. soybean production regions. Compositional analyses included important nutrients (protein, fat, carbohydrates, fiber, ash, moisture, amino acids, fatty acids, and vitamin E) and antinutrients, consistent with OECD guidelines. In each assessment, MON 87701 was compared to an appropriate conventional control soybean variety that had a genetic background similar to MON 87701 but did not possess the introduced trait. In addition, the same analytes were assessed in 20 conventional soybean varieties to establish a 99% tolerance interval for each of the analytes.

In a further assessment, the overall dataset was evaluated for evidence of biologically relevant changes from a food and feed safety and nutritional perspective. Overall, statistical analyses of both forage and seed showed no significant difference ($p \geq 0.05$) between MON 87701 and the conventional soybean control for 40 of 55 comparisons from the combined-site analyses and 243 of 275 comparisons from the individual-site analyses. For the analytes where differences were noted ($p<0.05$), the magnitude of differences was generally low (most <5%), the differences were not observed consistently across all sites (individual-site analyses), and the mean values for MON 87701 were within the calculated 99% tolerance interval for the population of commercial conventional soybean varieties grown concurrently at the same field sites. Vitamin E levels were significantly higher than the control in the combined-site analysis and in four of five individual-site analyses, but were within the calculated 99% tolerance interval and, therefore, are not considered to be biologically meaningful. Forage and seed analytical component values were also comparable to published scientific literature and the ILSI-CCD database, further supporting the conclusion that soybean forage and seed produced from MON 87701 are compositionally equivalent to those of conventional soybean.

Collectively, these data and the history of safe use of soybean as a common source of processed human foods and animal feeds support a conclusion of "no concerns" for every criterion specified in the flowcharts outlined in the FDA's Food Policy document (Figure VII-1). MON 87701 is not materially different in composition, safety or nutrition from conventional soybean, other than the introduced protein conferring insect-protection. Sales or consumption of soybean seed or processed products derived from MON 87701 would be fully consistent with the FDA's Food Policy, the Federal Food, Drug and Cosmetic Act, and current practice for the development and introduction of new soybean varieties and biotechnology traits.

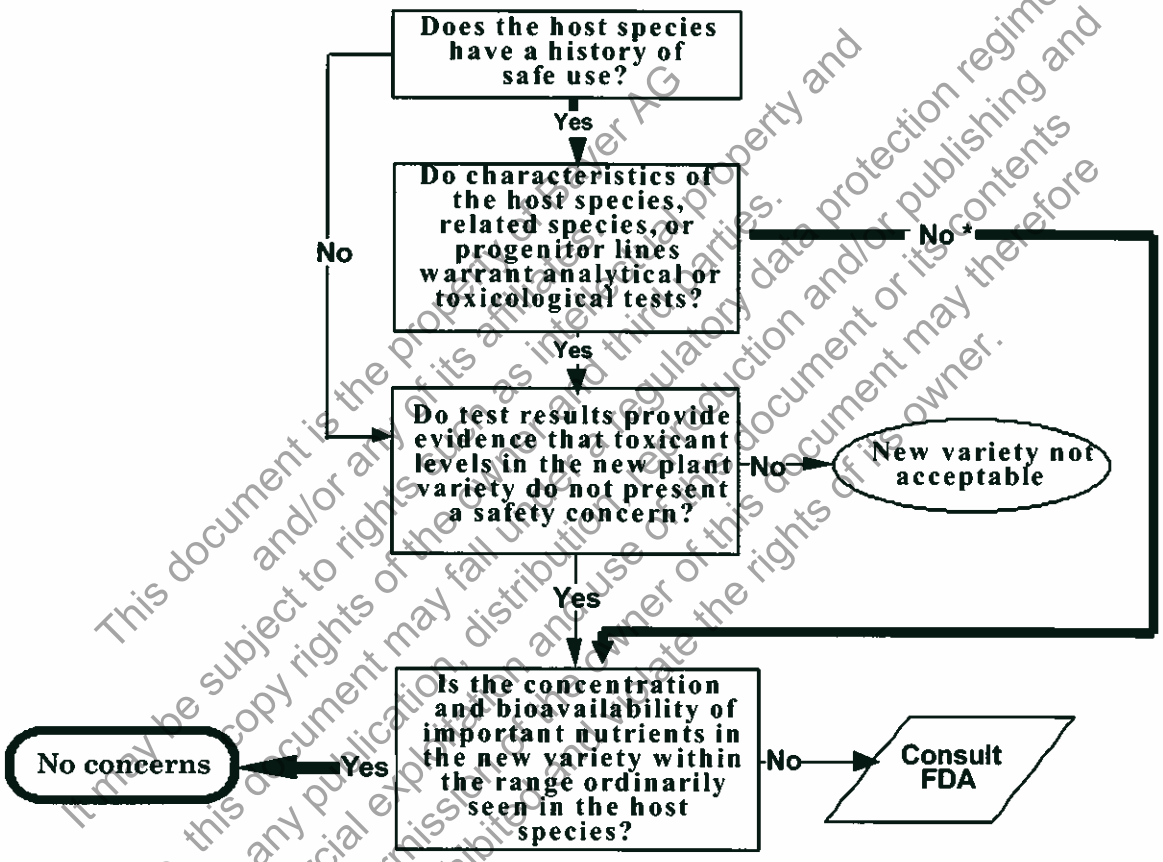

Figure VII-1. Safety Assessment of New Varieties: The Host Plant

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APPENDICES

Appendix A. Materials and Methods Used for Molecular Analyses of MON 87701

Materials

The DNA used in molecular analyses was isolated from leaf tissue of MON 87701 collected in 2007 harvested from Production Plan 07-01-59-05 (Seed lot: GLP-0705-18705-S). Additional DNA extracted from various MON 87701 generations of leaf tissues were used in generation stability analyses. The control DNA was isolated from the leaf tissue of a conventional soybean variety, A5547. The reference substance, plasmid PV-GMIR9, was used in the transformation process to develop MON 87701. Digested whole plasmid and probe templates generated from this plasmid served as positive hybridization controls. The plasmid was isolated prior to the study and its identity confirmed by restriction enzyme digestion. The 1 kb DNA extension ladder and λ DNA/*Hin*d III fragments from Invitrogen (Carlsbad, CA) were used for size estimations on agarose gels for Southern analyses. Additionally, the 500 bp ladder from Invitrogen and GeneRuler 1kb DNA ladder Plus from Fermentas were used for size estimations on agarose gels.

Characterization of the Materials

The quality of the source materials from MON 87701 and A5547 were verified by PCR analysis to confirm the presence or absence of MON 87701 except the materials used in the generational stability analyses where the identity of the materials was confirmed by the generation stability Southern blots themselves. The stability of the genomic DNA was confirmed in each Southern analysis by observation of the digested DNA sample on an ethidium bromide-stained agarose gel.

DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA from the test and control substances was isolated from soybean leaf tissue. The leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted from the processed leaf tissue using the following method. Approximately 5-6 grams of soybean leaf tissue was processed in liquid nitrogen using a mortar and pestle on dry ice. To each sample, 25 milliliters (ml) of a pre-warmed lysis solution was added (24.25 ml pre-warmed [50-60°C] CTAB extraction buffer [2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl] pH 8.0, 0.5 ml 2-mercaptoethanol [2-ME], and 0.25 ml of 10 mg/ml proteinase K for a final concentration of 2% 2-ME and 100 µg/ml proteinase K). The tube was incubated for at least 60 minutes at 50-60°C, with periodic shaking. Twenty ml of a phenol: chloroform: isoamyl alcohol (PCI 25:24:1) mixture was added to each tube and vigorously mixed by hand. The tubes were centrifuged for 10 minutes at 13,000 x g at 15-25°C and the supernatant was transferred to a pre-spun 50 ml MaXtract High Density conical tube (Qiagen, Carlsbad, CA). Twenty ml of PCI 25:24:1 was added to each tube and vigorously mixed by hand. The tubes were centrifuged for 10 minutes at 1500 x g at 15-25°C. This was repeated for a total of two MaXtract High Density extractions. After the last extraction, the upper aqueous phase was transferred to a clean 50 ml conical tube and approximately two times the volume of -20°C 100% ethanol was added. The tube was gently inverted

by hand several times to mix. To precipitate the DNA, the tubes were placed in a -20°C freezer for at least 30 minutes. To pellet the DNA, the tubes were centrifuged at 13,000 x g for 20 minutes at 1-9°C. The DNA was rinsed at least twice with 70% ethanol and residual ethanol was removed by heating at 37-65°C. The pellets were redissolved in 3 ml of TE (10 mM Tris HCl, 1 mM EDTA), pH 8.0. The tubes were incubated at 60-70°C for at least 1 hour to resuspend the pellets completely. The tubes were then centrifuged at 15,000 x g for 10 minutes at 15-25°C to remove undissolved material. The supernatants were transferred to a 13 ml Sarstedt tube and approximately 4 µl of 100 mg/ml RNase A was added to each tube. The tubes were then incubated at 60°C for 15 minutes. To remove residual polysaccharide compounds, the DNA was PEG precipitated according to draft SOP with the exception of using a smaller volume of TE buffer to resuspend the pellet, which created a more concentrated DNA solution for use in the Southern analyses.

Genomic DNA from the test substance samples used in the insert stability analyses was isolated according to draft SOP. Some of the genomic DNA from the test substance used in the T-DNA I copy number analyses was also isolated according to this SOP, except that the amount of processed leaf tissue was increased and the other volumes of material were increased accordingly. This was acknowledged in the raw data as a protocol deviation. All extracted DNA was stored in a 4°C refrigerator and/or -20°C freezer until use.

Quantification of Genomic DNA

Quantification of DNA samples was performed using a Hoefer DyNA Quant 200 Fluorometer with Roche molecular size marker IX as a DNA calibration standard.

Restriction Enzyme Digestion of Genomic DNA

Ten micrograms (µg) of genomic DNA extracted from the test and control substances was digested with the appropriate restriction enzymes according to the draft SOP in a total volume of ~500 µl using ~100 units of the restriction enzyme except for the reactions presented in Figure IV-6 which used ~50 units of restriction enzyme. For the purpose of running positive hybridization controls, ~10 µg of genomic DNA extracted from the control substance was digested and the appropriate positive hybridization control(s) were added to these digests and loaded.

DNA Probe Preparation for Southern Blot Analyses

Probes were prepared by PCR amplification of the PV-GMIR9 template using a standard procedure based on Sambrook and Russell (2001). The probes were designed based on the nucleotide content (%GC) so that the entire probe would hybridize under the conditions used. Approximately 25 ng of each probe template were radiolabeled with either ^{32}P-deoxycytidine triphosphate (dCTP) or ^{32}P-deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using the random priming method (RadPrime DNA Labeling System, Invitrogen) or by PCR. Probe locations relative to the genetic elements in plasmid PV-GMIR9 are depicted in Figure IV-2.

Southern Blot Analyses of Genomic DNA

Digested genomic DNA isolated from test and control materials was evaluated using Southern blot analyses. When multiple probes were used for the analysis, the appropriate

probe templates were used as positive hybridization controls (Figure IV-2). The plasmid DNA was digested with *Bgl* II / *Nco* I and added to conventional soybean genomic DNA as an additional positive hybridization control. The DNA was then separated by agarose gel electrophoresis. Southern blots were hybridized and washed at 50°C, 55°C, 60°C, or 65°C, depending on the melting temperature of the probe. The table below lists the temperature and radiolabeling conditions of the probes used in this study. Multiple exposures of each blot were then generated using Kodak Biomax MS film in conjunction with one Kodak Biomax MS intensifying screen in a -80°C freezer.

Probe	DNA Probe	Labeling Method	Probe labeled with dNTP (^{32}P)	Hybridization/ Wash Temperature (°C)
1	Backbone Probe 1	RadPrime	dATP	60
2	Backbone Probe 2	RadPrime	dATP	60
3	Backbone Probe 3	RadPrime	dATP	60
4	Backbone Probe 4	RadPrime	dCTP	65
5	T-DNA II Probe 5	RadPrime	dATP	55
6	T-DNA II Probe 6	RadPrime	dATP	55
7	T-DNA I Probe 7	RadPrime	dATP	50
8	T-DNA I Probe 8	RadPrime	dATP	60
9	T-DNA I Probe 9	RadPrime	dATP	55
10	T-DNA I Probe 10	RadPrime	dATP	60
11	T-DNA I Probe 11	RadPrime	dATP	55

DNA Sequence Analyses of the Insert

Overlapping PCR products were generated that span the insert and adjacent 5' and 3' flanking genomic DNA sequence in MON 87701 (Figure A-1). These products were sequenced to determine the nucleotide sequence of the insert in MON 87701, as well as determining the nucleotide sequence of the genomic DNA flanking the 5' and 3' ends of the insert. The PCR analysis was performed to amplify nine overlapping DNA fragments (Products A, B, C, D, E, F, G, H, and I) spanning the entire length of the insert. The table on the next page lists the PCR reaction and cycling conditions used in this study.

Reference:

Sambrook, J., and D. Russell. Eds., (2001). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Product Name	Product A		Product B		Product C		Product D	Product E		Product F	Product G		Product H	Product I
DNA Template (ng) in Reaction Volume (µl)	30 ng in 50 µl		48 ng in 25 µl		96 ng in 25 µl		48 ng in 25 µl	96 ng in 25 µl		48 ng in 25 µl	48 ng in 25 µl		96 ng in 25 µl	30 ng in 50 µl
Reaction Conditions	1*		2*		2		2	2			2		2	1
Cycling Conditions	Cycles	Temp Time	Cycles	Temp Time	Cycles	Temp Time	** Same Cycling conditions as Product C	Cycles	Temp Time	** Same Cycling conditions as Product C	Cycles	Temp Time	** Same Cycling conditions as Product E	** Same Cycling conditions as Product A
	1 cycle	95°C 2 min	1 cycle	94°C 2 min	1 cycle	94°C 2 min		1 cycle	94°C 2 min		1 cycle	94°C 2 min		
		94°C 30 sec		94°C 45 sec		94°C 45 sec			94°C 45 sec		10 cycles	94°C 45 sec		
	40 cycles	65°C 30 sec	35 cycles	69°C 45 sec	35 cycles	68°C 45 sec		35 cycles	65°C 45 sec		Decrease 1°C per cycle	70°C 45 sec		
		72°C 1.5 min		72°C 5 min		72°C 5 min			72°C 5 min			72°C 5 min		
	1 cycle	72°C 5 min	1 cycle	72°C 10 min	1 cycle	72°C 10min		1 cycle	72°C 10 min			94°C 45sec		
											35 cycles	60°C 45 sec		
												72°C 5 min		
											1 cycle	72°C 10 min		

1* 2 Mm $MgSO_4$, 0.2 µM of each primer, 0.1 mM each dNTP, 1M Betaine, and 0.2 U KOD Hot Start DNA Polymerase

2* 1 Mm $MgSO_4$, 0.8µM of each primer, 0.1 mM each dNTP, 1M Betaine, and 0.02 U KOD Hot Start DNA Polymerase

Aliquots of each PCR product were separated on 0.8 % (w/v) agarose E-gels (Invitrogen) and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. To remove residual excess primer following PCR amplification, Products B and C were treated with a 2 µl mixture of Exonuclease I (EXO) (USB Cleveland, OH) and Shrimp Alkaline Phosphatase (SAP) (USB) (0.1 Units (U)/ µl each) per 5µl of PCR product and cycled as follows: one cycle at 37°C for 15 minutes and one cycle at 80°C for 15 minutes. Not all products were treated with EXO-SAP prior to sequencing as documented in the raw data. The PCR products were sequenced using multiple primers, including some of the primers used for PCR amplification. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry (ABI, Foster City, CA).

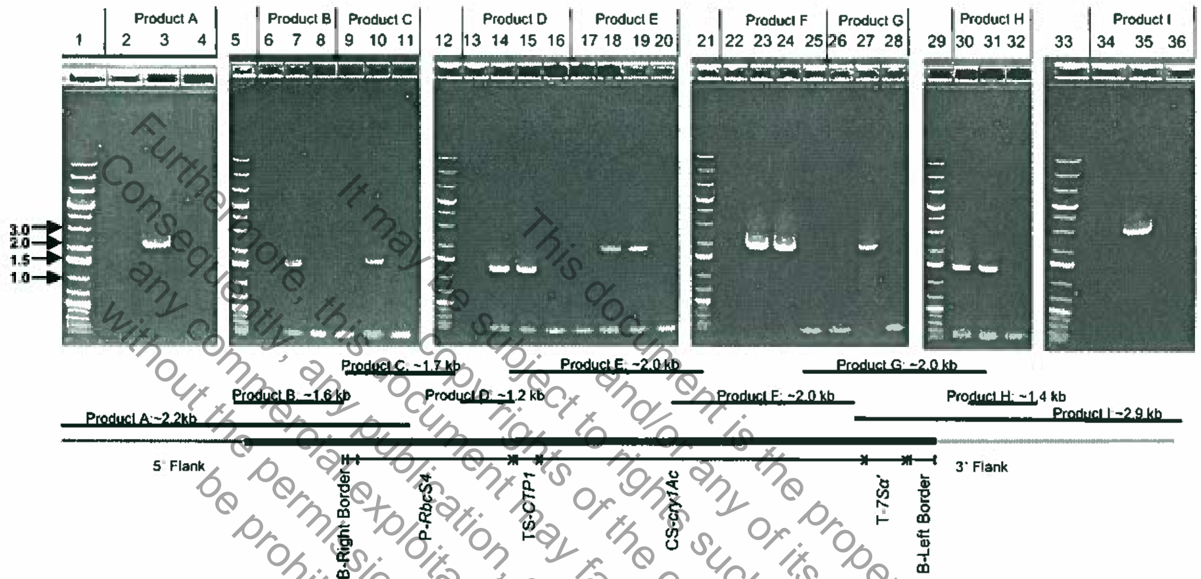

Figure A-1. Overlapping PCR Analysis Across the Insert in MON 87701

PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 87701 were performed on MON 87701 genomic DNA extracted from leaf tissue (Lanes 3, 6, 11, 15, 21, 25, and 30). Lanes 2, 6, 9, 13, 17, 22, 26, 30, and 34 contain reactions with conventional soybean control DNA extracted from leaf tissue. Lanes 4, 8, 11, 16, 20, 25, 28, 32, and 36 are reactions containing no template DNA. Lanes 15, 19, and 24 contain reactions with PV-GMIR9 control DNA. Lanes 1, 5, 12, 21, 29, and 33 contain Fermentas GeneRuler™ 1 kb Plus DNA Ladder. Lanes are marked to show which product has been loaded and is visualized on the agarose E-gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87701 that appears at the bottom of the figure. Three to six µl of each of the PCR products was loaded on the gel. PCR amplicons reported in this figure were not necessarily used in sequencing, but are representative of the study data.

Lanes	Lanes	Lanes	Lanes
1.) GeneRuler™ 1 kb Plus DNA Ladder	10.) MON 87701 genomic DNA	19.) PV-GMIR9 control DNA	28.) No template DNA control
2.) Conventional soybean control DNA	11.) No template DNA control	20.) No template DNA control	29.) GeneRuler™ 1 kb Plus DNA Ladder
3.) MON 87701 genomic DNA	12.) GeneRuler™ 1 kb Plus DNA Ladder	21.) GeneRuler™ 1 kb Plus DNA Ladder	30.) Conventional soybean control DNA
4.) No template DNA control	13.) Conventional soybean control DNA	22.) Conventional soybean control DNA	31.) MON 87701 genomic DNA
5.) GeneRuler™ 1 kb Plus DNA Ladder	14.) MON 87701 genomic DNA	23.) MON 87701 genomic DNA	32.) No template DNA control
6.) Conventional soybean control DNA	15.) PV-GMIR9 control DNA	24.) PV-GMIR9 control DNA	33.) GeneRuler™ 1 kb Plus DNA Ladder
7.) MON 87701 genomic DNA	16.) No template DNA control	25.) No template DNA control	34.) Conventional soybean control DNA
8.) No template DNA control	17.) Conventional soybean control DNA	26.) Conventional soybean control DNA	35.) MON 87701 genomic DNA
9.) Conventional soybean control DNA	18.) MON 87701 genomic DNA	27.) MON 87701 genomic DNA	36.) No template DNA control

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

Appendix B. Materials and Methods Used for Characterization of Cry1Ac Protein Produced in MON 87701

Materials

The MON 87701-produced Cry1Ac protein (Orion lot 10000801) was purified as described below from harvested seed of MON 87701 prior to the initiation of this study. The seed used for the isolation of Cry1Ac protein, lot GLP-0612-17898-S, was produced under protocol IP036 by the Monsanto Trait Development group. The identity of the harvested seed containing MON 87701 was confirmed by event-specific PCR; a copy of the Certificate of Analysis for this seed lot is archived in the Monsanto Regulatory archives with the records documenting protein isolation. The purified MON 87701-produced protein was stored in a -80 °C freezer in a buffer solution containing 50 mM CAPS, pH 10.8, 1 mM EDTA, 2 mM DTT, 1 mM benzamidine HCl, ~30 mM NaCl, ~1% ethylene glycol, and a trace amount of PMSF. The records describing the purification of this MON 87701-produced protein are archived under the Orion lot 10000801 in the Monsanto Regulatory archives.

The *E. coli*-produced Cry1Ac reference protein (Orion lot 10000804) was purified from the fermentation of *E. coli* transformed plasmid. The DNA sequence encoding this Cry1Ac reference protein was confirmed both prior to and following fermentation of *E. coli*. Records pertaining to the purification of this *E. coli*-produced reference protein are archived under Orion lot 10000804. The *E. coli*-produced Cry1Ac reference standard was previously characterized (APS Characterization Plan 20-100133) and a copy of the Certificate of Analysis (COA) is included as in Monsanto archives. The *E. coli*-produced Cry1Ac protein was stored in a -80 °C freezer in a buffer solution (50 mM CAPS, 1 mM benzamidine-HCl, 1 mM EDTA, and 2.5 mM DTT, pH 10.25) at a total protein concentration of 1.4 mg/ml.

The *E. coli*-produced Cry1Ac protein was used as a reference protein for the immunoblot assay, the functional activity assay, and the purity and molecular weight evaluation, and as a negative control in the glycosylation analysis.

Description of Assay Controls

Protein molecular weight standards (BioRad, Hercules, CA) were used to calibrate SDS-PAGE gels and verify protein transfer to PVDF membranes. A peptide mixture (CalMix2 from the Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis. A PTH-amino acid standard mixture (Applied Biosystems) was used to calibrate the sequencer for N-terminal sequence analysis. Dilutions of an amino acid standard (NIST) were used to generate a standard curve for determining protein concentration. Transferrin and horseradish peroxidase (both from Sigma, St. Louis, MO) were used as positive controls for glycosylation analysis. CandyCane Glycoprotein Molecular Weight Standards (Molecular Probes, Eugene, OR) were used as molecular weight markers and positive and negative controls for glycosylation analysis.

Protein Purification

The MON 87701-produced Cry1Ac protein was purified from harvested seed of MON 87701 prior to initiation of this study. The purification procedure was not performed under a GLP protocol or plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. The Cry1Ac protein was purified at 4 °C from an extract of ground seed using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

Approximately one kilogram of MON 87701 seed was ground to a powder using a Perten Laboratory Mill. Ground material was stored in a -80 °C freezer until use. To de-fat the seed powder, two ~500 g batches were extracted four times with warm hexane (~50 °C) added at a ratio of ~3 ml of hexane per gram of ground seed, and then air dried. The final weight of the de-fatted seed powder was ~760 g.

The Cry1Ac protein was purified from a total of four ~100 g aliquots of the ground, de-fatted MON 87701 seed in four separate runs that were pooled to generate the final MON 87701-produced Cry1Ac protein sample.

Each run included the following series of extraction and chromatography steps:

PBS wash – To promote extraction of neutral pH-soluble proteins, seed powder was stirred in cold PBS pH 7.0, 1 mM benzamidine HCl, 0.5 mM PMSF, 1% PVPP at 7.5 ml/g powder for about 1 h. The Cry1Ac-containing washed ground seed pellets were collected by centrifugation.

CAPS solubilization – Cry1Ac protein was extracted from the washed ground seed pellet with CAPS solubilization buffer (100 mM CAPS, pH 10.8, 1 mM benzamidine HCl, 0.5 mM PMSF, 1 mM EDTA, 10 mM DTT) added at 5 ml/g of starting powder. The suspension was stirred for 1-2 h, and solubilized proteins, including Cry1Ac, were separated from insoluble material by centrifugation.

$(NH_4)_2SO_4$ precipitation and re-solubilization – An ammonium sulfate precipitate was prepared by the addition of ammonium sulfate salt to the CAPS solubilization supernatant to a final saturation of 40%. After mixing for 2-4 h, precipitated proteins were collected by centrifugation, and were re-solubilized in 50 mM Bis-Tris propane, pH 9.0, 150 mM NaCl, 1 mM EDTA, 2 mM benzamidine HCl, 1 mM PMSF, 5 mM DTT at 0.75 ml per starting ml of CAPS supernatant. Insoluble material was removed by centrifugation at 37000×g for 1 h, and the supernatant was diluted with 50 mM Bis-Tris propane, pH 9.0, 1 mM EDTA, 2 mM benzamidine HCl, 1 mM DTT to bring the NaCl concentration to 50 mM.

Anion exchange chromatography – The diluted Cry1Ac-containing protein solution (18-26 column volumes, depending on the run) was loaded at a flow rate of 1.4-2.4 ml/min onto a CaptoQ (GE Healthcare, Piscataway, NJ) anion exchange column (100 ml, 50 x 50 mm) equilibrated with 50 mM Bis-Tris propane, pH 9.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine HCl buffer. After loading, the column was washed with 2.7-3.5 column volumes of the equilibration buffer. Proteins were then eluted in two steps, the first consisting of 4-7 column volumes of 50 mM Bis-Tris propane, pH 9.0, 300 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine HCl buffer, and the second

consisting of 3-4 column volumes of 50 mM Bis-Tris propane, pH 9.0, 600 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine HCl buffer. Cry1Ac protein was predominantly present in the second elution step, which was collected as a single fraction. All wash and elution steps were carried out at a flow rate of 6 ml/min.

Immunoaffinity chromatography – For immunoaffinity chromatography, resin was prepared by binding and then chemically cross-linking a monoclonal anti-Cry1Ac antibody to protein A agarose (Sigma, St. Louis, MO). The Cry1Ac-containing fraction from the anion exchange column (~300-400 ml, depending on the run) was loaded on to the immunoaffinity column (6 ml; 20 × 15 mm, h × d) equilibrated with 5-10 column volumes of 50 mM Bis-Tris propane, pH 9.0, 600 mM NaCl, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl, 1 mM PMSF. To maximize Cry1Ac binding to the immunoaffinity column, the load solution was recirculated through the column overnight. Following the load, the column was washed with 4-6 column volumes of the equilibration buffer. Proteins were then eluted in two elution steps, the first consisting of 4-7 column volumes of 50 mM Bis-Tris propane, pH 9.0, 800 mM NaCl, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl, 1 mM PMSF buffer, and the second consisting of ~7 column volumes of 50 mM Bis-Tris propane, pH 9.0, 800 mM NaCl, 30% (v/v) ethylene glycol, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl, 1 mM PMSF. Cry1Ac protein was predominantly present in the second elution step, which was collected as several ~ 4 ml fractions. Equilibration, load, wash, and first elution step were carried out at a flow rate of ~2 ml/min; the flow rate for the final elution step was ~0.7 ml/min. Fractions collected from the final elution step were evaluated for the presence and amount of Cry1Ac by quantitative immunoblot, and fractions with the highest amounts of Cry1Ac protein were pooled for each run.

All operations described above were carried out at 4°C. Following the final immunoaffinity chromatography run, the four batches of purified Cry1Ac protein were pooled. The pooled sample (~140 ml) was concentrated ~9-fold by diafiltration using a polysulfone hollow fiber cartridge with a 30 kDa molecular weight cut-off, diluted ~10-fold with a buffer containing 50 mM CAPS, pH 10.8, 1 mM EDTA, 2 mM DTT, 1 mM benzamidine HCl, and re-concentrated by diafiltration using the same cartridge. The final concentrated solution of the Cry1Ac protein (~1 ml) was diluted with the same buffer to a final volume of ~4 ml. This material was submitted to the APS program and assigned lot number 22-100135. The lot number was later reassigned as Orion lot 10000801 due to adoption of a new tracking database. The physical appearance of the protein solution was a clear liquid.

<u>Molecular Weight and Purity Estimation-SDS-PAGE</u>

Aliquots of the *E. coli*-produced and MON 87701-produced Cry1Ac proteins were mixed with 5 × sample buffer (0.31 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 25% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue) to a final total protein concentration of 22 ng/µl and 17 ng/µl, respectively. The MON 87701-produced protein was analyzed in duplicate at 95, 189, and 284 ng of total protein per lane. The *E. coli*-produced Cry1Ac reference standard was loaded at 198 ng total protein, in a single lane. The Broad Range Molecular Weight marker (Bio-Rad, Hercules, CA) was loaded at 360 ng total protein. All samples were heated in a thermo-block at 95.8 °C for 5 min and

applied to a pre-cast tris-glycine 4-20% gradient polyacrylamide gel. Electrophoresis was performed at a constant voltage of 125 V for 105 min.

The gel was stained using a Silver Staining Kit from Owl Separation Systems (Portsmouth, NH) according to the manufacturer's protocol. The gel was fixed for 15 min in 100 ml of Fixing Solution I. This was followed by incubation for 15 min in 100 ml of Fixing Solution II. Next, the gel was incubated in 100 ml of Pretreatment Solution for 10 min, followed by a 5 min wash in 100 ml of deionized water. The gel was stained using 100 ml of Silver Staining Solution for 12 min, followed by three 3-5 min washes, each in 100 ml of deionized water. Next the gel was incubated in 100 ml of Developer for 5 min, followed by addition of 5 ml of Stopper Solution to the Developer and incubation for 15 min. Finally, the gel was washed twice for 10 min each in 100 ml of deionized water. All incubations occurred at room temperature with gentle shaking.

Immunoblot Analysis-Immunoreactivity

Immunoblot analysis was performed to confirm the identity of the MON 87701-produced Cry1Ac protein and compare immunoreactivity of the MON 87701-produced and *E. coli*-produced proteins. The MON 87701-produced Cry1Ac protein and the *E. coli*-produced Cry1Ac reference protein (each corrected for the purity of the full-length protein), were loaded on gels at 10, 20, or 30 ng per lane. Each protein was mixed with 5× sample buffer, heated at 96.2 °C for 5 min, and applied to a pre-cast tris-glycine 4-20% gradient polyacrylamide gel. Electrophoresis was performed at a constant voltage of 200 V for 60 min. Precision Plus Dual Color molecular weight marker (Bio-Rad, Hercules, CA) was used to verify electrotransfer of protein to the membrane and estimate the size of the immunoreactive bands. Electrotransfer to a PVDF membrane was performed at a constant voltage of 100 V for 44 min.

The membrane was blocked overnight with 5% (w/v) NFDM in 1× PBST. The membrane was probed with a 1:500 dilution of goat affinity-purified anti-Cry1Ac antibody (Orion lot 10000963) in PBST containing 1% (w/v) NFDM for 1 h. Excess antibody was removed using three 5 min washes with PBST. Finally, the membrane was probed with HRP-conjugated anti-goat IgG (Pierce, Rockford, IL) at a dilution of 1:10000 in PBST containing 1% (w/v) NFDM for 2 h. Excess HRP-conjugate was removed using three washes, each at least 5 min, with PBST. The blocking step was performed at 4°C. All other incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (GE Healthcare, Piscataway, NJ) and exposed (1, 2, and 5 min) to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor.

Analysis of the film was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software.

MALDI-TOF Tryptic Mass Map Analysis

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry was used to confirm the identity of the MON 87701-produced Cry1Ac protein. The MON 87701-produced Cry1Ac protein was subjected to electrophoresis on an SDS-polyacrylamide gel. The protein sample was mixed with 5× DTT-containing

sample buffer (250 mM Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.5 M DTT) heated at 98.6°C for 5 min, and loaded across eight lanes of a pre-cast tris-glycine 4-20% gradient polyacrylamide gel. Precision Plus Dual Color molecular weight marker was loaded to enable estimation of molecular weight. Electrophoresis was performed at a constant voltage of 125 V for 105 min. Proteins were stained with Brilliant Blue G Colloidal stain (Sigma, St. Louis, MO) for 1 h, and destained according to manufacturer's protocol with 3 h of destaining in Destain Solution B prior to gel scanning.

The band representing full-length MON 87701-produced Cry1Ac protein was excised from several lanes of the gel, destained, reduced, and alkylated. Briefly, each excised gel band was destained for 30 min by incubation in 100 µl of destain solution in a microfuge tube. Following destaining, each excised gel band was incubated in 100 µl of 100 mM ammonium bicarbonate buffer for 40 min at room temperature. Each gel band was reduced in 100 µl of 10 mM DTT solution for 2 h at 37°C. Each band was alkylated by the addition of 100 µl of 20 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 25 min in the dark. Each gel band was subsequently washed in 200 µl of 25 mM ammonium bicarbonate buffer for 15-45 min at room temperature. This step was repeated two additional times, following which each gel band was dried using a Speed Vac concentrator. Three gel bands were combined and rehydrated with 60 µl of 0.02 µg/µl trypsin in 25 mM ammonium bicarbonate, 10% acetonitrile, and the sample was incubated for about 1 h at room temperature. Next, excess liquid was removed and the sample was incubated overnight at 37 °C in 120 µl of 25 mM ammonium bicarbonate, 10% acetonitrile. The following day, the sample was sonicated for 5 min, and the supernatant was transferred to a new tube and dried using a Speed Vac concentrator (Extract 1). The gel material was resuspended in 90 µl 60% acetonitrile, 0.1% TFA, 0.1% octyl-β-D-glucopyranoside solution, and sonicated for 5-10 min. After transfer of the supernatant to a new tube, this step was repeated one time, and the combined supernatants were dried using a Speed Vac concentrator (Extract 2). Extracts 1 and 2 were each resuspended in 20 µl 0.1% TFA and then dried using a Speed Vac concentrator. Extract 1 was resuspended in 5 µl of 50% acetonitrile, 0.1% TFA, while Extract 2 was resuspended in 10 µl of the same solution. Each extract was sonicated for 5 min. The extracts were then ready for loading onto the MALDI-TOF sample plate.

Mass spectral analyses were performed as follows. Mass calibration of the instrument was performed using an external peptide mixture (CalMix 2; Applied Biosystems, Foster City, CA). Extract 1 and Extract 2 samples (0.1-0.25 µl) were co-crystallized with 0.75 µl each of the following matrix solutions: α-cyano-4-hydroxy cinnamic acid (α-cyano), dihydroxybenzoic acid (DHB), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) at separate locations on the analysis plate. The samples in α-cyano matrix were analyzed in the 500 to 6000 Da range using 200 shots at a laser intensity setting of 2511. The laser intensity setting is a unit-less MALDI-TOF instrument-specific value. The samples in DHB matrix were analyzed in the 550 to 6000 Da range using 200 shots at a laser intensity setting of 3101. The samples in sinapinic acid matrix were analyzed in the 900 to 8000 Da range using 200 shots at a laser intensity setting of 3247. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993), except above 3000 Da, where mass-averaged values were

observed. GPMAW32 software (Applied Biosystems, Foster City, CA) was used to generate a theoretical trypsin digest of the expected Cry1Ac protein sequence, which was based upon the nucleotide sequence of the inserted cry1Ac gene and the N-terminal sequence analysis that identified the amino terminus of the protein. Masses were calculated for each theoretical fragment and compared to the raw mass data. Experimental masses (MH+) were assigned to ion peaks in the 500 to 1000 Da range if there were two or more isotopically resolved ion peaks, and in the 1000 to 8000 Da range if there were three or more isotopically resolved ion peaks in the spectra. Ion peaks were not assessed if the ion peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal ±2 Da from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data.

N-terminal Sequence Analysis

N-terminal sequence analysis was used to confirm the identity of the MON 87701-produced Cry1Ac protein. The MON 87701-produced Cry1Ac protein was subjected to electrophoresis on an SDS-polyacrylamide gel. MON 87701-produced Cry1Ac protein was mixed with 5 × DTT-containing sample buffer, heated at 98.6 °C for 5 min and then loaded across eight lanes of a pre-cast tris-glycine 4-20% gradient polyacrylamide gel. Precision Plus Dual Color molecular weight marker was used to estimate molecular weights and verify protein transfer to a PVDF membrane. Electrophoresis was performed at a constant voltage of 125 V for 105 min. Electrotransfer to a PVDF membrane was performed at a constant voltage of 25 V for 2 h. After transfer the blot was washed in deionized water three times for 2-5 min each, then briefly (≤2 min) stained in Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad, Hercules, CA). The blot was destained in Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad, Hercules, CA) for ~5 min, and the blot image was captured using a Bio-Rad GS-800 densitometer with the supplied Quantity One software.

Two bands were excised from the stained membrane: a band with a molecular weight of ~133 kDa, corresponding to full-length Cry1Ac protein, and a band with a molecular weight of ~75 kDa that, by purity analysis, represented ~10% of the total protein. N-terminal sequence analysis was performed on each of the excised bands for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785A Programmable Absorbance Dectector and Procise Control Software (version 1.1a) was used. Chromatographic data were collected using Atlas software (Thermo Scientific, Woburn, MA). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (β-lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the test protein to verify that the sequencer met acceptable performance criteria for repetitive yield and sequence identity.

Glycosylation Analysis

Glycosylation analysis was used to determine whether the MON 87701-produced Cry1Ac protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 87701-produced Cry1Ac protein, the *E. coli*-produced Cry1Ac reference protein, and the positive controls, transferrin and horseradish peroxidase (both from Sigma, St. Louis, MO), were each mixed with 5× sample buffer. These samples were heated at 96 °C for 4 min, cooled, and loaded on a tris-glycine 4-20% gradient polyacrylamide gel. Both *E. coli*- and MON 87701-produced Cry1Ac proteins were loaded at 50 and 100 ng purity-corrected for the full-length protein. The Precision Plus Dual Color pre-stained protein molecular weight was loaded to verify electrotransfer of the proteins to the membrane and as markers for molecular weight, and the CandyCane Glycoprotein Molecular Weight Standard (Molecular Probes, Eugene, OR) was loaded as markers for molecular weight and to provide additional positive and negative controls for glycosylation. Electrophoresis was performed at a constant voltage of 150 V for 15 min, then 200 V for 55 min. Electrotransfer to a PVDF membrane was performed at a constant voltage of 25 V for 80 min.

Carbohydrate detection was performed directly on the PVDF membrane using the Pro-Q Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes, Eugene, OR). All steps were performed at room temperature. The PVDF membrane was fixed in two changes of 25 ml each of a solution containing 50% methanol and 5% glacial acetic acid, with the first fix step for 60 min and the second overnight. Two 15 min washes (50 ml each) of 3% (v/v) glacial acetic acid (wash solution) were followed by a 20 min oxidation in 25 ml of the kit-supplied oxidizing solution. After oxidation, three 15 min washes in wash solution prepared the membrane for staining. The blot was incubated in 25 ml of Pro-Q Emerald Staining Solution prepared as recommended for blot staining. After 75 min of staining in the dark, two 15 min washes were followed by one 20 min wash, all in 50 ml of wash solution. The final wash cycles included two 1 min deionized water washes followed by three 25 ml, 5 min washes in 100% methanol. Last, the blot was washed for 10 min in deionized water. The blot was then scanned using the BioRad Molecular Imager FX using the Alexa 488 illumination setting in order to visualize fluorescent signal from the glycosylated proteins.

After glycosylation analysis, the blot was stained to visualize the proteins present on the membrane. The blot was stained for 2 min in Coomassie Brilliant Blue R-250 Staining Solution (BioRad). The blot was destained in Coomassie Brilliant Blue R-250 Destaining Solution (BioRad) for ~15 min, and the blot image was captured using a BioRad GS-800 densitometer with the supplied Quantity One software.

Functional Activity Assay

The functional activities of the MON 87701-produced Cry1Ac protein and the *E. coli*-produced Cry1Ac reference protein were compared using an insect bioassay. Aliquots of MON 87701-produced Cry1Ac protein and *E. coli*-produced Cry1Ac reference protein were transferred to the Monsanto Ecological Technology Center for testing in an assay using corn earworm (CEW; Helicoverpa zea), an insect species known to be susceptible to Cry1Ac protein (MacIntosh et al., 1990). Dose-response assays were performed for Cry1Ac proteins from both sources in parallel and assays were repeated on three separate

days to estimate the mean EC_{50} value, the effective concentration necessary to inhibit CEW growth by 50% relative to the control response.

CEW Bioassay

Materials:

Plant-Produced Cry1Ac protein, *E. coli*-produced Cry1Ac Reference Standard Protein and Control Substance:

The reference standard, an *E. coli*-produced Cry1Ac protein (Orion ID: 10000804) and a plant-produced Cry1Ac protein (Orion ID: 10000801) from the harvested seed of MON 87701, were received from the Monsanto Product Characterization Center (PCC). The total protein concentration of the *E. coli*-produced Cry1Ac protein aliquots was 1.4 mg/mL, with a purity of 80%, and a purity corrected concentration of 1.1 mg Cry1Ac/mL. The total protein concentration of the plant-produced Cry1Ac protein aliquots were 42 μg/mL with a purity of 77%, and a purity corrected concentration of 32 μg Cry1Ac/mL. The *E. coli*-produced Cry1Ac protein was suspended in 50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, 1 mM benzamidine-HCl buffer, while the plant-produced Cry1Ac protein was suspended in 50 mM CAPS, pH 10.8, 1 mM EDTA, 2 mM DTT, 1 mM benzamidine, and <1% ethylene glycol buffer. Additionally, the buffers used to store the *E. coli*-produced and the plant-produced proteins, 50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mMDTT, and 1 mM benzamidine-HCl (lot # G-826331-A), and "Soy Cry1Ac Sample Buffer" (lot # G-824555B) were received from the PCC. The plant-produced and *E. coli*-produced Cry1Ac proteins were stored in a -80°C freezer and the buffers were stored in a 4°C refrigerator.

Methods:

Insects. CEW were obtained from Benzon Research Inc (Carlisle, PA). Insect eggs were incubated at temperatures ranging from 10° C to 27° C, to achieve the desired hatch time.

Bioassays. CEW were used to measure biological activity of the MON 87701-produced and *E. coli*-produced Cry1Ac protein samples. The bioassay was replicated three times on separate days with separate batches of insects. The MON 87701-produced and *E. coli*-produced substances were run in parallel during each bioassay. Each bioassay replicate for the *E. coli*-produced and MON 87701-produced Cry1Ac proteins consisted of a series of six dilutions yielding a dose series with a two-fold separation factor ranging from 0.00065 – 0.020 μg Cry1Ac protein/mL diet and two buffer controls. All dose levels, including the buffer controls, contained an equal volume and composition of buffer. The Cry1Ac protein dosing solutions were prepared by diluting the protein with purified water and incorporating the dilution into a Southland agar-based insect diet (Lake Village, AR). This dose series in diet was chosen to adequately characterize the dose-effect relationship on CEW weight gain for the proteins from both sources. The diet mixture was then dispensed in 1 mL aliquots into a 128 well tray (# BAW128, Bio-Serv, Frenchtown, NJ). Insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a

ventilated adhesive cover (# BACV16, Bio-Serv, Frenchtown, NJ) and the insects were allowed to feed for a period of approximately seven days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14 light:10 dark. The number of surviving insect and the combined weight of the surviving insects at each dose level was recorded at the end of the 7-day incubation period.

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Appendix C. Materials and Methods Used for the Analysis of the Levels of Cry1Ac Protein in MON 87701

Materials

Tissue samples analyzed in this study were produced from five field sites in the U.S. during the 2007 season from seed lot GLP-0612-17898-S for MON 87701 and GLP-0612-17895-S for control. The control line was A5547, which is a conventional variety and does not contain the *cry1Ac* coding region. Samples were stored in a -80°C freezer throughout the study. An *E. coli*-produced Cry1Ac protein (Monsanto APS; Orion lot # 10000780) was used as a reference standard for the assay.

Characterization of the Materials

The identities of the test and control substances were confirmed by analysis of the starting seed DNA by an event-specific polymerase chain reaction (PCR) method and the results were archived under the seed lot numbers. The seed samples harvested from the field were also verified by PCR and the resulting Verification of Identity was archived under the starting seed lot numbers, following the Monsanto standard operating procedure.

Field Design and Tissue Collection

Production Plan 07-01-71-01 was initiated during the 2007 planting season to generate test and control substances at various soybean-growing locations in the U.S. The field sites were as follows: Baldwin County, AL (site code AL); Jackson County, AR (site code AR); Clarke County, GA (site code GA); Jackson County, IL (site code IL); and Wayne County, NC (site code NC). These field sites were representative of soybean producing regions suitable for commercial soybean production. At each site, three replicated plots of soybean plants containing MON 87701, as well as the negative control, were planted using a randomized complete block field design. Over-season leaf (OSL 1-4), root, forage, and seed tissues were collected from each replicated plot at all field sites. The over season leaf samples were collected four times at different growth stages: (1) V3 – V4 stage, (2) V6 – V8 stage, (3) V10 – V12 stage, and (4) V14 – V16. Production Plan 07-01-71-02 was initiated during the 2007 planting season to generate test and control substances at Jackson County, IL (site code IL) in the U.S. At this site, plots of plants containing MON 87701, as well as the negative control, were planted using a single plot field design. Pollen/anther tissues were collected from each plot. Throughout both field productions, sample identity was maintained by using unique sample identifiers and proper chain-of-custody documentation. All tissue samples, except harvested seed, were stored in a -80°C freezer and shipped on dry ice to the Monsanto processing facility in Saint Louis, Missouri. Harvested seed samples were stored and shipped at ambient temperature.

Over-season leaf tissue samples were collected from the youngest set of fully expanded trifoliate leaves at the following growth stages: OSL1 at V3-V4 growth stage; OSL2 at V6-V8; OSL3 at V10-V12; and OSL4 at V14-V16. The root and forage tissues were collected at approximately the R6 growth stage, and the above-ground portion of the

plant was labeled as the forage, and the below ground portion was washed and labeled as root tissue. Harvested seed samples were collected at the R8 growth stage.

Tissue Processing and Protein Extraction

All tissue samples produced at the field sites were shipped to Monsanto's processing facility in Creve Coeur, MO. During the processing step, dry ice was combined with the individual samples, and vertical cutters or mixers were used to thoroughly grind and mix the tissues. Processed samples were transferred into capped 15 ml tubes and stored in a -80°C freezer until use.

The Cry1Ac proteins were extracted from soybean tissues using a Harbil mixer (Harbil Industries, Compton, CA) and the appropriate amount of Tris-borate buffer with L-ascorbic acid (TBA) [0.1 M Tris, 0.1 M $Na_2B_4O_7 \cdot 10H_2O$, 0.01 M $MgCl_2$, 0.05% (v/v) Tween-20 at pH 7.8, and 0.2% (w/v) L-ascorbic acid]. Insoluble material was removed from the extracts by using a Serum Filter System (Fisher Scientific, Pittsburgh, PA), except pollen, which were centrifuged. The extracts were aliquoted and stored in a -80°C freezer until analyzed.

Anti-Cry1Ac Antibodies

Mouse monoclonal antibody clone M19-N4-A6, also known as M19 (IgG1 isotype, kappa light chain; lot 7495955) specific for the Cry1Ac protein was purified from mouse ascites fluid using Protein-A Sepharose affinity chromatography and was used as the capture antibody in the Cry1Ac ELISA. The concentration of the purified IgG was determined to be 6.0 mg/ml by spectrophotometric methods. Production of the M19 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 10 mM sodium phosphate, 150 mM sodium chloride, and 15 mM sodium azide.

Goat antibodies (lot G-805044) specific for Cry1Ac were purified using Protein-G Agarose affinity chromatography. The concentration of the purified IgG was determined to be 3.7 mg/ml by spectrophotometric methods. The purified antibody was stored in 1X phosphate-buffered saline (PBS), pH 7.4, and coupled with biotin (Pierce, Rockford, IL) according to the manufacturer's instructions, and assigned lot number G-805045. The detection reagent was NeutrAvidin (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP).

Cry1Ac ELISA Method

The Cry1Ac ELISA was performed according to a draft SOP. Mouse anti-Cry1Ac antibody was diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) to a final concentration of 2.0 µg/ml, and immobilized onto 96-well microtiter plates followed by incubation in a 4°C refrigerator for ≥8 h. Prior to each step in the assay, plates were washed with 1× phosphate-buffered saline with Tween-20 (PBST). Cry1Ac protein standard or sample extract was added at 100 µl per well and incubated for 1 h at 37°C. The captured Cry1Ac protein was detected by the addition of 100 µl per well of biotinylated goat anti-Cry1Ac antibodies and NeutrAvidin-HRP (Pierce, Rockford, IL). Plates were developed by adding 100 µl per well of HRP substrate, 3,3',5,5'-tetramethyl-benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD).

The enzymatic reaction was terminated by the addition of 100 µl per well of 3 M H_3PO_4. Quantification of the Cry1Ac protein was accomplished by interpolation from a Cry1Ac protein standard curve that ranged from 1.0 – 32 ng/ml.

Moisture Analysis

A homogeneous tissue-specific site pool (TSSP) was prepared using the test and control samples of a given tissue type grown at a given site. These pools were prepared for all tissues, except pollen, in this study. All tissues, except pollen, were analyzed for moisture content using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO). The mean percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$DWCF = 1 - [Mean\ \%\ TSSP\ Moisture / 100]$$

The DWCF was used to convert protein levels assessed on a µg/g fresh weight (fwt) basis into levels reported on a µg/g dry weight (dwt) basis using the following calculation:

$$Protein\ Level\ in\ Dry\ Weight = \frac{(Protein\ Level\ Fresh\ Weight)}{(DWCF)}$$

The protein levels that were reported to be less than or equal to the limit of detection (LOD) or less than the limit of quantitation (LOQ) on a fresh weight basis were not reported on a dry weight basis.

Data Analyses

All Cry1Ac ELISA plates were analyzed on a SPECTRAmax Plus (Molecular Devices, Sunnyvale, CA) or SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. All protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620-650 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GXP version 5.0.1. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was reported on a "µg/g fwt" basis. For all proteins, this conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The protein values in "µg/g fwt" were also converted to "µg/g dwt" by applying the DWCF, except for pollen which was not analyzed for moisture content due to insufficient sample volume. Microsoft Excel 2002 (Version 10.68241.6839 SP3, Microsoft, Redmond, WA) was used to calculate the Cry1Ac protein levels in soybean tissues.

Appendix D. Summary of the Tryptic Masses of the Cry1Ac Protein Identified Using MALDI-TOF MS

Table D-1. Summary of the Tryptic Masses Identified for the Full-Length MON 87701-Produced Cry1Ac Protein Using MALDI-TOF Mass Spectrometry.[1]

1	2	3	4	5	6				
AC-1	AC-2	DHB-1	DHB-2	SA-1	SA-2	Expected	Diff[2]	Fragment	Sequence[3]
579.40		579.38				579.33	0.07 (1)	754-757	YQLR
589.39		589.37				589.28	0.11 (1)	1028-1032	VCPGR
						589.31	0.08 (1)	229-232	DWIR
611.42						611.36	0.06 (1)	941-945	VHSIR
621.46		621.45				621.37	0.09 (1)	1033-1037	GYILR
649.46		649.44				649.37	0.09 (1)	258-262	TYPIR
688.10						688.37	0.27 (1)	98-103	NQAISR
727.46		727.45				727.35	0.11 (1)	233-237	YNQFR
731.46		731.46				731.36	0.10 (1)	428-433	QGFSHR
764.51		764.49				764.39	0.12 (1)	92-97	IEEFAR
		781.51				781.38	0.13 (3)	197-202	YNDLTR
804.41		804.58				804.46	0.05 (1)	263-269	TVSQLTR
816.52		816.52				816.40	0.12 (1)	222-228	VWGPDSR
832.44		832.44	832.62			832.48	0.04 (1)	671-677	ELSEKVK
								743-749	IDESKLK
854.43	854.54	854.55				854.41	0.02 (1)	1119-1125	SYTDGRR
907.60	907.74	907.61				907.46	0.14 (1)	178-185	DVSVFGQR
940.66		940.67				940.51	0.15 (1)	365-372	TLSSTLYR
976.66	976.80	976.67				976.50	0.16 (1)	434-441	LSHVSMFR
1027.69						1027.53	0.16 (1)	696-703	DINRQPER
1038.66	1038.82	1038.68		1038.49	1038.65	1038.50	0.16 (1)	214-221	WYNTGLER
1066.62		1066.66		1066.55		1066.43	0.19 (1)	1126-1133	ENPCEFNR
1074.72		1074.75				1074.55	0.17 (1)	286-296	GSAQGIEGSIR
		1078.73				1078.55	0.18 (3)	687-695	NLLQDSNFK
1144.74	1144.92	1144.78	1145.00	1144.73	1144.84	1144.57	0.17 (1)	454-462	APMFSWIHR

Table D-1. Summary of the Tryptic Masses Identified for the Full-Length MON 87701-Produced Cry1Ac Protein Using MALDI-TOF Mass Spectrometry (cont'd.)

1 AC-1	2 AC-2	3 DHB-1	4 DHB-2	5 SA-1	6 SA-2	Expected	Diff[2]	Fragment	Sequence[3]
1203.87	1204.05	1203.90		1203.83		1203.68	0.19 (1)	354-364	IVAQLGQGVYR
1216.77	1216.96	1216.82				1216.60	0.17 (1)	505-515	LNSSGNNIQNR
1237.78	1237.97	1237.83	1238.08	1237.76		1237.60	0.18 (1)	186-196	WGFDAATINSR
1249.85		1249.88				1249.65	0.20 (1)	595-606	NFSGTAGVIIDR
1253.84	1254.04	1253.87		1253.83		1253.65	0.19 (1)	442-453	SGFSNSSVSIIR
1258.84	1259.04	1258.87				1258.65	0.19 (1)	203-213	LIGNYTDHAVR
1303.85	1304.05	1303.90	1304.23	1303.85	1304.04	1303.67	0.18 (1)	970-980	IFTAFSLYDAR
		1352.96				1352.71	0.25 (3)	1137-1148	DYTPLPVGYVTK
1398.90		1398.95				1398.67	0.23 (1)	120-131	EWEADPTNPALR
1424.88	1425.07	1424.90				1424.65	0.23 (1)	999-1010	GHVDVEEQNNQR
		1552.08		1552.01		1551.81	0.27 (3)	896-907	EKLEWETNIVYK
	1577.36	1577.19		1577.10		1576.81 1576.87	0.55 (2) 0.49 (2)	687-699 628-642	NLLQDSNFKDINR AVNALFTSTNQLGLK
1599.06						1598.71	0.35 (1)	1125-1136	RENPCEFNRGYR
1704.09	1704.37	1704.21	1704.57	1704.14		1703.88	0.21 (1)	516-530	GYIEVPIHFPSTSTR
	1795.38	1795.16	1795.59	1795.10		1794.87	0.51 (2)	704-721	GWGGSTGITIQGGDDVFK
1801.13	1801.40	1801.26	1801.64	1801.15		1800.87	0.26 (1)	758-772	GYIEDSQDLEIYSIR
1901.15	1901.47	1901.27	1901.67	1901.21	1901.50	1900.91	0.24 (1)	270-285	EIYTNPVLENFDGSFR
1902.15	1902.49	1902.28		1902.21	1902.47	1901.82 1902.96	0.33 (1) 0.81 (1)	1119-1133 104-119	SYTDGRRENPCEFNR LEGLSNLYQIYAESFR
1904.22	1904.49	1904.24	1903.77	1904.24	1904.48	1904.06	0.16 (1)	625-642	AQKAVNALFTSTNQLGLK
1956.29	1956.59	1956.39	1956.82	1956.33	1956.58	1956.01	0.28 (1)	1011-1027	SVLVVPEWEAEVSQEVR
		2088.38				2088.94	0.56 (1)	1100-1118	GYNEAPSVPADYASVYEEK
2098.42	2098.82	2098.55	2098.94	2098.50	2098.81	2098.15	0.27 (1)	865-883	LGNLEFLEEKPLVGEALAR
		2118.44		2118.41		2118.11	0.33 (3)	463-482	SAEFNNIIASDSITQIPAVK
	2142.76	2142.42	2143.20	2142.41	2142.83	2142.08	0.68 (2)	607-624	FEFIPVTATLEAEYNLER
2149.32	2149.71	2149.43				2149.05	0.27 (1)	408-427	SGTVDSLDEIPPQNNNVPPR
2195.47	2195.84	2195.63	2196.12	2195.51	2195.82	2195.16	0.31 (1)	239-257	ELTLTVLDIVSLFPNYDSR

Table D-1. Summary of the Tryptic Masses Identified for the Full-Length MON 87701-Produced Cry1Ac Protein Using MALDI-TOF Mass Spectrometry (cont'd.)

1 AC-1	2 AC-2	3 DHB-1	4 DHB-2	5 SA-1	6 SA-2	Expected	Diff[2]	Fragment	Sequence[3]
2211.41	2211.76	2211.49	2212.03	2211.48	2211.80	2211.12	0.29 (1)	483-504	GNFLFNGSVISGPGFTGGDLVR
						2211.13	0.28 (1)	434-453	LSHVSMFRSGFSNSSVSIIR
		2278.60				2278.14	0.46 (3)	203-221	LIGNYTDHAVRWYNTGLER
						2278.21	0.39 (3)	516-534	GYIEVPIHFPSTSTRYRVR
			2376.24	2375.60		2375.24	1.00 (4)	777-799	HETVNVPGTGSLWPLSAQSPIGK
2616.65	2617.12	2616.80	2617.31	2616.79	2617.19	2616.36	0.29 (1)	946-969	EAYLPELSVIPGVNAAIFEELEGR
				3284.93		3284.61 Ma[4]	0.32 (5)	104-131	LEGLSNLYQIYAESFREWEADPTNPALR
				3318.05		3318.71 Ma	0.66 (5)	258-285	TYPIRTVSQLTREIYTNPVLENFDGSFR
				3365.91		3365.71 Ma	0.20 (5)	911-940	ESVDALFVNSQYDQLQADTNIAMIHAADKR
			3374.20			3374.77 Ma	0.57 (4)	595-624	NFSGTAGVIIDRFEFIPVTATLEAEYNLER
	3731.09	3732.34		3731.25	3732.14	3731.12 Ma	0.03 (2)	373-406	RPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYR
					4371.09	4370.75 Ma	0.34 (6)	704-742	GWGGSTGITIQGGDDVFKENYVTLSGTFDECYPTYLYQK
			4676.21		4676.70	4675.45 Ma	0.76 (4)	136-177	IQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLR
	5564.48	5564.43	5563.75			5564.43 Ma	0.05 (2)	136-185	IQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVS VFGQR
					6142.52	6141.69 Ma	0.83 (6)	537-594	YASVTPIHLNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESA NAFTSSLGNIVGVR

[1] Only experimental masses that matched expected masses are listed in the table. All mass values shown were rounded to two decimal places. Columns 1-6 represent experimentally observed masses from Extract 1 or Extract 2 of trypsinized protein mixed with matrices α-cyano-4-hydroxy cinnamic acid (AC), dihydroxybenzoic acid (DHB), or 3,5-dimethoxy-4-hydroxycinnamic acid (SA).
[2] Diff represents the difference between the experimental mass and the expected mass; the number in parenthesis indicates the column containing the experimental mass used to calculate the difference.
[3] Sixty-eight unique sequences are shown. Two of the 70 fragments identified were methionine-oxidized versions of two sequences shown.
[4] Ma indicates mass averaged value. Unless Ma is indicated, expected mass is monoisotopic mass.

Appendix E. Materials and Methods Used in the Safety Assessment of the Cry1Ac Protein

1. Digestibility Assessment of Cry1Ac in Simulated Gastric and Intestinal Fluids

1.1. Test Substance

The test substance was the *Escherichia coli*-produced full-length Cry1Ac protein (historical APS lot 20-100133, current Orion lot 10000804), which was purified from the fermentation of *E. coli* transformed with plasmid pMON 107800. The construct for the *E. coli*-produced Cry1Ac was engineered to encode the same Cry1Ac protein as that found in MON 87701, which contains four amino acids from the C-terminal end of a chloroplast transit peptide preceding the anticipated N-terminus of the protein.

The DNA sequence encoding the Cry1Ac protein was confirmed both prior to and following fermentation of *E. coli*. Records pertaining to the purification of this *E. coli*-produced Cry1Ac protein are archived under APS lot 20-100133. The Cry1Ac protein is stored in a -80°C freezer in a buffer containing 50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, and 1 mM benzamidine HCl.

1.2. Characterization of Test Substance

The characterization of the physicochemical and functional properties of the test substance was performed under characterization plan 20-100133 and is described in the current Certificate of Analysis under Orion lot number 10000804. The Cry1Ac protein has a total protein concentration of 1.4 mg/ml as determined by amino acid analysis, a purity of 80%, and an apparent molecular weight of 131.7 kDa as determined by SDS-PAGE. The N-terminal sequence of the *E. coli* produced full-length Cry1Ac protein was also confirmed during characterization.

2. Test Systems

Two test systems, SGF and SIF, were utilized independently to test stability of the full-length Cry1Ac protein and then in a sequential digestibility assay where the Cry1Ac protein was first exposed to SGF followed by exposure to SIF.

2.1. Simulated Gastric Fluid (SGF)

SGF contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH. The SGF was prepared using a highly purified form of pepsin (catalog number P-6887, Sigma Company, St. Louis, MO) according to the draft SOP. The SGF was formulated so that ten units of pepsin activity per µg of the Cry1Ac protein would be present in the digestion reactions. The amount of pepsin powder used to prepare SGF was calculated from the specific activity reported on the product label. Activity was assessed using the SGF activity assay according to the draft SOP where one unit of activity is defined as a change in $A_{280\,nm}$ of 0.001 per min at 37 °C, and is measured as trichloroacetic acid (TCA) soluble products using hemoglobin as the substrate. The SGF activity assay was used to confirm the activity of the preparation before initiating the digestions of the

Cry1Ac protein. The digestion of the Cry1Ac protein was monitored by SDS-PAGE stained gels and western blot analysis using a Cry1Ac specific antibody.

2.1.1. Justification for Selection of the SGF Test System

In vitro digestion models are used widely to assess the nutritional value of ingested proteins based on their amino acid bioavailability. The correlation between protein allergenicity and protein stability in an *in vitro* pepsin digestion assay has been previously established (Astwood et al., 1996). The pepsin digestibility assay protocol that was used in this study was standardized by the International Life Science Institute (ILSI) in a multi-laboratory test and the results demonstrated that the *in vitro* pepsin digestion assay is reproducible when a common protocol is followed (Thomas et al., 2004).

2.2. Simulated Intestinal Fluid (SIF)

SIF contained a mixture of enzymes, known as pancreatin, in a buffer adjusted to neutral pH. The SIF was prepared using pancreatin (catalog number P1500, Sigma Company, St. Louis, MO) according to the draft SOP. The SIF was formulated so that 55.3 µg of pancreatin powder would be present per µg of Cry1Ac protein in the digestion reactions. The activity of the SIF was confirmed using an SIF activity assay (draft SOP) prior to initiating the digestions of the Cry1Ac protein. The digestion of the Cry1Ac protein was monitored by western blot analysis using a Cry1Ac specific antibody.

2.2.1. Justification for Selection of the SIF Test System

In vitro digestion models are used widely to assess the digestibility of ingested substances. SIF is frequently used for *in vitro* studies to assess the digestibility of food components (Yagami et al., 2000; Okunuki et al., 2002).

3. Experimental Design

3.1. Digestibility of the Cry1Ac Protein in SGF

Digestibility of the Cry1Ac protein in SGF was evaluated over time by analyzing specimens from targeted incubation time points. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Specimen Code(s)
0 min	SGF T0, SGF P0, SGF N0
0.5 min	SGF T1
2 min	SGF T2
5 min	SGF T3
10 min	SGF T4
20 min	SGF T5
30 min	SGF T6
60 min	SGF T7, SGF P7, SGF N7

SGF was prepared to contain approximately 2279 U/ml of pepsin activity by dilution of a stock SGF solution with SGF buffer lacking pepsin (10 mM HCl, 2 mg/ml NaCl, pH ~1.2). The digestion mixture was prepared by adding 126 µl of the solution containing Cry1Ac protein to a tube containing 774 µl of pre-heated (36.5 °C, 10 min) SGF which

corresponds to 176.4 µg of Cry1Ac protein and 1764 U of pepsin, respectively. The tube contents were mixed by vortexing then placed immediately in a 36.6 °C water bath. Specimens (100 µl) were removed at 0.5, 2, 5, 10, 20, 30 and 60 min (corresponding to specimen codes SGF T1 through SGF T7). Each 100 µl specimen was placed immediately in a tube containing quenching mixture, consisting of 35 µl of 0.7 M sodium carbonate buffer (0.7 M Na_2CO_3, pH 11), and 35 µl of 5× Laemmli buffer [5× LB, 312.5 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) Bromophenol Blue, and 50% (v/v) glycerol, pH 6.8].

The SGF T0 specimen was prepared in a separate tube. The SGF T0 sample used 86 µl of SGF (196 U of pepsin) quenched by the addition of 35 µl of 0.7 M sodium carbonate buffer, and 35 µl of 5× LB prior to the addition of 14 µl (19.6 µg) of the Cry1Ac protein.

All specimens, after the addition of the corresponding quenching buffers, were heated to 75-100 °C for 5-10 minutes, frozen on dry ice and stored in a -80 °C freezer until analysis.

3.1.1. SGF Experimental Control Specimens

Experimental control specimens were prepared to determine the stability of the Cry1Ac protein in the test system buffer lacking pepsin (10 mM HCl, 2 mg/ml NaCl, pH ~1.2). The SGF P0 control was prepared in a similar manner as described in Section 6.1 for SGF T0 with an incubation time of 0 min. The SGF P7 control was prepared in a manner similar to SGF P0, except the protein and 10 mM HCl, 2 mg/ml NaCl were incubated for 60 min before quenching with carbonate buffer and 5× LB.

Experimental control specimens were also prepared to determine the stability of the test system lacking the Cry1Ac protein. Protein storage buffer (50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, 1 mM Benzamidine-HCl) was added to SGF in place of the Cry1Ac protein. The SGF N0 control was prepared in a similar manner as described in Section 6.1 for SGF T0 with an incubation time of 0 min. The SGF N7 control was also prepared in a manner similar to SGF N0, except storage buffer and SGF were incubated for 60 min before quenching with carbonate buffer and 5× LB.

All quenched specimens were heated to 75-100 °C for 5-10 min, frozen on dry ice, and stored in a –80 °C freezer until analysis.

3.2. Digestibility of the Cry1Ac Protein in SGF Followed by SIF

Digestibility of the full-length Cry1Ac protein in SGF followed by SIF was evaluated over time by analyzing specimens at targeted incubation time points. The Cry1Ac protein was digested first in SGF (phase I) as described in Section 5.1 for 2 min at which point the reaction was stopped by quenching with 0.7 M sodium carbonate buffer. The SGF digested and quenched Cry1Ac was then placed in the SIF assay (phase II) and digested as described in Section 5.2. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Specimen Code(s)
SGF system (Phase I)	
0 min	SEQ 0min
2 min	SEQ 2min
SIF system (Phase II)	
0 min	SEQ T0, SEQ P0, SEQ N0
0.5 min	SEQ T1
2 min	SEQ T2
5 min	SEQ T3
10 min	SEQ T4
30 min	SEQ T5
1 h	SEQ T6
2 h	SEQ T7, SEQ P7, SEQ N7

For phase I, the SGF was prepared to contain approximately 2632 U/ml of pepsin activity. The digestion in SGF was prepared by adding 143 µl of the Cry1Ac protein to a tube containing 760 µl of pre-heated (36.6 °C, 5 min) SGF, corresponding to 200 µg of Cry1Ac protein and 2000 U of pepsin, respectively. The tube contents were mixed by vortexing and immediately placed in a 36.5 °C water bath. The tube was removed after 2 min, and the reaction was immediately quenched by adding 316 µl of 0.7 M sodium carbonate buffer. After quenching, an aliquot of 120 µl was removed for analysis, and mixed with 30 µl of 5× LB, and heated to 75-100 °C for 5-10 min, frozen on dry ice, and designated as SEQ 2min.

For phase II (SIF digestion), 625 µl of the quenched SGF reaction mixture was added to 550 µl of pre-heated (36.0 °C, 5 min) SIF, corresponding to 100 µg SGF digested and quenched Cry1Ac protein (based on the predigested concentration) and 5.5 mg of pancreatin. The tube contents were mixed by vortexing and immediately placed in a 37.5 °C water bath. Digestion aliquots (100 µl) were removed from the tube at 1, 2, 5, 10, 30 min, 1, and 2 h (corresponding to specimen codes SEQ T1 through SEQ T7) and immediately placed in a tube containing 25 µl of 5× LB, heated to 75-100 °C for 5-10 min, and frozen on dry ice for complete quenching.

The SEQ 0min specimen for the SGF digestion phase was prepared in a separate tube by first quenching 76 µl of SGF (200 U of pepsin) with 32 µl of sodium carbonate buffer, and 31 µl of 5× LB buffer and heating to 75-100 °C for 5-10 min prior to the addition of 14 µl (20 µg)of the Cry1Ac protein.

The SEQ T0 specimen for the SIF digestion phase was prepared in a separate tube by first quenching 55 µl of SIF (0.55 mg) with 30 µl of 5× LB buffer and heating to 75-100 °C for 5-10 min prior to the addition of 63 µl (10 µg, based on the pre-digestion concentration) of the SGF digested and quenched Cry1Ac protein.

3.2.1. SGF Followed by SIF Experimental Control Specimens

Experimental control specimens for the SIF digestion phase were prepared to determine the stability of the Cry1Ac protein fragment in the SIF test system buffer lacking pancreatin enzymes (50 mM potassium phosphate monobasic, pH adjusted to 7.5 with sodium hydroxide). The SEQ P0 control was prepared in a similar manner as described in Section 3.2 for SEQ T0 with an incubation time of 0 min. The SEQ P7 control was also prepared in a similar manner, except the SGF digested and quenched Cry1Ac protein and 50 mM KH_2PO_4 were incubated for 2h before quenching with 5× LB.

Experimental control specimens were also prepared to characterize the test system (SIF) lacking the SGF digested and quenched Cry1Ac protein. Protein storage buffer (50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, 1 mM Benzamidine-HCl) was added to SIF in place of the SGF digested Cry1Ac protein. The SEQ N0 control was prepared in a similar manner as described in Section 6.2 for SEQ T0 with an incubation time of 0 min. The SEQ N7 control was also prepared in a similar manner, except storage buffer and 1× SIF were incubated for 2h before quenching with 5× LB.

All quenched specimens were heated to 75-100 °C for 5-10 min, frozen on dry ice, and stored in a –80 °C freezer until analysis.

3.3. Digestibility of the Cry1Ac Protein in SIF

Digestibility of the full-length Cry1Ac protein in SIF was evaluated over time by analyzing specimens at multiple incubation time points. A numerical code using the numbers 0 through 8 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Specimen Code
0 min	SIF T0, SIF P0, SIF N0
5 min	SIF T1
15 min	SIF T2
30 min	SIF T3
1 h	SIF T4
2 h	SIF T5
4 h	SIF T6
8 h	SIF T7
24 h	SIF T8, SIF P8, SIF N8

The digestion was prepared by adding 200 µl of the test substance to a tube containing 1.55 ml of pre-heated (36.2 °C, 5 min) SIF, corresponding to 280 µg of Cry1Ac protein and 15.5 mg of pancreatin, respectively. The tube contents were mixed by vortexing and immediately placed in a 36.1 °C water bath. Digestion specimens (100 µl) were removed at 5, 15, 30 min, 1, 2, 4, 8, and 24 h (corresponding to specimen time points SIF T1 through SIF T8) and immediately placed in a tube containing 25 µl of 5× LB, heated to 75-100 °C for 5-10 min, and frozen on dry ice for complete quenching.

The SIF TO specimen was prepared in a separate tube by first quenching 110 µl of SIF (1.1 mg) with 31 µl of 5× LB buffer and heating to 75-100 °C for 5-10 min prior to the addition of 14 µl (20 µg) of the Cry1Ac protein.

3.3.1. SIF Experimental Control Specimens

Experimental control specimens were prepared to determine the stability of the Cry1Ac protein in the test system buffer lacking pancreatin (50 mM potassium phosphate, pH 7.5). The SIF P0 control was prepared in a similar manner as described in Section 6.3 for SIF T0 with an incubation time of 0 min. The SIF P8 control was also prepared in a similar manner, except the protein and 50 mM KH_2PO_4 were incubated for 24h before quenching with 5× LB.

Experimental control specimens were also prepared to characterize the test system (SIF) lacking the Cry1Ac protein. Protein storage buffer (50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, 1 mM Benzamidine-HCl) was added to SIF in place of the Cry1Ac protein. The SIF N0 control was prepared in a similar manner as described in Section 6.3 for SIF T0 with an incubation time of 0 min. The SIF N8 control was also prepared in a similar manner, except the protein storage buffer and 1× SIF were incubated for 24h before quenching with 5× LB.

All quenched specimens were heated to 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a –80 ºC freezer until analysis.

4. Specimen Retention

All specimens will be retained in a –80 ºC freezer for one year, after which it is concluded that they will no longer afford analytical evaluation and may be discarded.

5. Analytical Methods

Activities of the SGF and SIF were assessed using pepsin and pancreatin activity assays respectively. The digestibility of the Cry1Ac protein in SGF, and in SGF followed by SIF, were assessed using stained SDS-PAGE and western blot analysis. The digestibility of the Cry1Ac protein in SIF was assessed using western blot analysis. The limit of detection (LOD) of the Cry1Ac protein was visually estimated from stained SDS-PAGE and western blot data. The identity of a transiently stable fragment of ~4 kDa in SGF digestion was determined by N-terminal sequencing.

5.1. SGF Activity Assay

The SGF activity assay was conducted according to the draft SOP to confirm the suitability of the test system before its use with the Cry1Ac protein. The assay is based on the ability of pepsin to digest denatured hemoglobin. Undigested hemoglobin was precipitated with TCA, and the amount of soluble peptides was estimated by measuring the absorbance at 280 nm. The amount of soluble peptides is directly proportional to the amount of protease activity. One unit of pepsin activity in this assay is defined as the amount of pepsin that will produce a change in absorbance at 280 nm of 0.001 per min at pH 1.2-2.0 at 37 ± 2 ºC. The SGF solution was formulated to contain 0.03 mg of powder per ml of SGF buffer. Acceptable specific activity (units/mg pepsin powder) for the SGF was equal to the specific activity determined by the manufacturer, ±1000 units/mg.

Because digestions of the Cry1Ac protein in SGF and in SGF followed by SIF were performed on the same day, only one SGF activity assay was conducted to confirm the suitability of the SGF.

The SGF activity assay was conducted as follows: The SGF solution was diluted to 0.03 mg of solid material (pepsin) per ml of SGF [the dilution factor (DF) was 26.7]. Acidified hemoglobin [2% (w/v), 5 ml] was added to each of three replicates of the test sample and blank samples and pre-warmed at 37 ± 2 °C for 5-10 min prior to starting the reactions. Diluted SGF (1 ml) was added to each test sample and both test and blank samples were incubated at 36.9°C for an additional 10 min. The reactions were stopped by the addition of 10 ml of 5% (v/v) chilled TCA to the test and blank samples. Diluted SGF (1 ml) was then added to the blank samples. Samples were mixed and then incubated for another 5-10 min at 36.9°C. Precipitated protein was removed by filtering the test and the blank samples using 0.8 µm syringe filters. Samples of the clarified test and blank samples were read at 280 nm in a Beckman DU-650 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) The activities of pepsin were calculated using the following equation:

$$\frac{MeanTest_{A280nm} - MeanBlank_{A280nm}}{0.001 \times 10\text{min} \times 1ml} \times DF$$

where 0.001 is the change in the absorbance at 280 nm per min at pH 1.2-2.0 and 37 ± 2 °C produced by one unit of pepsin activity; 10 min is the reaction time, 1 ml is the amount of SGF added to the reaction; and, DF is the dilution factor for the SGF.

5.2. SIF Activity Assay

The SIF activity assay was conducted according to the draft SOP to confirm the suitability of the test system before its use with the Cry1Ac protein. One unit of pancreatin activity in this assay is defined as an increase in the absorbance at 574 nm of 0.001 per min at 37 ± 2 °C. An acceptable specific activity for the SIF was defined as 11,000 ± 3,000 U/ml.

The assay is based on the estimation of the amount of soluble peptides present in a trichloroacetic acid (TCA) solution after pancreatin digestion of resorufin-labeled casein (Roche Molecular Biochemicals, Mannheim, Germany). Undigested resorufin-labeled casein is precipitated with TCA and the amount of soluble peptide is estimated in the supernatant by measuring the absorbance at 574 nm. The amount of soluble peptide is directly proportional to the amount of proteolytic activity.

Because digestions of Cry1Ac in SIF and in SGF followed by SIF were performed on two separate days, an SIF activity assay was conducted on the day of each digestion to confirm the suitability of the SIF.

The SIF activity assay was conducted as follows: 50 µl of 0.4% (w/v) resorufin-labeled casein and 50 µl of incubation buffer (200 mM Tris, pH 7.8, 20 mM $CaCl_2$) was added to each of three activity replicates and three blank replicates and preheated for 1 – 3 minutes at 36.9 and 37.7°C for Cry1Ac Pancreatin Activity 1 and Cry1Ac Pancreatin Activity 2, respectively. To initiate the reaction, each of the three activity replicates were incubated with 100 µl 0.05× SIF (1× SIF was diluted to 0.05× SIF before the activity assay was initiated), and the three blank replicates were incubated with 100 µl 50 mM KH_2PO_4, pH 7.5 in place of SIF for 15 min at 36.9 and 37.7°C for Cry1Ac Pancreatin Activity 1 and Cry1Ac Pancreatin Activity 2, respectively. The reaction was quenched by the addition of 480 µl of chilled 5% (v/v) TCA to activity and blank replicates. The supernatants

recovered after centrifugation (400 µl) were neutralized by the addition of 600 µl assay buffer (500 mM Tris-HCl, pH 8.8), and the absorbance of the clarified activity and blank replicates was read at 574 nm using a Beckman DU-650 spectrophotometer. The activity of SIF was calculated using the following equation:

$$\frac{Mean\ Activity_{A574nm} - Mean\ Blank_{A574nm}}{0.001 \times 15\,\text{min} \times 0.1\,ml \times 0.05}$$

where 0.001 is the change in the absorbance at 574 nm per min at 37 ± 2 °C produced by one unit of pancreatin activity, 15 min is the reaction time, 0.1 ml is the amount of 0.05× SIF added to the reaction, and 0.05 is the SIF dilution factor.

5.3. SDS-PAGE and Colloidal Brilliant Blue G Staining

Specimens containing 1× LB from the SGF and from SGF followed by SIF digestions of the Cry1Ac protein were separated by SDS-PAGE using pre-cast tricine 10-20% polyacrylamide gradient mini-gels and tricine running buffer (Invitrogen, Carlsbad, CA) according to the draft SOP. The Cry1Ac protein was loaded at 0.8 µg per lane based on pre-digestion total protein concentration. All experimental controls were loaded at the same volumes as those containing Cry1Ac protein so that they would be comparable. Prior to loading on the gels, all specimens were heated for 5 min at 95.6 °C and 95.4 °C for SGF and SGF followed by SIF digestions of the Cry1Ac protein, respectively. Mark 12 molecular weight markers (Invitrogen, Carlsbad, CA) were loaded to estimate the relative molecular weight of proteins and peptides visualized by staining. Electrophoresis was performed at a constant voltage of 125 V for 90 (SGF digestion) and 75 (SGF followed by SIF digestion) min. After electrophoresis, proteins were visualized by staining the gels with Colloidal Brilliant Blue G (Sigma, St. Louis, MO).

The Colloidal Brilliant Blue G staining procedure was selected because it is an effective method for detecting nanogram quantities of a protein in a gel (Neuhoff et al., 1988). After separation of the proteins, the gels were fixed in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol for 30 min and stained for ~ 16 h in 1× Brilliant Blue G-Colloidal stain solution containing 20% (v/v) methanol. The gels were destained for 30 s in 10% (v/v) acetic acid, 25% (v/v) methanol and then completely destained for ~4 h (SGF) and ~ 5 h (SGF followed by SIF) in a 25% (v/v) methanol solution. Images were captured using a Bio-Rad GS-800 densitometer (BioRad, Hercules, CA). The results of the *in vitro* digestibility of Cry1Ac in SGF and SGF followed by SIF were determined by visual examination of the stained gels.

The LOD of the Cry1Ac protein was visually estimated from the Colloidal Brilliant Blue G stained SDS-PAGE data. Various dilutions of the SGF T0 specimen were loaded onto a gel and separated by SDS-PAGE. The SDS-PAGE gel that was used to estimate the LOD was run concurrently with the gel used to assess Cry1Ac protein digestibility in SGF. The dilutions of the SGF T0 specimen were loaded at volumes that represented approximately 0.8, 0.4, 0.1, 0.05, 0.02, 0.01, 0.005, 0.0025, 0.001, and 0.0005 µg total protein per lane.

5.4. Western Blot Analysis

Specimens from the SGF, SGF followed by SIF, and SIF digestions of the Cry1Ac protein were separated by SDS-PAGE using pre-cast tricine 10-20% polyacrylamide

gradient mini-gels with tricine running buffer. The protein amount loaded in each lane was based on pre-digestion concentrations of the Cry1Ac protein. The digestion samples were diluted with 1× LB to a concentration of ~2 ng/µl, and ~10 ng of the Cry1Ac protein digestion specimens were loaded in each lane. The experimental controls were loaded in as amounts equal to the digestion specimens. All specimens from the SGF, SIF, and SGF followed by SIF digestions were heated to 95.9, 95.7 and 95.4 °C for 3, 3, and 5 min respectively, prior to loading on the gels. Electrophoresis was performed at a constant voltage of 125 V for 90, 90, and 75 min on specimens from SGF, SIF and SGF followed by SIF digestions, respectively. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes with a pore size of 0.45 µm (Invitrogen, Carlsbad, CA) for 60 min at a constant current of 300 mA. Prestained molecular weight markers (Precision Plus Dual color Protein Standards, Bio-Rad, Hercules, CA) were used to verify electrotransfer of the proteins to the membranes.

Proteins transferred to nitrocellulose membranes were analyzed by western blot. The membranes were blocked for ~16 h at ~4 °C with 5% (w/v) non-fat dry milk (NFDM) in a phosphate buffered saline - Tween® 20 (PBST) buffer. All subsequent incubations were performed at room temperature. Goat anti–Cry1Ac affinity purified antibody (lot 10000963) was incubated with the membranes for 60 min at a dilution of 1:500 in 1% (w/v) NFDM in PBST. Excess antibody was removed by three 5 min washes with PBST. The membranes were incubated with HRP–conjugated rabbit anti-goat IgG (lot G819862-B) at a dilution of 1:10,000 in 1% (w/v) NFDM in PBST for 90 min (only 60 min for the SGF followed by SIF digestion), and washed three times for 5 min (10 min for the SGF followed by SIF digestion) with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ) and exposed to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare, Piscataway, NJ). Films were developed using a Konica SRX101A automated film processor (Konica, Tokyo, Japan). The films were scanned using a Bio-Rad GS-800 densitometer to produce electronic images to be used as figures for reporting purposes.

The approximate molecular weights of the proteins observed on the western blot were visually estimated relative to the positions of the molecular weight markers.

The LOD for the western blot analysis procedure was estimated for the Cry1Ac protein by loading various dilutions of the SGF and SIF zero time point (SGF T0 and SIF T0, respectively) digestion specimens on separate gels. These gels were run concurrently with the SGF and SIF digestion western blot gels, respectively, and subjected to the same western blot procedure as described above. The following approximate total protein loadings (based upon the pre-digestion Cry1Ac concentration) of the SGF T0 and SIF T0 specimens were used for the western blot LOD analysis: 10, 5, 2.5, 1, 0.5, 0.2, 0.1, 0.05, and 0.025 ng per lane.

5.5. N-Terminal Sequencing

N-terminal sequencing by Edman degradation was used to determine the N-terminal sequence of the transiently stable fragment from the SGF digestion with apparent molecular weight of ~4 kDa.

5.5.1. Protein Blot for N-Terminal Sequence Analysis

The specimen SGF T1 was used to further characterize the transiently stable fragment of ~4 kDa. This specimen corresponds to the 30 s digestion of the Cry1Ac protein which provided a sufficient amount of the fragment for sequencing.

The SGF T1 specimen was heated at 95.6 °C for 3 min, and loaded in triplicate at 3.6 µg per lane onto a tricine 10-20% polyacrylamide gradient 10-well gel. Precision Plus pre-stained molecular weight markers were loaded in parallel to verify electrotransfer of the protein to the membrane and estimate the size of the stained bands observed. Electrophoresis was performed at a constant voltage of 125 V for 90 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 60 min at a constant current of 300 mA. The blot was stained with Coomassie Blue R-250 stain (Bio-Rad, Hercules, CA) and then destained for ≥ 5 min with Coomassie Blue R-250 destain (Bio-Rad, Hercules, CA) to visualize the markers and the stable fragments. The blot was scanned using a Bio-Rad GS-800 densitometer to produce an electronic image.

5.5.2. N-Terminal Sequencing

The band corresponding to the transiently stable fragment of ~4 kDa was excised from the blot. N-terminal sequence analysis was performed according to the current version of BR-EQ-0265 for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785 Programmable Absorbance Detector and Procise™ Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas[99] software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for the analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the analysis of the two protein bands to verify that the sequencer met performance criteria for repetitive yield and sequence identity. Identity of the fragment was established by comparing identified amino acids to the expected Cry1Ac protein sequence.

References:

Astwood, J.D., Leach, J.N., and Fuchs, R.L. (1996) Allergenicity of foods derived from transgenic plants. *Nature Biotech.* 14, 1269-1273.

Neuhoff, V., Norbert, A., Taube, D., and Wolfgang, E. (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis 9*, 255-262.

Okunuki, H., Techima, R., Shigeta, T., Sakushima, J., Akiyama, H., Yukihiro, G., Toyoda, M., and Sawada, J. (2002) Increased digestibility of two products in genetically modified food (CP4-EPSPS and Cry1Ab) after preheating. *J. Food Hyg. Soc. Japan* 43, 68-73.

Thomas, K., Aalbers, M., Bannon, G.A., Bartels, M., Dearman, R.J., Esdaile, D.J., Fu, T.J., Glatt, C.M., Hadeld, N., Hatzos, C., Hee, S.L., Heylings, J.R., Goodman, R.E., Henry, B., Herouet, C., Holsapple, M., Ladics, G.S., Landry, T.D., MacIntosh, S.C., Rice, E.A., Privalle, L.S., Steiner, H.Y., Teshima, R., van Ree, R., Woolhiser, M., and Zawodny, J. (2004) A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology* 39, 87–98.

Yagami, T., Haishima, Y., Nakamura, A., Hiroyuki, O., and Ikesawa, Z. (2000) Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J. Allergy Clin. Immunol.* 106, 752-762.

Appendix F. Materials and Methods Used for Compositional Analysis of Soybean Harvested Seed and Forage Collected from 2007 U.S. Field Trials

Materials

MON 87701, a conventional soybean control (A5547), and conventional reference soybean varieties were grown at five U.S. locations in 2007. MON 87701 and the control were grown from seed lots GLP-0612-17898-S and GLP-0612-17895-S, respectively. The control material, A5547, has background genetics representative of MON 87701 but does not contain the *Cry1Ac* gene coding sequence or produce the Cry1Ac protein. In addition, twenty conventional soybean varieties produced alongside of MON 87701 were included for the generation of a 99% tolerance interval. These varieties, locations, and seed lot numbers are listed below:

Material Name	Seed Lot Number	Site Code
A5843	GLP-0702-18243-S	AL
A5959	GLP-0702-18245-S	AL
CMA 5804AOC	GLP-0702-18244-S	AL
H6686	GLP-0702-18247-S	AL
UA 4805	GLP-0702-18123-S	AR
Ozark	GLP-0702-18124-S	AR
Anand	GLP-0702-18122-S	AR
Hornbeck C5894	GLP-0702-18125-S	AR
A5560	GLP-0702-18242-S	GA
CMC 5901COC	GLP-0702-18246-S	GA
LEE 74	GLP-0702-18248-S	GA
A5403	GLP-0702-18241-S	GA
A4922	GLP-0702-18234-S	IL
H4994	GLP-0702-18235-S	IL
H5218	GLP-0702-18236-S	IL
A5427	GLP-0702-18238-S	IL
DP 5989	GLP-0702-18126-S	NC
Hutcheson	GLP-0703-18396-S	NC
USG 5601T	GLP-0703-18402-S	NC
Fowler	GLP-0703-18395-S	NC

Characterization of the Materials

The identities of the forage and harvested seed samples from MON 87701, control, and reference soybean varieties were verified prior to their use in the study by confirming the chain-of-custody documentation supplied with the forage and harvested seed collected from the field plots. The harvested seed of MON 87701, the conventional soybean control, and reference soybean varieties was also characterized by event-specific polymerase chain reaction (PCR) analysis, for the presence or absence of MON 87701.

Field Production of the Samples

The field design and tissue collection process have been described previously in Appendix C with the addition of reference soybean varieties as described above. A total of twenty different conventional soybean varieties were planted at five field locations with four different varieties grown at each site. Seed were planted in a randomized complete block design with three replicates per block for each of MON 87701, the conventional soybean control and reference soybean varieties. All the samples were grown under normal agronomic field conditions for their respective geographic regions.

Summary of Analytical Methods

Harvested soybean seed and forage samples from MON 87701, the control, and conventional reference soybean varieties were shipped on dry ice to EPL Bio-Analytical Services (EPL-BAS), 9095 W. Harristown Blvd. Niantic, Illinois for compositional analyses. Analyses were performed using methods that are currently used to evaluate the nutritional quality of food and feed.

SOYBEAN FORAGE ANALYTICAL METHOD SUMMARIES:

Ash (SOP-SE-2)

Subsamples of ground forage (3 grams) are ignited in a muffle furnace for three hours at 650°C. The weight of the ash residue remaining after ignition is determined gravimetrically. There is no analytical reference substance for this analysis. Ash results are expressed on a percent fresh weight basis.

Reference:

AOAC International Method 923.03 (2000). In Official Methods of Analysis of AOAC International, 17th Edition. Association of Official Analytical Chemists International, Gaithersburg, MD.

Carbohydrates (CHO)

Total carbohydrate content is calculated by difference using the fresh weight-derived data and the formula presented below. There is no analytical reference substance for this analysis.

Carbohydrates (%) = 100 – Moisture (%) -Ash (%) - Fat (%) - Protein (%)

Reference:

United States Department of Agriculture (1973). "Energy Value of Foods," *Agriculture Handbook No. 74,* pp. 2-11.

Crude Fat (SOP-SE-1)

Subsamples of ground forage (2 grams) are dried in an oven for at least 2 hours. The crude fat content is determined gravimetrically after acid hydrolysis and extraction with mixed ethers. There is no analytical reference substance for this analysis. Fat results are expressed on a percent fresh weight basis.

Reference:
AOAC International Method 922.06 (2000). In Official Methods of Analysis of AOAC International, 17th Edition. Association of Official Analytical Chemists International, Gaithersburg, MD.

Moisture (SOP-SE-25)
Moisture content is determined gravimetrically. Subsamples (2 grams) of ground forage are dried to a constant weight in a forced air oven at 135°C for at least 2 hours. Moisture results are expressed on a percent of fresh weight basis. There is no analytical reference substance for this analysis.

Reference:
AOAC International Method 930.15 (2000). In Official Methods of Analysis of AOAC International, 17th Edition. Association of Official Analytical Chemists International, Gaithersburg, MD.

Crude Protein (SOP-SE-20)
Protein content is determined using an automated Kjeldahl technique. A Foss-Tecator 2300 Kjeltec Analyzer Unit is used. Samples are manually digested on a heating block using sulfuric acid and a selenium catalyst then transferred to the analyzer unit where the digests are distilled and titrated. The protein content is calculated by multiplying the amount of nitrogen in the sample by 6.25. Ammonium sulfate is used as an analytical reference substance to verify the accuracy of the distillation step performed by the analyzer unit. The ammonium sulfate reference standard is obtained from Fisher Scientific (Fairlawn, NJ) and has a nitrogen content of 21.0%. The lot number is 043629 Protein results are expressed on a percent fresh weight basis.

Reference:
Foss-Tecator (1999). *Foss-Tecator Kjeltec 2300 Site Preparation, Installation, and Operating Guide*, Foss-Tecator AB, Box 70, S-263 21 Hoganos, Sweden.

Acid Detergent Fiber (SE-3)
Subsamples of ground forage are analyzed to determine the percentage of acid detergent fiber (ADF) by digesting with an acid detergent solution and washing with water. The remaining residue is dried and weighed to determine ADF content. Samples are analyzed with the Ankom Extraction Apparatus. There is no analytical reference substance for this analysis. ADF results are expressed on a percent fresh weight basis.

Reference:
Ankom Technology (1999). ANKOM200 Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.

Neutral Detergent Fiber (SE-9)
Subsamples of ground forage are analyzed to determine the percentage of neutral detergent fiber (NDF) by digesting with an neutral detergent solution, sodium sulfite and alpha amylase. The remaining residue is dried and weighed to determine NDF content. Samples are analyzed with the Ankom Extraction Apparatus. There is no analytical reference substance for this analysis. NDF results are expressed on a percent fresh weight basis.

Reference:
Ankom Technology (1999). ANKOM^{200} Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.

HARVESTED SOYBEAN SEED ANALYTICAL METHOD SUMMARIES:

Ash (SOP-SE-2)
Subsamples of ground soybean seed are ignited in a muffle furnace for three hours at 650°C. The weight of the ash residue remaining after ignition is determined gravimetrically. There is no analytical reference substance for this analysis. Ash results are expressed on a percent fresh weight basis.

Reference:
AOAC International Method 923.03 (2000). In Official Methods of Analysis of AOAC International, 17th Edition. Association of Official Analytical Chemists International, Gaithersburg, MD.

Carbohydrates (CHO)
Total carbohydrate content is calculated by difference using the fresh weight-derived data and the formula presented below. There is no analytical reference substance for this analysis.

Carbohydrates (%) = 100 – Moisture (%) -Ash (%) - Fat (%) - Protein (%)

Reference:
United States Department of Agriculture (1973). "Energy Value of Foods," *Agriculture Handbook No. 74,* pp. 2-11.

Crude Fat (SOP-SE-27)
Subsamples of ground soybean seed are extracted for 16 hours with pentane using soxhlet extraction apparatus. The pentane extract is evaporated to dryness and the crude fat residue is determined gravimetrically. There is no analytical reference standard for this analysis. Fat results are expressed on a percent fresh weight basis.

Reference:
AOAC International Method 960.39 (2000). In Official Methods of Analysis of AOAC International, 17th Edition. Association of Official Analytical Chemists International, Gaithersburg, MD.

Moisture (SOP-SE-4)

Moisture content is determined gravimetrically. Subsamples (2 grams) of ground seed are dried to a constant weight in a vacuum oven at 100°C and 25 inches of mercury pressure for 15 hours. Moisture results are expressed on a percent of fresh weight basis. There is no analytical reference substance for this analysis.

Reference:
AOAC International Method 925.09 (2000). In Official Methods of Analysis of AOAC International, 17th Edition. Association of Official Analytical Chemists International, Gaithersburg, MD.

Crude Protein (SOP-SE-20)

Protein content is determined using an automated Kjeldahl technique. A Foss-Tecator 2300 Kjeltec Analyzer Unit is used. Samples are manually digested on a heating block using sulfuric acid and a selenium catalyst then transferred to the analyzer unit where the digests are distilled and titrated. The protein content is calculated by multiplying the amount of nitrogen in the sample by 6.25. Ammonium sulfate is used as an analytical reference substance to verify the accuracy of the distillation step performed by the analyzer unit. The ammonium sulfate reference substance is obtained from Fisher Scientific (Fairlawn, NJ) lot number 043629 and has a nitrogen content of 21.0%. Protein results are expressed on a percent fresh weight basis.

Reference:
Foss-Tecator (1999) *Foss-Tecator Kjeltec 2300 Site Preparation, Installation, and Operating Guide*, Foss-Tecator AB, Box 70, S-263 21 Hoganos, Sweden.

Acid Detergent Fiber (SE-3)

Subsamples of ground soybean seed are analyzed to determine the percentage of acid detergent fiber (ADF) by digesting with an acid detergent solution and washing with water. The remaining residue is dried and weighed to determine ADF content. Samples are analyzed with the Ankom Extraction Apparatus. There is no analytical reference substance for this analysis. ADF results are expressed on a percent fresh weight basis.

Reference:
Ankom Technology (1999). ANKOM200 Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.

Neutral Detergent Fiber (SE-9)

Subsamples of ground soybean seed are analyzed to determine the percentage of neutral detergent fiber (NDF) by digesting with a neutral detergent solution, sodium sulfite and alpha amylase. The remaining residue is dried and weighed to determine NDF content. Samples are analyzed with the Ankom Extraction Apparatus. There is no analytical reference substance for this analysis. ADF results are expressed on a percent fresh weight basis.

Reference:
Ankom Technology (1999). ANKOM200 Fiber Analyzer Operator's Manual, Ankom Technology, 140 Turk Hill Park, Fairport, NY 14450.

Tryptophan (SE-22)

Subsamples of ground soybean seed are analyzed to determine the amount of tryptophan by hydrolyzing with 4M LiOH and diluting to 50 mL with deionized water. Samples are filtered and analyzed by reverse phase High Performance Liquid Chromatography (HPLC) with ultra-violet (UV) detection. L-Tryptophan is used as the analytical reference substance to verify the accuracy of the method and HPLC. The L-Tryptophan analytical reference substance is purchased from Sigma, has a purity of >99% and lot number 026K0375. The limit of quantitation (LOQ) is 0.125 %. Tryptophan results are expressed on a percent fresh weight basis.

Reference:

Tagers, S.R.; Pesti, G.M. 1990. "Determination of Tryptophan from Feedstuffs Using Reverse Phase High-Performance Liquid Chromatography." Journal of Micronutrient Analysis. 7:27-35.

Amino Acids (SE-58)

Subsamples of ground soybean seed are analyzed to determine the amount of the 15 amino acids by converting the free acids, after acid hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives. Samples are then analyzed by reverse phase Ultra Performance Liquid Chromatography (UPLC) with UV detection. The following amino acids are used as analytical reference substances to verify the accuracy of the method and UPLC:

- L-Alanine – lot number 443129/1 and purity 100%. LOQ is 0.375%.
- L-Arginine Hydrochloride – lot number 095K0089 and purity >99%. LOQ is 0.310%.
- L-Aspartic Acid (Free Acid) – lot number 093K01502 and purity >99%. LOQ is 0.375%.
- L-Glutamic Acid (Free Acid) – lot number 085K0713 and purity >99%. LOQ is 0.375%.
- Glycine (Free Base) – lot number 034K0166 and purity >99%. LOQ is 0.375%.
- L-Histidine Monohydrochloride Monohydrate – lot number 114K0378 and purity >99%. LOQ is 0.278%.
- L-Isoleucine – lot number 065K0231 and purity >99%. LOQ is 0.375%.
- L-Leucine – lot number 045K0387 and purity >99%. LOQ is 0.375%
- L-Lysine Monohydrochloride – lot number 067K0078 and purity 99.4%. LOQ is 0.300%.
- L-Phenylalanine – lot number 1166794 and purity 100%. LOQ is 0.375%.
- L-Proline- lot number 106K0128 and purity 98.9%. LOQ is 0.370%.
- L-Serine – lot number 077K0015 and purity 99.5%. LOQ is 0.375%.
- L-Threonine – lot number 095K0374 and purity >99%. LOQ is 0.375%.
- L-Tyrosine (Free Base) – lot number 075K0015 and purity 100%. LOQ is 0.375%.
- L-Valine – lot number 095K0378 and purity >99%. LOQ is 0.375%.

- L-Alpha-Amino-N-Butyric Acid - lot number 126K2666 and purity 100%. No LOQ.

The amino acid analytical reference substances are purchased from Sigma. The amino acids results are expressed on a percent fresh weight basis.

References:

Hong, Ji Liu (1994). "Determination of Amino Acids by Precolumn Derivatization 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and Ultra-Performance Liquid Chromatography with Ultraviolet Detection." Journal of Chromatography A, 670 (1994): 59-66.

Waters Method, Analysis of Amino Acids in Feeds and Foods Using Modification of the ACCQ•Tag Method ™ for Amino Acid Analysis.

Cystine and Methionine (SE-59)

Subsamples of ground soybean seed are analyzed to determine the amount of cystine and methionine by converting the cystine to cysteic acid and methionine to methionine sulfone after acid oxidation and hydrolysis, to the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatives. Samples are then analyzed by reverse phase UPLC with UV detection. The LOQ is 0.0417 %. The following analytical reference substances are used to verify the method and UPLC:

- L-Cysteic Acid - lot number 1215898 and purity 99.3%
- L-Methionine Sulfone - lot number 116K1146 and purity 100%
- L-Cystine - lot number 037K0148 and purity 100%
- L-Methionine - lot number 074K0372 and purity >99%

The analytical reference substances are purchased from Sigma. The cystine and methionine results are expressed on a percent fresh weight basis.

References:

Hong, Ji Liu (1994). "Determination of Amino Acids by Precolumn Derivatization 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate and Ultra-Performance Liquid Chromatography with Ultraviolet Detection." Journal of Chromatography A, 670 (1994): 59-66.

Waters Method, Analysis of Amino Acids in Feeds and Foods Using Modification of the ACCQ•Tag Method ™ for Amino Acid Analysis.

Fatty Acid (SE-45)

Subsamples of ground soybean seed are analyzed to determine the fatty acid content by using a soxhlet extraction apparatus. The fatty acids are derivatized into methyl esters with boron trifluoride/methanol. The methyl esters are then assayed by Gas Chromatography (GC) with Flame Ionization Detection (FID). The following analytical reference substances are used to verify the method and the GC:

- Fatty Acid Methyl Ester (FAME) Standard (Major Acids) 0.5% C12:0 (LOQ = 0.00505%), 0.5% C14:0 (LOQ = 0.00509%), 10% C16:0 (LOQ = 0.102%), 0.5% C16:1 (LOQ = 0.00512%), 0.5% C17:0 (LOQ = 0.00513%), 0.5% C17:1 (LOQ = 0.00513%), 4% C18:0 (LOQ = 0.0412%), 20% C18:1 (LOQ = 0.206%), 51% C18:2 (LOQ = 0.00926%), 9% C18:3 (LOQ = 0.00165%), 0.5% C20:0 (LOQ = 0.00517%), 0.5% C20:1 (LOQ = 0.00517%), 0.5% C20:2 (LOQ = 0.00517%), 1% C22:0 (LOQ = 0.0104%), and 1% C24:0 (LOQ = 0.0104%), lot number N15-P.
- FAME Reference Standard (Minor Acids), 10% C8:0 (LOQ = 0.0197%), 10% C10:0 LOQ = 0.0200%), 10% C14:1 (LOQ = 0.0203%), 10% C15:0 (LOQ = 0.0204%), 10% C15:1 (LOQ = 0.0204%), 10% C17:1 (0.00513%), 10% C18:2 (0.00926%), 10% C18:3 (GLA) (LOQ = 0.0206), 10% C20:3 (LOQ = 0.0207%), 10% C20:4 (LOQ = 0.0207%), and 10% C22:1 (LOQ = 0.0207%, lot number N15-P.
- Tridecanoic Acid (C13:0) - lot number N-13A-JY10-Q and purity >99%. No LOQ.
- Methyl Tridecanoate - lot number N-13M-MA12-R and purity >99%. No LOQ.

The analytical reference substances are purchased from Nu-Chek Prep. The fatty acid results are reported on a percent fresh weight basis.

Reference:

AOAC International Method 939.05 (2000). In Official Methods of Analysis of the AOAC International, 17th Edition. Association of Official Analytical Chemists International, Gaithersburg, Maryland.

Trypsin Inhibitor (SE-12)

Subsamples of ground soybean seed are analyzed to determine trypsin inhibitor content by extracting with sodium hydroxide. Trypsin is added and reacted with the trypsin inhibitor. The amount of trypsin present in the sample is measured using a spectrophotometer, and the amount of inhibitor is calculated based on how much trypsin remains. The trypsin reference substance was purchased from MP Biomedicals. The activity is 245 µ/mg and the lot number is 5432H. There is no LOQ. The trypsin results are reported on a percent fresh weight basis.

Reference:

Anonymous 1997. Trypsin Inhibitor Activity. Official Methods and Recommended Practices of AOCS, Ba 12-75.

Phytic Acid (SE-10)

Subsamples of ground soybean seed are analyzed to determine the amount of phytic acid by extracting the phytic acid with dilute hydrochloric acid and isolating it using an ion-exchange solid phase extraction column. Once isolated and eluted, the phytic acid is analyzed for elemental phosphorus by inductively coupled plasma optical emission spectroscopy (ICP-OES). The phytic acid content is then calculated from the phosphorus concentration. The LOQ is 0.355%. The following analytical reference substances are used to verify the method and the ICP-OES:

- Phosphorus Standard - lot number SC7061617 and concentration 10,050 µg/mL

- Yttrium Standard – lot number SC7192512 and concentration 1001 µg/mL
- Phytic Acid Standard - lot number 035K0590 and purity 97%.

The phosphorus and yttrium were purchased from SCP Science Solution and the phytic acid from Sigma. Phytic acid is reported on a percent fresh weight basis.

Reference:

Anonymous 1988. Phytic Acid in Foods. Official Methods of Analysis of AOAC International, Vol. 2.32.5.18.

Lectins (SE-49)

Subsamples of ground soybean seed are analyzed to determine the amount of lectin by extracting the lectin with potassium phosphate buffer. Lectin was assayed using a hemagglutination test using rabbit red blood cells. The amount of hemagglutination was measured by the amount of turbidity using a spectrophotometer. There are no reference substances and LOQ for the assay. Lectin is reported on a percent fresh weight basis.

Reference:

Leiner, Irvin, E. 1954. *The Photometric Determination of the Hemagglutination Activity of Soyin and Crude Soybean Extracts.* Scientific Journal Series, Minnesota Agricultural Experiment Station.

Isoflavones (SE-56)

Subsamples of ground soybean seed are analyzed to determine the amount of aglycones by extracting the aglycones with ethanol and hydrochloric acid. The extract is cleaned up using a C18 Sep-PAK and assayed by reverse phase HPLC with UV detection. The LOQ is 10.00 mg/Kg. The following analytical reference substances were used to verify the method and HPLC:

- Daidzein - lot number DA-120 and purity >99%,
- Genistein - lot number CH-147 and purity >99%
- Glycitein - lot number 0306103 and purity 97%

The analytical reference substances daidzein and genistein are purchased from LC Laboratories and glycitein was purchased from Indofine Chemical Company, Inc. Isoflavones are reported on a percent fresh weight basis.

References:

Pettersson, H., and Kiessling, K.H., "Liquid Chromatographic Determination of the Plant Estrogens Coumestrol and Isoflavones in Animal Feed." Association of Analytical Chemist Journal, 67 (3):503-506 (1984).

Seo, A., and Morr, C.V., "Improved High Performance Liquid Chromatographic Analysis of Phenolic Acids and Isoflavoids from Soybean Protein Products." J. Agric. Food Chem, 32: 530-533 (1984).

Stachyose/Raffinose (SOP SE-40)

Subsamples of ground soybean seed are analyzed to determine the amount of stachyose and raffinose by extracting with methanol/DI water, partitioning with chloroform and evaporating to dryness. The sample residue is redissolved in DI water and analyzed by reverse phase HPLC with refractive index detection. The following analytical reference substances were used to verify the method and HPLC:

- Stachyose Hydrate - lot number 065K3775 and purity 98%. The LOQ is 0.260%
- Raffinose Pentahydrate - lot number 035K1371 and purity 99%. The LOQ is 0.200%.

The analytical reference substances were purchased from Sigma. Stachyose and raffinose are reported on a percent fresh weight basis.

References:

Anonymous 1985, "Determination of Simple Sugars in Cereal Products – HPLC Method." Approved Methods of the Association of Cereal Chemists, Volume II, 80-04.

Johansen, Helle Nygaard; Glisto, Vibe; Knudsen, Erik Bach. 1996. Influence of Extraction Solvent and Temperature on the Quantitative Determination of Oligosaccharides from Plant Materials by High-Performance Liquid Chromatography. J. Agric. Food Chem., 44, 1470-1474.

Vitamin E (α-tocopherol) (SOP-SE-42)

Subsamples of ground soybean seed are analyzed to determine the amount α-tocopherol by extracting with hexane. The hexane extract is analyzed by HPLC with fluorescence detection. The following analytical reference substance was used to verify the method and HPLC: Alpha Tocopherol. The analytical reference substance was purchased from Sigma and has a purity of 97%. The lot number is 066K0667. The LOQ is 2.00 mg/Kg. Alpha tocopherol is reported on a percent fresh weight basis.

Reference:

Anonymous 1984, "High Performance Liquid Chromatography of the Tocols in Corn Grain." JAOCS, Vol. 61 No. 7, July 1984.

Data Processing and Statistical Analysis

After compositional analyses were performed at EPL-BAS, data spreadsheets were forwarded to Monsanto Company. The data were reviewed, formatted, and sent to Certus International, Inc. for statistical analysis.

The following formulas were used for re-expression of composition data for statistical analysis:

Component	From (X)	To	Formula[1]
Proximates (excluding Moisture), Fiber, Phytic Acid, Raffinose, Stachyose, Amino Acids (AA)	% FW	% DW	X/d
Isoflavones	mg/kg FW	mg/kg DW	X/d
Trypsin Inhibitor	TIU/mg FW	TIU/mg DW	X/d
Vitamin E	mg/kg FW	mg/100g DW	X/(10*d)
Fatty Acids (FA)	% FW	% Total FA	$(100)X_j/\Sigma X$, for each FA_j where ΣX is over all the FA
[1] 'X' is the individual sample value; 'd' is the fraction of the sample that is dry matter.			

In order to complete a statistical analysis for a compositional component in this study, at least 50% of the values for an analyte had to be greater than the assay LOQ. Analytes with more than 50% of observations below the assay LOQ were excluded from summaries and analysis. The following nine analytes with more than 50% of observations below the assay LOQ were excluded from statistical analysis: 8:0 caprylic acid, 12:0 lauric acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 18:3 gamma linolenic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, and 22:1 erucic acid.

Otherwise, individual analyses that were below the LOQ were assigned a value equal to half the LOQ. The following components were assigned values:

Component	Units	Obs. Below LOQ N	Obs. Below LOQ (%)	Total N	LOQ	Value Assigned
Seed Fatty Acid						
17:1 Heptadecenoic Acid	% FW	8	12.7	63	0.0051	0.0026
20:2 Eicosadienoic Acid	% FW	9	14.3	63	0.0052	0.0026

A PRESS residual is the difference between any value and its predicted value from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ± 3. Extreme data points that are also outside of the ± 6 studentized PRESS residual range are considered for exclusion, as outliers, from the final analyses. The following results had PRESS residual values outside of ± 6 range:

Site	Rep	Description	Analyte	ID	Sent Value	Value	PRESS Std Residual
Forage Proximate							
GA	2	CMC 5901COC	Moisture	07017101-00329	38.6	38.6000	-11.4687
Seed Proximate							
NC	2	MON 87701	Total Fat	07017101-00627	33.7	36.3656	9.6571

Both identified values were considered outliers and were removed from further analysis. Because moisture content is required for unit re-expression of forage composition data, all additional forage composition data associated with this sample with an outlier moisture value were removed from the dataset for statistical evaluation.

The outlier test procedure was reapplied to all remaining moisture and total fat data to detect potential outliers that were masked in the first analysis. No further PRESS residuals were outside of ± 6 range.

All soybean compositional analysis components were statistically analyzed using a mixed model analysis of variance. The five replicated sites were analyzed both separately and combined. Individual replicated site analyses used model (1).

(1) $Yij = U + Ti + Bj + eij,$

where Yij = unique individual observation, U = overall mean, Ti = substance effect, Bj = random block effect, and eij = residual error.

Combined site analyses used model (2).

(2) $Yijk = U + Ti + Lj + B(L)jk + LTij + eijk,$

where Yijk = unique individual observation, U = overall mean, Ti = substance effect, Lj = random location effect, B(L)jk = random block within location effect, LTij = random location by substance interaction effect, and eijk = residual error.

A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured.

For each compositional component, 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of commercial conventional substances. Each tolerance interval estimate was based upon one observation per unique reference substance. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

SAS® software was used to generate all summary statistics and perform all analyses. Report tables present p-values from SAS as either <0.001 or the actual value truncated to three decimal places.

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Appendix G. Individual Site Composition Tables for Soybean Harvested Seed and Forage Collected from 2007 U.S. Field Trials

Table G-1. Statistical Summary of Site AL Soybean Forage and Proximate Content for MON 87701 vs the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	34.43 (3.22) [30.04 - 38.83]	39.77 (2.63) [37.44 - 42.06]	-5.34 (4.15) [-12.02 - 1.39]	-18.56, 7.88	0.288	(27.99 - 47.33) [14.93, 56.87]
Neutral Detergent Fiber	46.71 (7.87) [42.49 - 49.59]	48.02 (6.52) [42.05 - 59.19]	-1.31 (9.17) [-9.60 - -0.31]	-30.47, 27.86	0.895	(30.96 - 54.55) [21.51, 66.01]
Proximate (% DW, unless noted)						
Ash	5.52 (0.51) [5.05 - 5.98]	7.27 (0.42) [6.24 - 8.13]	-1.76 (0.66) [-1.47 - -1.19]	-3.85, 0.34	0.076	(4.77 - 8.54) [2.46, 10.14]
Carbohydrates	69.48 (1.72) [68.29 - 71.06]	66.97 (1.46) [63.68 - 69.20]	2.51 (1.86) [1.86 - 4.62]	-3.41, 8.43	0.270	(60.61 - 77.26) [56.93, 85.88]
Moisture (% FW)	74.24 (0.91) [72.70 - 75.40]	76.93 (0.78) [75.00 - 78.10]	-2.69 (0.95) [-2.30 - -2.30]	-5.70, 0.32	0.065	(66.50 - 80.20) [57.84, 88.56]
Protein	19.86 (1.92) [19.72 - 19.92]	19.84 (1.57) [17.94 - 23.29]	0.026 (2.41) [-3.57 - 1.98]	-7.65, 7.70	0.992	(12.68 - 22.92) [7.05, 27.27]
Total Fat	4.90 (0.56) [3.96 - 5.85]	5.98 (0.46) [5.61 - 6.72]	-1.08 (0.72) [-2.76 - 0.25]	-3.37, 1.22	0.232	(3.48 - 7.88) [1.11, 9.11]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-2. Statistical Summary of AL Site Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.83 (0.036) [1.83 - 1.84]	1.81 (0.030) [1.81 - 1.82]	0.018 (0.047) [0.018 - 0.020]	-0.13, 0.17	0.731	(1.66 - 1.93) [1.49, 2.02]
Arginine	2.97 (0.081) [2.95 - 3.00]	2.79 (0.067) [2.72 - 2.89]	0.18 (0.11) [0.056 - 0.28]	-0.15, 0.52	0.180	(2.54 - 2.99) [2.22, 3.25]
Aspartic Acid	5.28 (0.11) [5.23 - 5.26]	5.27 (0.095) [5.15 - 5.34]	0.013 (0.12) [-0.11 - 0.11]	-0.36, 0.39	0.916	(4.74 - 5.50) [4.22, 5.96]
Cystine	0.67 (0.044) [0.65 - 0.67]	0.61 (0.037) [0.58 - 0.63]	0.060 (0.049) [0.044 - 0.066]	-0.095, 0.21	0.308	(0.53 - 0.68) [0.45, 0.77]
Glutamic Acid	8.24 (0.17) [8.18 - 8.21]	8.18 (0.14) [8.08 - 8.26]	0.057 (0.20) [-0.076 - 0.13]	-0.57, 0.69	0.791	(7.53 - 8.72) [6.60, 9.37]
Glycine	1.88 (0.042) [1.86 - 1.89]	1.80 (0.034) [1.76 - 1.85]	0.078 (0.054) [0.0092 - 0.12]	-0.094, 0.25	0.244	(1.67 - 1.99) [1.49, 2.09]
Histidine	1.17 (0.030) [1.16 - 1.18]	1.12 (0.024) [1.09 - 1.15]	0.055 (0.038) [0.014 - 0.090]	-0.067, 0.18	0.248	(1.04 - 1.24) [0.94, 1.31]
Isoleucine	1.98 (0.046) [1.98 - 1.99]	1.91 (0.037) [1.88 - 1.96]	0.071 (0.059) [0.019 - 0.10]	-0.12, 0.26	0.313	(1.73 - 2.02) [1.54, 2.14]

Table G-2. Statistical Summary of Site AL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	3.35 (0.074) [3.34 - 3.36]	3.20 (0.060) [3.13 - 3.29]	0.15 (0.095) [0.053 - 0.23]	-0.15, 0.45	0.205	(2.93 - 3.32) [2.64, 3.52]
Lysine	2.95 (0.091) [2.92 - 2.99]	2.83 (0.074) [2.67 - 2.91]	0.13 (0.12) [0.022 - 0.077]	-0.25, 0.50	0.357	(2.35 - 3.15) [2.05, 3.47]
Methionine	0.58 (0.038) [0.56 - 0.58]	0.53 (0.032) [0.51 - 0.56]	0.042 (0.043) [0.018 - 0.049]	-0.095, 0.18	0.403	(0.49 - 0.62) [0.42, 0.68]
Phenylalanine	2.44 (0.089) [2.40 - 2.48]	2.20 (0.073) [2.08 - 2.38]	0.24 (0.12) [0.016 - 0.41]	-0.13, 0.60	0.130	(1.97 - 2.44) [1.66, 2.64]
Proline	2.15 (0.044) [2.15 - 2.16]	2.10 (0.036) [2.09 - 2.12]	0.054 (0.057) [0.029 - 0.072]	-0.13, 0.23	0.414	(1.92 - 2.25) [1.73, 2.35]
Serine	2.17 (0.045) [2.15 - 2.19]	2.09 (0.037) [2.05 - 2.13]	0.080 (0.058) [0.018 - 0.14]	-0.11, 0.27	0.262	(1.96 - 2.30) [1.75, 2.38]
Threonine	1.70 (0.038) [1.69 - 1.72]	1.62 (0.031) [1.58 - 1.68]	0.078 (0.049) [0.010 - 0.13]	-0.079, 0.24	0.210	(1.54 - 1.74) [1.40, 1.83]
Tryptophan	0.51 (0.012) [0.50 - 0.52]	0.51 (0.0096) [0.50 - 0.52]	0.00042 (0.014) [-0.0056 - -0.0055]	-0.044, 0.044	0.977	(0.47 - 0.55) [0.43, 0.59]

Table G-2. Statistical Summary of Site AL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.32 (0.031) [1.28 - 1.33]	1.20 (0.026) [1.17 - 1.22]	0.12 (0.032) [0.11 - 0.12]	0.016, 0.22	0.034	(1.04 - 1.31) [0.85, 1.48]
Valine	2.07 (0.043) [2.06 - 2.07]	1.99 (0.035) [1.96 - 2.04]	0.075 (0.056) [0.029 - 0.11]	-0.10, 0.25	0.271	(1.83 - 2.13) [1.64, 2.22]
Fatty Acid (% Total FA)						
10:0 Capric Acid	0.19 (0.012) [0.18 - 0.19]	0.21 (0.010) [0.18 - 0.23]	-0.025 (0.016) [-0.032 - -0.0018]	-0.076, 0.026	0.215	(0.15 - 0.27) [0.065, 0.34]
14:0 Myristic Acid	0.10 (0.0013) [0.10 - 0.10]	0.10 (0.0010) [0.10 - 0.11]	-0.00044 (0.0015) [-0.00076 - 0.0021]	-0.0052, 0.0043	0.790	(0.064 - 0.097) [0.052, 0.12]
16:0 Palmitic Acid	12.07 (0.11) [12.05 - 12.09]	12.04 (0.090) [11.96 - 12.08]	0.032 (0.14) [0.013 - 0.095]	-0.42, 0.48	0.836	(9.80 - 12.38) [8.88, 13.53]
16:1 Palmitoleic Acid	0.10 (0.0043) [0.091 - 0.11]	0.092 (0.0036) [0.091 - 0.095]	0.0079 (0.0052) [-0.00090 - 0.015]	-0.0087, 0.025	0.225	(0.073 - 0.14) [0.037, 0.15]
17:0 Heptadecanoic Acid	0.10 (0.00085) [0.10 - 0.10]	0.099 (0.00074) [0.099 - 0.099]	0.0025 (0.00080) [0.0017 - 0.0028]	-0.00008, 0.0050	0.053	(0.076 - 0.10) [0.066, 0.11]
17:1 Heptadecenoic Acid	0.048 (0.00083) [0.047 - 0.048]	0.047 (0.00068) [0.046 - 0.047]	0.00076 (0.0011) [-0.00020 - 0.0016]	-0.0027, 0.0042	0.529	(0.020 - 0.064) [0.0058, 0.083]

Table G-2. Statistical Summary of Site AL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
18:0 Stearic Acid	4.42 (0.096) [4.38 - 4.45]	4.51 (0.078) [4.34 - 4.60]	-0.091 (0.12) [-0.14 - 0.037]	-0.48, 0.30	0.512	(3.21 - 5.24) [1.88, 6.25]
18:1 Oleic Acid	26.17 (1.17) [25.70 - 26.64]	27.43 (0.96) [26.26 - 28.78]	-1.26 (1.52) [-2.14 - -1.55]	-6.08, 3.57	0.468	(16.69 - 35.16) [5.01, 42.01]
18:2 Linoleic Acid	49.75 (1.07) [49.32 - 50.17]	48.50 (0.88) [47.18 - 49.32]	1.24 (1.38) [1.16 - 2.14]	-3.16, 5.64	0.435	(44.17 - 57.72) [38.57, 66.94]
18:3 Linolenic Acid	5.60 (0.12) [5.55 - 5.65]	5.47 (0.10) [5.34 - 5.68]	0.13 (0.16) [0.16 - 0.31]	-0.38, 0.64	0.473	(4.27 - 8.81) [2.69, 10.81]
20:0 Arachidic Acid	0.54 (0.013) [0.54 - 0.55]	0.55 (0.011) [0.53 - 0.57]	-0.0059 (0.017) [-0.016 - 0.0079]	-0.061, 0.049	0.754	(0.36 - 0.55) [0.23, 0.64]
20:1 Eicosenoic Acid	0.24 (0.015) [0.21 - 0.28]	0.28 (0.012) [0.27 - 0.28]	-0.031 (0.018) [-0.065 - -0.00053]	-0.088, 0.027	0.189	(0.21 - 0.30) [0.16, 0.33]
20:2 Eicosadienoic Acid	0.033 (0.0060) [0.020 - 0.045]	0.044 (0.0049) [0.040 - 0.047]	-0.011 (0.0077) [-0.020 - -0.0013]	-0.035, 0.014	0.253	(0.016 - 0.054) [0.0029, 0.083]
22:0 Behenic Acid	0.64 (0.019) [0.62 - 0.65]	0.63 (0.016) [0.61 - 0.65]	0.0064 (0.025) [-0.00071 - 0.018]	-0.074, 0.086	0.816	(0.38 - 0.59) [0.30, 0.67]

Table G-2. Statistical Summary of Site AL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	14.83 (0.61) [13.66 - 16.00]	14.28 (0.49) [14.00 - 14.72]	0.55 (0.78) [-1.07 - 1.88]	-1.94, 3.04	0.534	(12.79 - 17.98) [11.13, 20.21]
Neutral Detergent Fiber	17.37 (1.02) [15.06 - 19.33]	17.49 (0.93) [16.02 - 18.38]	-0.13 (0.84) [-0.97 - 1.25]	-2.80, 2.55	0.890	(13.32 - 23.57) [7.24, 28.70]
Proximate (% DW, unless noted)						
Ash	5.89 (0.073) [5.87 - 5.90]	5.79 (0.059) [5.69 - 5.88]	0.10 (0.094) [0.025 - 0.18]	-0.20, 0.40	0.358	(4.32 - 5.62) [3.74, 6.45]
Carbohydrates	29.12 (0.78) [29.10 - 29.14]	30.31 (0.64) [29.88 - 30.56]	-1.19 (1.01) [-1.39 - -0.74]	-4.41, 2.03	0.324	(31.97 - 38.00) [28.17, 40.99]
Moisture (% FW)	6.94 (1.08) [6.86 - 7.01]	6.85 (0.89) [6.42 - 7.63]	0.082 (1.40) [-0.62 - 0.35]	-4.37, 4.54	0.957	(5.48 - 11.70) [1.45, 12.81]
Protein	42.12 (0.53) [42.01 - 42.23]	41.51 (0.43) [41.07 - 41.87]	0.60 (0.68) [0.37 - 0.40]	-1.56, 2.77	0.440	(38.14 - 42.66) [35.30, 45.38]
Total Fat	22.92 (0.33) [22.69 - 23.08]	22.40 (0.27) [22.25 - 22.55]	0.52 (0.39) [0.28 - 0.84]	-0.73, 1.77	0.275	(17.90 - 23.56) [14.74, 25.18]
Vitamin (mg/100g DW)						
Vitamin E	7.78 (0.20) [7.58 - 7.98]	6.98 (0.16) [6.86 - 7.21]	0.79 (0.26) [0.70 - 1.12]	-0.022, 1.61	0.053	(1.65 - 8.08) [0, 11.09]

Table G-2. Statistical Summary of Site AL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg FW)	0.17 (0.20) [0.062 - 0.33]	0.32 (0.17) [0.28 - 0.36]	-0.15 (0.22) [-0.22 - -0.037]	-0.86, 0.55	0.542	(0.090 - 2.47) [0, 3.40]
Phytic Acid	2.25 (0.11) [2.22 - 2.29]	2.39 (0.090) [2.23 - 2.66]	-0.14 (0.14) [-0.015 - 0.019]	-0.59, 0.32	0.412	(1.10 - 2.32) [0.54, 3.05]
Raffinose	0.51 (0.076) [0.49 - 0.56]	0.49 (0.063) [0.43 - 0.55]	0.021 (0.089) [-0.054 - 0.075]	-0.26, 0.31	0.827	(0.52 - 1.62) [0.038, 2.24]
Stachyose	1.84 (0.11) [1.83 - 1.89]	2.37 (0.095) [2.27 - 2.55]	-0.53 (0.13) [-0.72 - -0.38]	-0.93, -0.13	0.024	(1.97 - 5.55) [0.99, 7.93]
Trypsin Inhibitor (TIU/mg DW)	33.46 (2.35) [32.26 - 32.53]	31.07 (2.02) [26.21 - 34.20]	2.39 (2.43) [-0.54 - 6.33]	-5.33, 10.12	0.396	(20.84 - 37.24) [13.58, 46.02]
Isoflavone (mg/kg DW)						
Daidzein	202.56 (28.49) [188.96 - 216.15]	216.48 (23.26) [198.95 - 237.23]	-13.93 (36.78) [-9.99 - 2.88]	-130.99, 103.14	0.730	(213.98 - 1273.94) [0, 1585.14]
Genistein	229.96 (22.24) [214.73 - 245.19]	253.03 (18.16) [244.95 - 259.82]	-23.07 (28.71) [-30.22 - -14.64]	-114.43, 68.29	0.480	(148.06 - 1024.50) [0, 1352.86]
Glycitein	71.34 (5.26) [61.08 - 79.67]	64.86 (4.82) [61.28 - 67.07]	6.47 (4.15) [-0.19 - 12.60]	-6.73, 19.68	0.216	(32.42 - 208.45) [0, 272.12]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-3. Statistical Summary of Site AR Soybean Forage Fiber and Proximate Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	37.57 (2.54) [31.80 - 41.20]	36.11 (2.54) [31.86 - 39.42]	1.46 (3.59) [-0.066 - 2.66]	-8.52, 11.43	0.705	(27.99 - 47.33) [14.93, 56.87]
Neutral Detergent Fiber	49.83 (2.23) [46.69 - 55.99]	38.62 (2.23) [37.23 - 40.51]	11.21 (3.15) [6.31 - 18.76]	2.45, 19.96	0.023	(30.96 - 54.55) [21.51, 66.01]
Proximate (% DW, unless noted)						
Ash	5.83 (0.41) [5.29 - 6.52]	6.61 (0.41) [5.58 - 7.23]	-0.78 (0.58) [-1.72 - 0.085]	-2.40, 0.84	0.251	(4.77 - 8.54) [2.46, 10.14]
Carbohydrates	70.56 (1.12) [68.75 - 72.89]	70.59 (1.12) [69.06 - 72.99]	-0.030 (1.58) [-0.31 - 0.33]	-4.42, 4.36	0.985	(60.61 - 77.26) [56.93, 85.88]
Moisture	72.57 (0.68) [71.60 - 73.30]	72.47 (0.68) [72.20 - 72.60]	0.10 (0.82) [-1.00 - 0.70]	-2.18, 2.38	0.908	(66.50 - 80.20) [57.84, 88.56]
Protein	18.53 (0.85) [17.10 - 20.03]	17.37 (0.85) [16.54 - 18.45]	1.16 (1.20) [-0.031 - 1.92]	-2.17, 4.49	0.389	(12.68 - 22.92) [7.05, 27.27]
Total Fat	5.20 (0.44) [4.61 - 5.99]	5.33 (0.44) [4.31 - 6.39]	-0.13 (0.48) [-1.41 - 0.70]	-1.47, 1.21	0.798	(3.48 - 7.88) [1.11, 9.11]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-4. Statistical Summary of Site AR Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.70 (0.023) [1.66 - 1.77]	1.67 (0.023) [1.65 - 1.69]	0.026 (0.033) [-0.034 - 0.099]	-0.065, 0.12	0.474	(1.66 - 1.93) [1.49, 2.02]
Arginine	2.63 (0.064) [2.57 - 2.69]	2.61 (0.064) [2.53 - 2.66]	0.020 (0.091) [-0.061 - 0.092]	-0.23, 0.27	0.838	(2.54 - 2.99) [2.22, 3.25]
Aspartic Acid	4.85 (0.091) [4.69 - 5.12]	4.82 (0.091) [4.76 - 4.91]	0.028 (0.13) [-0.16 - 0.31]	-0.33, 0.38	0.837	(4.74 - 5.50) [4.22, 5.96]
Cystine	0.62 (0.021) [0.58 - 0.66]	0.61 (0.021) [0.58 - 0.63]	0.0092 (0.018) [-0.011 - 0.043]	-0.042, 0.060	0.642	(0.53 - 0.68) [0.45, 0.77]
Glutamic Acid	7.57 (0.12) [7.35 - 7.94]	7.50 (0.12) [7.37 - 7.66]	0.071 (0.18) [-0.23 - 0.46]	-0.42, 0.56	0.705	(7.53 - 8.72) [6.60, 9.37]
Glycine	1.73 (0.017) [1.70 - 1.76]	1.69 (0.017) [1.67 - 1.70]	0.040 (0.024) [0.0088 - 0.056]	-0.028, 0.11	0.180	(1.67 - 1.99) [1.49, 2.09]
Histidine	1.11 (0.014) [1.09 - 1.12]	1.07 (0.014) [1.05 - 1.09]	0.038 (0.020) [0.027 - 0.054]	-0.018, 0.095	0.132	(1.04 - 1.24) [0.94, 1.31]
Isoleucine	1.79 (0.027) [1.74 - 1.86]	1.75 (0.027) [1.72 - 1.79]	0.034 (0.038) [-0.044 - 0.11]	-0.070, 0.14	0.413	(1.73 - 2.02) [1.54, 2.14]

Table G-4. Statistical Summary of Site AR Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	2.99 (0.041) [2.93 - 3.10]	2.93 (0.041) [2.87 - 2.97]	0.066 (0.059) [-0.038 - 0.17]	-0.097, 0.23	0.325	(2.93 - 3.32) [2.64, 3.52]
Lysine	2.68 (0.086) [2.58 - 2.83]	2.61 (0.086) [2.45 - 2.74]	0.068 (0.12) [-0.12 - 0.39]	-0.27, 0.40	0.603	(2.35 - 3.15) [2.05, 3.47]
Methionine	0.55 (0.018) [0.51 - 0.57]	0.53 (0.018) [0.51 - 0.57]	0.011 (0.022) [-0.013 - 0.049]	-0.051, 0.073	0.648	(0.49 - 0.62) [0.42, 0.68]
Phenylalanine	2.06 (0.056) [2.03 - 2.08]	2.02 (0.056) [1.98 - 2.06]	0.042 (0.079) [0.011 - 0.095]	-0.18, 0.26	0.625	(1.97 - 2.44) [1.66, 2.64]
Proline	1.99 (0.027) [1.95 - 2.07]	1.96 (0.027) [1.93 - 1.99]	0.034 (0.038) [-0.043 - 0.11]	-0.071, 0.14	0.417	(1.92 - 2.25) [1.73, 2.35]
Serine	2.00 (0.021) [1.97 - 2.06]	1.95 (0.021) [1.92 - 1.97]	0.055 (0.026) [0.010 - 0.10]	-0.015, 0.13	0.095	(1.96 - 2.30) [1.75, 2.38]
Threonine	1.58 (0.021) [1.55 - 1.62]	1.54 (0.021) [1.51 - 1.55]	0.045 (0.030) [0.0080 - 0.071]	-0.038, 0.13	0.209	(1.54 - 1.74) [1.40, 1.83]
Tryptophan	0.51 (0.015) [0.48 - 0.54]	0.49 (0.015) [0.46 - 0.52]	0.021 (0.021) [-0.039 - 0.075]	-0.039, 0.080	0.393	(0.47 - 0.55) [0.43, 0.59]

Table G-4. Statistical Summary of Site AR Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.08 (0.052) [1.04 - 1.13]	1.12 (0.052) [1.03 - 1.17]	-0.037 (0.074) [-0.11 - 0.095]	-0.24, 0.17	0.641	(1.04 - 1.31) [0.85, 1.48]
Valine	1.89 (0.025) [1.85 - 1.96]	1.85 (0.025) [1.82 - 1.88]	0.036 (0.035) [-0.033 - 0.098]	-0.062, 0.13	0.365	(1.83 - 2.13) [1.64, 2.22]
Fatty Acid (% Total FA)						
10:0 Capric Acid	0.18 (0.023) [0.14 - 0.22]	0.23 (0.023) [0.19 - 0.26]	-0.053 (0.024) [-0.11 - -0.012]	-0.12, 0.012	0.085	(0.15 - 0.27) [0.065, 0.34]
14:0 Myristic Acid	0.083 (0.00059) [0.082 - 0.084]	0.084 (0.00059) [0.083 - 0.085]	-0.00087 (0.00084) [-0.0021 - 0.00099]	-0.0032, 0.0015	0.359	(0.064 - 0.097) [0.052, 0.12]
16:0 Palmitic Acid	11.65 (0.053) [11.60 - 11.70]	11.65 (0.053) [11.50 - 11.73]	-0.0067 (0.076) [-0.14 - 0.20]	-0.22, 0.20	0.933	(9.80 - 12.38) [8.88, 13.53]
16:1 Palmitoleic Acid	0.082 (0.0033) [0.073 - 0.088]	0.085 (0.0033) [0.078 - 0.089]	-0.0030 (0.0047) [-0.016 - 0.0071]	-0.016, 0.010	0.558	(0.073 - 0.14) [0.037, 0.15]
17:0 Heptadecanoic Acid	0.096 (0.00074) [0.095 - 0.097]	0.095 (0.00074) [0.095 - 0.096]	0.00052 (0.0010) [-0.00053 - 0.0017]	-0.0024, 0.0034	0.640	(0.076 - 0.10) [0.066, 0.11]
17:1 Heptadecenoic Acid	0.037 (0.0042) [0.023 - 0.046]	0.044 (0.0042) [0.043 - 0.045]	-0.0070 (0.0055) [-0.020 - 0.0031]	-0.022, 0.0084	0.275	(0.020 - 0.064) [0.0058, 0.083]

Table G-4. Statistical Summary of Site AR Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
18:0 Stearic Acid	4.01 (0.048) [3.97 - 4.07]	4.11 (0.048) [4.03 - 4.16]	-0.095 (0.041) [-0.16 - -0.058]	-0.21, 0.019	0.082	(3.21 - 5.24) [1.88, 6.25]
18:1 Oleic Acid	20.21 (0.33) [19.78 - 20.96]	20.62 (0.33) [20.34 - 21.14]	-0.41 (0.43) [-1.36 - 0.59]	-1.59, 0.78	0.395	(16.69 - 35.16) [5.01, 42.01]
18:2 Linoleic Acid	54.22 (0.30) [53.57 - 54.63]	53.79 (0.30) [53.50 - 54.07]	0.43 (0.29) [-0.23 - 0.98]	-0.37, 1.24	0.207	(44.17 - 57.72) [38.57, 66.94]
18:3 Linolenic Acid	8.29 (0.062) [8.14 - 8.41]	8.17 (0.062) [8.12 - 8.26]	0.12 (0.087) [-0.12 - 0.27]	-0.12, 0.36	0.234	(4.27 - 8.81) [2.69, 10.81]
20:0 Arachidic Acid	0.42 (0.0035) [0.41 - 0.42]	0.42 (0.0035) [0.41 - 0.43]	-0.0016 (0.00087) [-0.0026 - -0.00088]	-0.0040, 0.00084	0.145	(0.36 - 0.55) [0.23, 0.64]
20:1 Eicosenoic Acid	0.22 (0.010) [0.19 - 0.23]	0.21 (0.010) [0.18 - 0.22]	0.0095 (0.014) [-0.026 - 0.046]	-0.030, 0.049	0.543	(0.21 - 0.30) [0.16, 0.33]
20:2 Eicosadienoic Acid	0.030 (0.0069) [0.021 - 0.046]	0.037 (0.0069) [0.020 - 0.047]	-0.0070 (0.0086) [-0.024 - 0.0015]	-0.031, 0.017	0.461	(0.016 - 0.054) [0.0029, 0.083]
22:0 Behenic Acid	0.47 (0.0035) [0.46 - 0.48]	0.46 (0.0035) [0.45 - 0.46]	0.014 (0.0046) [0.0047 - 0.024]	0.0013, 0.027	0.037	(0.38 - 0.59) [0.30, 0.67]

Table G-4. Statistical Summary of Site AR Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	16.56 (0.60) [15.87 - 16.91]	15.99 (0.60) [14.46 - 17.77]	0.57 (0.61) [-0.86 - 1.41]	-1.12, 2.25	0.403	(12.79 - 17.98) [11.13, 20.21]
Neutral Detergent Fiber	16.40 (1.30) [15.95 - 17.25]	18.91 (1.30) [15.02 - 22.45]	-2.51 (1.82) [-6.43 - 0.93]	-7.57, 2.55	0.240	(13.32 - 23.57) [7.24, 28.70]
Proximate (% DW, unless noted)						
Ash	4.92 (0.077) [4.77 - 5.11]	4.80 (0.077) [4.72 - 4.89]	0.11 (0.086) [0.047 - 0.21]	-0.13, 0.35	0.260	(4.32 - 5.62) [3.74, 6.45]
Carbohydrates	38.33 (0.78) [36.06 - 39.61]	37.81 (0.78) [37.06 - 39.23]	0.53 (1.05) [-1.07 - 2.56]	-2.40, 3.46	0.642	(31.97 - 38.00) [28.17, 40.99]
Moisture (% FW)	7.57 (0.97) [5.93 - 10.70]	6.02 (0.97) [5.44 - 6.63]	1.55 (1.37) [-0.54 - 4.70]	-2.26, 5.36	0.321	(5.48 - 11.70) [1.45, 12.81]
Protein	39.01 (0.43) [38.09 - 40.46]	38.64 (0.43) [38.41 - 38.82]	0.37 (0.61) [-0.73 - 2.05]	-1.34, 2.07	0.583	(38.14 - 42.66) [35.30, 45.38]
Total Fat	17.79 (0.48) [17.33 - 18.48]	18.73 (0.48) [17.24 - 19.57]	-0.94 (0.59) [-1.82 - 0.090]	-2.58, 0.70	0.185	(17.90 - 23.56) [14.74, 25.18]
Vitamin (mg/100g DW)						
Vitamin E	6.88 (0.12) [6.77 - 7.08]	5.03 (0.12) [4.88 - 5.12]	1.85 (0.17) [1.66 - 2.20]	1.36, 2.33	<0.001	(1.65 - 8.08) [0, 11.09]

Table G-4. Statistical Summary of Site AR Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg FW)	1.53 (0.66) [0.88 - 2.01]	0.80 (0.66) [0.30 - 1.05]	0.74 (0.93) [0.59 - 0.96]	-1.84, 3.32	0.470	(0.090 - 2.47) [0, 3.40]
Phytic Acid	1.70 (0.082) [1.61 - 1.78]	2.01 (0.082) [1.90 - 2.14]	-0.30 (0.11) [-0.53 - -0.17]	-0.61, 0.0017	0.050	(1.10 - 2.32) [0.54, 3.05]
Raffinose	1.51 (0.069) [1.44 - 1.54]	1.51 (0.069) [1.40 - 1.61]	-0.0048 (0.098) [-0.073 - 0.040]	-0.28, 0.27	0.963	(0.52 - 1.62) [0.038, 2.24]
Stachyose	5.68 (0.17) [5.47 - 5.82]	5.72 (0.17) [5.36 - 5.98]	-0.043 (0.24) [-0.50 - 0.38]	-0.71, 0.62	0.865	(1.97 - 5.55) [0.99, 7.93]
Trypsin Inhibitor (TIU/mg DW)	23.48 (1.42) [23.07 - 23.96]	26.68 (1.42) [23.09 - 30.95]	-3.21 (2.01) [-6.99 - 0.32]	-8.79, 2.38	0.186	(20.84 - 37.24) [13.58, 46.02]
Isoflavone (mg/100g DW)						
Daidzein	767.90 (29.71) [747.32 - 793.95]	658.21 (29.71) [619.71 - 732.57]	109.69 (33.77) [29.86 - 171.61]	15.93, 203.46	0.031	(213.98 - 1273.94) [0, 1585.14]
Genistein	807.35 (25.42) [771.77 - 840.99]	680.07 (25.42) [662.77 - 714.36]	127.28 (25.83) [94.92 - 178.22]	55.57, 198.99	0.007	(148.06 - 1024.50) [0, 1352.86]
Glycitein	182.99 (10.76) [172.51 - 191.49]	163.24 (10.76) [140.43 - 191.71]	19.75 (15.22) [-19.20 - 51.06]	-22.51, 62.02	0.264	(32.42 - 208.45) [0, 272.12]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-5. Statistical Summary of Site GA Soybean Forage Fiber and Proximate Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	31.26 (2.13) [30.21 - 31.87]	32.76 (2.13) [27.42 - 36.30]	-1.50 (2.54) [-4.43 - 2.79]	-8.55, 5.54	0.585	(27.99 - 47.33) [14.93, 56.87]
Neutral Detergent Fiber	42.64 (3.78) [37.02 - 52.38]	42.08 (3.78) [34.23 - 46.58]	0.56 (5.34) [-8.40 - 5.81]	-14.26, 15.39	0.920	(30.96 - 54.55) [21.51, 66.01]
Proximate (% DW, unless noted)						
Ash	5.58 (0.18) [5.50 - 5.69]	5.25 (0.18) [5.10 - 5.38]	0.33 (0.25) [0.17 - 0.42]	-0.37, 1.03	0.262	(4.77 - 8.54) [2.46, 10.14]
Carbohydrates	71.49 (0.93) [70.41 - 72.44]	72.52 (0.93) [70.55 - 73.86]	-1.03 (1.32) [-2.23 - -0.14]	-4.68, 2.62	0.478	(60.61 - 77.26) [56.93, 85.88]
Moisture (% FW)	71.13 (0.34) [70.60 - 71.70]	70.13 (0.34) [69.40 - 70.80]	1.00 (0.49) [-0.20 - 1.70]	-0.35, 2.35	0.108	(66.50 - 80.20) [57.84, 88.56]
Protein	16.76 (0.64) [15.94 - 17.94]	15.58 (0.64) [14.20 - 16.67]	1.19 (0.91) [0.077 - 2.22]	-1.34, 3.71	0.261	(12.68 - 22.92) [7.05, 27.27]
Total Fat	6.22 (0.46) [5.94 - 6.51]	6.61 (0.46) [5.74 - 7.23]	-0.39 (0.65) [-1.00 - 0.20]	-2.19, 1.42	0.584	(3.48 - 7.88) [1.11, 9.11]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-6. Statistical Summary of Site GA Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.69 (0.019) [1.68 - 1.70]	1.65 (0.019) [1.63 - 1.67]	0.042 (0.027) [0.0042 - 0.063]	-0.033, 0.12	0.193	(1.66 - 1.93) [1.49, 2.02]
Arginine	2.80 (0.036) [2.72 - 2.91]	2.57 (0.036) [2.55 - 2.60]	0.22 (0.050) [0.16 - 0.31]	0.085, 0.36	0.011	(2.54 - 2.99) [2.22, 3.25]
Aspartic Acid	4.83 (0.072) [4.80 - 4.87]	4.73 (0.072) [4.59 - 4.90]	0.097 (0.10) [-0.039 - 0.23]	-0.19, 0.38	0.396	(4.74 - 5.50) [4.22, 5.96]
Cystine	0.62 (0.013) [0.61 - 0.63]	0.60 (0.013) [0.56 - 0.64]	0.022 (0.018) [-0.018 - 0.052]	-0.027, 0.072	0.279	(0.53 - 0.68) [0.45, 0.77]
Glutamic Acid	7.63 (0.098) [7.53 - 7.69]	7.39 (0.098) [7.21 - 7.60]	0.24 (0.14) [0.092 - 0.45]	-0.15, 0.62	0.159	(7.53 - 8.72) [6.60, 9.37]
Glycine	1.74 (0.020) [1.73 - 1.78]	1.67 (0.020) [1.64 - 1.72]	0.070 (0.028) [0.059 - 0.089]	0.0065, 0.15	0.063	(1.67 - 1.99) [1.49, 2.09]
Histidine	1.15 (0.011) [1.13 - 1.16]	1.09 (0.011) [1.08 - 1.12]	0.057 (0.015) [0.043 - 0.074]	0.015, 0.098	0.019	(1.04 - 1.24) [0.94, 1.31]
Isoleucine	1.81 (0.016) [1.77 - 1.84]	1.74 (0.016) [1.71 - 1.77]	0.074 (0.022) [0.035 - 0.12]	0.012, 0.14	0.029	(1.73 - 2.02) [1.54, 2.14]

Table G-6. Statistical Summary of Site GA Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	3.04 (0.024) [2.98 - 3.09]	2.91 (0.024) [2.87 - 2.96]	0.13 (0.033) [0.087 - 0.18]	0.043, 0.22	0.014	(2.93 - 3.32) [2.64, 3.52]
Lysine	2.75 (0.051) [2.67 - 2.79]	2.60 (0.051) [2.54 - 2.66]	0.15 (0.073) [0.11 - 0.20]	-0.056, 0.35	0.114	(2.35 - 3.15) [2.05, 3.47]
Methionine	0.53 (0.018) [0.48 - 0.55]	0.51 (0.018) [0.47 - 0.54]	0.017 (0.026) [-0.059 - 0.080]	-0.055, 0.089	0.548	(0.49 - 0.62) [0.42, 0.68]
Phenylalanine	2.24 (0.059) [2.11 - 2.35]	2.06 (0.059) [1.99 - 2.20]	0.18 (0.069) [0.13 - 0.25]	-0.013, 0.37	0.060	(1.97 - 2.44) [1.66, 2.64]
Proline	2.00 (0.014) [1.99 - 2.02]	1.94 (0.014) [1.93 - 1.94]	0.069 (0.020) [0.055 - 0.089]	0.014, 0.12	0.025	(1.92 - 2.25) [1.73, 2.35]
Serine	2.02 (0.031) [2.00 - 2.04]	1.94 (0.031) [1.91 - 1.99]	0.076 (0.044) [0.046 - 0.11]	-0.045, 0.20	0.157	(1.96 - 2.30) [1.75, 2.38]
Threonine	1.60 (0.018) [1.56 - 1.62]	1.53 (0.018) [1.50 - 1.59]	0.061 (0.024) [0.028 - 0.11]	-0.0065, 0.13	0.066	(1.54 - 1.74) [1.40, 1.83]
Tryptophan	0.52 (0.0098) [0.50 - 0.54]	0.51 (0.0098) [0.50 - 0.53]	0.0044 (0.014) [-0.026 - 0.026]	-0.034, 0.043	0.764	(0.47 - 0.55) [0.43, 0.59]

Table G-6. Statistical Summary of Site GA Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.18 (0.044) [1.08 - 1.27]	1.09 (0.044) [1.02 - 1.12]	0.097 (0.062) [-0.043 - 0.25]	-0.076, 0.27	0.195	(1.04 - 1.31) [0.85, 1.48]
Valine	1.91 (0.017) [1.88 - 1.94]	1.84 (0.017) [1.80 - 1.87]	0.073 (0.023) [0.034 - 0.12]	0.0078, 0.14	0.035	(1.83 - 2.13) [1.64, 2.22]
Fatty Acid (% Total FA)						
10:0 Capric Acid	0.20 (0.017) [0.18 - 0.24]	0.18 (0.017) [0.16 - 0.19]	0.022 (0.025) [0.0012 - 0.048]	-0.046, 0.091	0.414	(0.15 - 0.27) [0.065, 0.34]
14:0 Myristic Acid	0.094 (0.0023) [0.092 - 0.095]	0.097 (0.0023) [0.092 - 0.10]	-0.0031 (0.0033) [-0.0085 - 0.0020]	-0.012, 0.0060	0.395	(0.064 - 0.097) [0.052, 0.12]
16:0 Palmitic Acid	11.51 (0.12) [11.32 - 11.81]	11.93 (0.12) [11.79 - 12.11]	-0.42 (0.17) [-0.72 - 0.026]	-0.90, 0.062	0.072	(9.80 - 12.38) [8.88, 13.53]
16:1 Palmitoleic Acid	0.10 (0.0043) [0.097 - 0.11]	0.10 (0.0043) [0.094 - 0.11]	-0.0016 (0.0057) [-0.0042 - 0.0031]	-0.017, 0.014	0.798	(0.073 - 0.14) [0.037, 0.15]
17:0 Heptadecanoic Acid	0.086 (0.0023) [0.084 - 0.088]	0.087 (0.0023) [0.082 - 0.092]	-0.00085 (0.0026) [-0.0064 - 0.0021]	-0.0081, 0.0065	0.763	(0.076 - 0.10) [0.066, 0.11]
17:1 Heptadecenoic Acid	0.040 (0.0041) [0.039 - 0.041]	0.032 (0.0041) [0.019 - 0.040]	0.0078 (0.0057) [0.00006 - 0.022]	-0.0081, 0.024	0.244	(0.020 - 0.064) [0.0058, 0.083]

Table G-6. Statistical Summary of Site GA Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
18:0 Stearic Acid	5.21 (0.12) [5.07 - 5.36]	5.12 (0.12) [4.78 - 5.36]	0.085 (0.14) [-0.042 - 0.29]	-0.31, 0.48	0.581	(3.21 - 5.24) [1.88, 6.25]
18:1 Oleic Acid	23.10 (0.67) [22.70 - 23.71]	22.28 (0.67) [20.85 - 23.50]	0.82 (0.92) [0.21 - 2.04]	-1.72, 3.37	0.419	(16.69 - 35.16) [5.01, 42.01]
18:2 Linoleic Acid	50.98 (0.59) [50.39 - 51.53]	51.56 (0.59) [50.31 - 52.88]	-0.57 (0.74) [-1.35 - 0.083]	-2.62, 1.47	0.478	(44.17 - 57.72) [38.57, 66.94]
18:3 Linolenic Acid	7.23 (0.14) [7.16 - 7.35]	7.24 (0.14) [7.01 - 7.57]	-0.016 (0.20) [-0.40 - 0.20]	-0.56, 0.53	0.937	(4.27 - 8.81) [2.69, 10.81]
20:0 Arachidic Acid	0.57 (0.012) [0.56 - 0.58]	0.55 (0.012) [0.51 - 0.57]	0.018 (0.014) [-0.0035 - 0.047]	-0.021, 0.057	0.264	(0.36 - 0.55) [0.23, 0.64]
20:1 Eicosenoic Acid	0.24 (0.0027) [0.23 - 0.24]	0.22 (0.0027) [0.22 - 0.22]	0.012 (0.0038) [0.0040 - 0.016]	0.0013, 0.022	0.035	(0.21 - 0.30) [0.16, 0.33]
20:2 Eicosadienoic Acid	0.043 (0.0022) [0.039 - 0.049]	0.041 (0.0022) [0.039 - 0.044]	0.0013 (0.0028) [-0.0019 - 0.0049]	-0.0066, 0.0092	0.678	(0.016 - 0.054) [0.0029, 0.083]
22:0 Behenic Acid	0.60 (0.011) [0.58 - 0.62]	0.55 (0.011) [0.52 - 0.58]	0.046 (0.014) [0.019 - 0.078]	0.0075, 0.084	0.029	(0.38 - 0.59) [0.30, 0.67]

Table G-6. Statistical Summary of Site GA Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	14.21 (0.35) [13.53 - 14.97]	14.92 (0.35) [14.38 - 15.77]	-0.71 (0.40) [-1.07 - -0.25]	-1.82, 0.40	0.152	(12.79 - 17.98) [11.13, 20.21]
Neutral Detergent Fiber	16.23 (0.46) [15.51 - 17.15]	16.14 (0.46) [15.03 - 17.07]	0.095 (0.65) [-1.56 - 2.12]	-1.70, 1.89	0.889	(13.32 - 23.57) [7.24, 28.70]
Proximate (% DW, unless noted)						
Ash	4.81 (0.079) [4.70 - 4.98]	4.80 (0.079) [4.70 - 4.86]	0.0072 (0.11) [-0.14 - 0.11]	-0.30, 0.32	0.951	(4.32 - 5.62) [3.74, 6.45]
Carbohydrates	35.10 (1.46) [34.68 - 35.36]	38.48 (1.46) [35.52 - 43.48]	-3.37 (2.04) [-8.12 - -0.26]	-9.04, 2.29	0.173	(31.97 - 38.00) [28.17, 40.99]
Moisture (% FW)	6.37 (0.40) [5.86 - 7.14]	7.00 (0.40) [6.16 - 8.03]	-0.63 (0.51) [-0.96 - -0.050]	-2.04, 0.77	0.279	(5.48 - 11.70) [1.45, 12.81]
Protein	39.33 (1.08) [38.79 - 39.77]	35.93 (1.08) [32.29 - 38.13]	3.39 (1.42) [1.64 - 6.49]	-0.55, 7.34	0.075	(38.14 - 42.66) [35.30, 45.38]
Total Fat	20.79 (0.45) [20.29 - 21.20]	20.79 (0.45) [19.39 - 21.68]	-0.0030 (0.63) [-1.39 - 1.80]	-1.76, 1.76	0.996	(17.90 - 23.56) [14.74, 25.18]
Vitamin (mg/100g DW)						
Vitamin E	9.16 (0.22) [8.51 - 9.62]	7.77 (0.22) [7.64 - 7.94]	1.38 (0.32) [0.57 - 1.97]	0.51, 2.26	0.011	(1.65 - 8.08) [0, 11.09]

Table G-6. Statistical Summary of Site GA Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg FW)	0.90 (0.20) [0.68 - 1.10]	0.84 (0.20) [0.54 - 1.28]	0.055 (0.097) [-0.18 - 0.20]	-0.21, 0.32	0.600	(0.090 - 2.47) [0, 3.40]
Phytic Acid	1.50 (0.11) [1.39 - 1.71]	1.52 (0.11) [1.31 - 1.66]	-0.020 (0.16) [-0.26 - 0.11]	-0.47, 0.43	0.907	(1.10 - 2.32) [0.54, 3.05]
Raffinose	1.60 (0.098) [1.53 - 1.66]	1.69 (0.098) [1.47 - 1.85]	-0.086 (0.14) [-0.32 - 0.19]	-0.47, 0.30	0.566	(0.52 - 1.62) [0.038, 2.24]
Stachyose	4.75 (0.14) [4.40 - 4.96]	4.96 (0.14) [4.74 - 5.19]	-0.21 (0.17) [-0.35 - -0.067]	-0.67, 0.25	0.269	(1.97 - 5.55) [0.99, 7.93]
Trypsin Inhibitor (TIU/mg DW)	23.28 (0.86) [21.65 - 25.24]	29.27 (0.86) [27.29 - 30.69]	-6.00 (1.13) [-7.75 - -4.60]	-9.12, -2.87	0.005	(20.84 - 37.24) [13.58, 46.02]
Isoflavone (mg/kg DW)						
Daidzein	796.62 (53.29) [725.52 - 921.29]	748.07 (53.29) [742.75 - 753.38]	48.55 (75.37) [-27.87 - 178.54]	-160.71, 257.81	0.554	(213.98 - 1273.94) [0, 1585.14]
Genistein	736.27 (36.05) [667.09 - 811.59]	708.78 (36.05) [676.11 - 760.87]	27.49 (50.98) [-9.02 - 50.72]	-114.04, 169.03	0.618	(148.06 - 1024.50) [0, 1352.86]
Glycitein	181.56 (11.88) [171.02 - 195.97]	202.27 (11.88) [179.22 - 227.25]	-20.71 (12.27) [-49.56 - -4.37]	-54.79, 13.37	0.166	(32.42 - 208.45) [0, 272.12]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-7. Statistical Summary of Site IL Soybean Forage Fiber and Proximate Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	36.67 (2.60) [31.94 - 44.08]	36.80 (2.60) [33.62 - 39.28]	-0.13 (3.68) [-5.57 - 10.46]	-10.35, 10.08	0.972	(27.99 - 47.33) [14.93, 56.87]
Neutral Detergent Fiber	48.78 (4.07) [46.12 - 53.23]	51.06 (4.07) [43.04 - 64.19]	-2.29 (5.75) [-18.07 - 7.27]	-18.26, 13.69	0.711	(30.96 - 54.55) [21.51, 66.01]
Proximate (% DW, unless noted)						
Ash	6.56 (0.45) [5.92 - 7.46]	6.48 (0.45) [6.38 - 6.54]	0.079 (0.54) [-0.59 - 0.92]	-1.43, 1.59	0.891	(4.77 - 8.54) [2.46, 10.14]
Carbohydrates	74.57 (0.94) [71.98 - 76.73]	74.18 (0.94) [74.04 - 74.26]	0.39 (1.33) [-2.28 - 2.50]	-3.30, 4.08	0.782	(60.61 - 77.26) [56.93, 85.88]
Moisture	75.83 (0.32) [75.20 - 76.80]	76.63 (0.32) [76.30 - 77.10]	-0.80 (0.46) [-1.60 - 0.50]	-2.07, 0.47	0.154	(66.50 - 80.20) [57.84, 88.56]
Protein	14.53 (0.68) [13.56 - 15.74]	14.87 (0.68) [14.48 - 15.40]	-0.34 (0.97) [-1.18 - 1.26]	-3.03, 2.35	0.742	(12.68 - 22.92) [7.05, 27.27]
Total Fat	4.28 (0.39) [3.60 - 4.87]	4.43 (0.39) [4.23 - 4.64]	-0.15 (0.55) [-0.81 - 0.23]	-1.69, 1.38	0.796	(3.48 - 7.88) [1.11, 9.11]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-8. Statistical Summary of Site IL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.72 (0.036) [1.66 - 1.80]	1.63 (0.036) [1.59 - 1.71]	0.087 (0.043) [0.079 - 0.095]	-0.033, 0.21	0.115	(1.66 - 1.93) [1.49, 2.02]
Arginine	2.61 (0.052) [2.49 - 2.70]	2.44 (0.052) [2.37 - 2.56]	0.17 (0.058) [0.11 - 0.25]	0.0060, 0.33	0.045	(2.54 - 2.99) [2.22, 3.25]
Aspartic Acid	4.87 (0.14) [4.68 - 5.20]	4.63 (0.14) [4.46 - 4.96]	0.24 (0.16) [0.23 - 0.25]	-0.21, 0.69	0.214	(4.74 - 5.50) [4.22, 5.96]
Cystine	0.63 (0.023) [0.58 - 0.65]	0.66 (0.023) [0.62 - 0.69]	-0.032 (0.033) [-0.11 - 0.032]	-0.12, 0.059	0.378	(0.53 - 0.68) [0.45, 0.77]
Glutamic Acid	7.53 (0.20) [7.28 - 8.02]	7.16 (0.20) [6.89 - 7.64]	0.36 (0.24) [0.32 - 0.39]	-0.31, 1.04	0.207	(7.53 - 8.72) [6.60, 9.37]
Glycine	1.75 (0.022) [1.71 - 1.80]	1.68 (0.022) [1.64 - 1.72]	0.073 (0.030) [0.042 - 0.10]	-0.011, 0.16	0.074	(1.67 - 1.99) [1.49, 2.09]
Histidine	1.11 (0.012) [1.09 - 1.13]	1.05 (0.012) [1.03 - 1.08]	0.052 (0.017) [0.039 - 0.072]	0.0054, 0.098	0.036	(1.04 - 1.24) [0.94, 1.31]
Isoleucine	1.78 (0.031) [1.74 - 1.85]	1.69 (0.031) [1.64 - 1.75]	0.099 (0.039) [0.075 - 0.12]	-0.0084, 0.21	0.062	(1.73 - 2.02) [1.54, 2.14]

Table G-8. Statistical Summary of Site IL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	2.97 (0.053) [2.88 - 3.08]	2.80 (0.053) [2.73 - 2.93]	0.18 (0.067) [0.15 - 0.23]	-0.0079, 0.37	0.056	(2.93 - 3.32) [2.64, 3.52]
Lysine	2.78 (0.088) [2.70 - 2.90]	2.57 (0.088) [2.49 - 2.74]	0.21 (0.12) [0.16 - 0.26]	-0.13, 0.55	0.162	(2.35 - 3.15) [2.05, 3.47]
Methionine	0.53 (0.028) [0.49 - 0.56]	0.56 (0.028) [0.53 - 0.59]	-0.033 (0.039) [-0.094 - 0.028]	-0.14, 0.076	0.445	(0.49 - 0.62) [0.42, 0.68]
Phenylalanine	2.08 (0.048) [1.94 - 2.21]	1.94 (0.048) [1.91 - 1.99]	0.14 (0.066) [0.018 - 0.31]	-0.043, 0.32	0.101	(1.97 - 2.44) [1.66, 2.64]
Proline	1.99 (0.040) [1.94 - 2.09]	1.90 (0.040) [1.85 - 1.99]	0.092 (0.049) [0.083 - 0.10]	-0.045, 0.23	0.135	(1.92 - 2.25) [1.73, 2.35]
Serine	2.01 (0.034) [1.94 - 2.08]	1.92 (0.034) [1.87 - 1.99]	0.089 (0.049) [0.061 - 0.12]	-0.046, 0.22	0.142	(1.96 - 2.30) [1.75, 2.38]
Threonine	1.59 (0.022) [1.56 - 1.63]	1.52 (0.022) [1.49 - 1.58]	0.070 (0.031) [0.052 - 0.10]	-0.015, 0.15	0.083	(1.54 - 1.74) [1.40, 1.83]
Tryptophan	0.53 (0.0088) [0.52 - 0.53]	0.51 (0.0088) [0.50 - 0.52]	0.019 (0.011) [0.012 - 0.023]	-0.011, 0.049	0.159	(0.47 - 0.55) [0.43, 0.59]

Table G-8. Statistical Summary of Site IL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.10 (0.014) [1.07 - 1.13]	1.01 (0.014) [0.98 - 1.04]	0.092 (0.015) [0.067 - 0.12]	0.050, 0.13	0.003	(1.04 - 1.31) [0.85, 1.48]
Valine	1.90 (0.033) [1.85 - 1.98]	1.80 (0.033) [1.76 - 1.87]	0.10 (0.040) [0.090 - 0.12]	-0.0073, 0.22	0.060	(1.83 - 2.13) [1.64, 2.22]
Fatty Acid (% Total FA)						
10:0 Capric Acid	0.18 (0.017) [0.16 - 0.19]	0.20 (0.017) [0.16 - 0.25]	-0.022 (0.024) [-0.092 - 0.028]	-0.089, 0.046	0.424	(0.15 - 0.27) [0.065, 0.34]
14:0 Myristic Acid	0.089 (0.00087) [0.086 - 0.090]	0.090 (0.00087) [0.089 - 0.091]	-0.00099 (0.0012) [-0.0036 - 0.0017]	-0.0044, 0.0024	0.465	(0.064 - 0.097) [0.052, 0.12]
16:0 Palmitic Acid	11.53 (0.048) [11.39 - 11.63]	11.71 (0.048) [11.69 - 11.72]	-0.17 (0.050) [-0.30 - -0.075]	-0.31, -0.034	0.025	(9.80 - 12.38) [8.88, 13.53]
16:1 Palmitoleic Acid	0.093 (0.0021) [0.092 - 0.097]	0.097 (0.0021) [0.097 - 0.097]	-0.0036 (0.0030) [-0.0053 - -0.00043]	-0.012, 0.0048	0.304	(0.073 - 0.14) [0.037, 0.15]
17:0 Heptadecanoic Acid	0.093 (0.00086) [0.093 - 0.094]	0.094 (0.00086) [0.092 - 0.094]	-0.00023 (0.0011) [-0.00061 - 0.00035]	-0.0032, 0.0027	0.841	(0.076 - 0.10) [0.066, 0.11]
17:1 Heptadecenoic Acid	0.043 (0.0038) [0.040 - 0.045]	0.042 (0.0038) [0.042 - 0.042]	0.00034 (0.0054) [-0.0019 - 0.0028]	-0.015, 0.015	0.952	(0.020 - 0.064) [0.0058, 0.083]

Table G-8. Statistical Summary of Site IL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
18:0 Stearic Acid	4.87 (0.047) [4.76 - 4.99]	5.00 (0.047) [4.92 - 5.06]	-0.13 (0.067) [-0.26 - -0.049]	-0.31, 0.059	0.130	(3.21 - 5.24) [1.88, 6.25]
18:1 Oleic Acid	22.33 (0.23) [21.78 - 22.97]	21.87 (0.23) [21.68 - 22.05]	0.46 (0.21) [0.096 - 0.91]	-0.13, 1.06	0.097	(16.69 - 35.16) [5.01, 42.01]
18:2 Linoleic Acid	51.78 (0.18) [51.32 - 52.33]	52.08 (0.18) [51.95 - 52.25]	-0.30 (0.19) [-0.63 - 0.078]	-0.84, 0.24	0.201	(44.17 - 57.72) [38.57, 66.94]
18:3 Linolenic Acid	7.64 (0.087) [7.45 - 7.74]	7.52 (0.087) [7.44 - 7.57]	0.12 (0.092) [0.012 - 0.19]	-0.13, 0.38	0.256	(4.27 - 8.81) [2.69, 10.81]
20:0 Arachidic Acid	0.51 (0.0055) [0.50 - 0.53]	0.52 (0.0055) [0.51 - 0.52]	-0.0036 (0.0065) [-0.014 - 0.0075]	-0.022, 0.014	0.603	(0.36 - 0.55) [0.23, 0.64]
20:1 Eicosenoic Acid	0.24 (0.011) [0.23 - 0.25]	0.23 (0.011) [0.22 - 0.23]	0.012 (0.015) [0.0017 - 0.018]	-0.030, 0.053	0.484	(0.21 - 0.30) [0.16, 0.33]
20:2 Eicosadienoic Acid	0.050 (0.0041) [0.047 - 0.054]	0.045 (0.0041) [0.044 - 0.047]	0.0045 (0.0057) [0.00080 - 0.011]	-0.011, 0.020	0.472	(0.016 - 0.054) [0.0029, 0.083]
22:0 Behenic Acid	0.54 (0.012) [0.53 - 0.56]	0.52 (0.012) [0.51 - 0.52]	0.026 (0.012) [0.014 - 0.042]	-0.0058, 0.058	0.085	(0.38 - 0.59) [0.30, 0.67]

Table G-8. Statistical Summary of Site IL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	15.69 (0.78) [14.93 - 16.18]	17.06 (0.78) [15.34 - 19.02]	-1.37 (0.77) [-2.84 - 0.62]	-3.51, 0.77	0.150	(12.79 - 17.98) [11.13, 20.21]
Neutral Detergent Fiber	16.74 (0.40) [16.41 - 17.18]	16.21 (0.40) [15.32 - 17.41]	0.53 (0.57) [-1.00 - 1.86]	-1.04, 2.11	0.399	(13.32 - 23.57) [7.24, 28.70]
Proximate (% DW, unless noted)						
Ash	5.42 (0.083) [5.20 - 5.55]	5.29 (0.083) [5.16 - 5.36]	0.13 (0.042) [0.034 - 0.21]	0.010, 0.25	0.039	(4.32 - 5.62) [3.74, 6.45]
Carbohydrates	36.65 (0.71) [35.60 - 37.72]	39.17 (0.71) [37.69 - 39.96]	-2.53 (0.72) [-3.34 - -2.09]	-4.53, -0.52	0.024	(31.97 - 38.00) [28.17, 40.99]
Moisture (% FW)	9.23 (1.03) [6.88 - 10.40]	7.45 (1.03) [6.66 - 8.74]	1.78 (1.12) [0.22 - 3.46]	-1.34, 4.90	0.187	(5.48 - 11.70) [1.45, 12.81]
Protein	38.91 (0.63) [37.73 - 40.58]	37.49 (0.63) [36.66 - 38.71]	1.43 (0.78) [1.08 - 1.87]	-0.73, 3.59	0.140	(38.14 - 42.66) [35.30, 45.38]
Total Fat	19.05 (0.47) [18.30 - 19.76]	18.05 (0.47) [17.78 - 18.30]	1.00 (0.66) [0.0042 - 1.98]	-0.83, 2.84	0.203	(17.90 - 23.56) [14.74, 25.18]
Vitamin (mg/100g DW)						
Vitamin E	6.72 (0.19) [6.36 - 7.27]	5.31 (0.19) [4.98 - 5.58]	1.41 (0.16) [1.17 - 1.69]	0.98, 1.84	<0.001	(1.65 - 8.08) [0, 11.09]

Table G-8. Statistical Summary of Site IL Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg FW)	0.98 (0.28) [0.21 - 1.65]	1.04 (0.28) [0.84 - 1.18]	-0.058 (0.39) [-0.88 - 0.47]	-1.15, 1.04	0.889	(0.090 - 2.47) [0, 3.40]
Phytic Acid	2.07 (0.085) [1.88 - 2.22]	2.05 (0.085) [1.91 - 2.13]	0.022 (0.12) [-0.25 - 0.31]	-0.31, 0.36	0.865	(1.10 - 2.32) [0.54, 3.05]
Raffinose	1.60 (0.065) [1.41 - 1.70]	1.58 (0.065) [1.52 - 1.67]	0.018 (0.092) [-0.16 - 0.18]	-0.24, 0.27	0.852	(0.52 - 1.62) [0.038, 2.24]
Stachyose	6.05 (0.29) [5.48 - 6.42]	6.10 (0.29) [5.53 - 6.65]	-0.046 (0.40) [-0.63 - 0.73]	-1.16, 1.07	0.915	(1.97 - 5.55) [0.99, 7.93]
Trypsin Inhibitor (TIU/mg DW)	27.09 (1.77) [22.34 - 31.92]	29.51 (1.77) [28.50 - 30.68]	-2.42 (1.96) [-6.16 - 2.58]	-7.85, 3.02	0.284	(20.84 - 37.24) [13.58, 46.02]
Isoflavone (mg/kg DW)						
Daidzein	890.96 (28.34) [834.82 - 983.26]	803.42 (28.34) [788.95 - 830.65]	87.54 (29.71) [45.87 - 152.61]	5.04, 170.04	0.042	(213.98 - 1273.94) [0, 1585.14]
Genistein	776.22 (31.97) [724.87 - 863.84]	725.36 (31.97) [701.70 - 744.59]	50.87 (42.57) [-19.72 - 162.14]	-67.32, 169.05	0.298	(148.06 - 1024.50) [0, 1352.86]
Glycitein	198.74 (14.52) [164.30 - 228.79]	204.69 (14.52) [177.84 - 219.15]	-5.95 (17.13) [-16.03 - 11.73]	-53.52, 41.62	0.746	(32.42 - 208.45) [0, 272.12]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-9. Statistical Summary of Site NC Soybean Forage Fiber and Proximate Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	44.66 (5.11) [37.12 - 58.25]	36.58 (6.26) [31.54 - 41.61]	8.09 (8.08) [-2.99 - 5.58]	-17.64, 33.82	0.390	(27.99 - 47.33) [14.93, 56.87]
Neutral Detergent Fiber	47.51 (2.97) [46.55 - 48.83]	48.85 (3.64) [42.62 - 55.09]	-1.35 (4.70) [-6.26 - 3.93]	-16.29, 13.60	0.792	(30.96 - 54.55) [21.51, 66.01]
Proximate (% DW, unless noted)						
Ash	5.65 (0.19) [5.42 - 6.03]	5.97 (0.23) [5.93 - 6.01]	-0.32 (0.30) [-0.51 - 0.028]	-1.28, 0.63	0.363	(4.77 - 8.54) [2.46, 10.14]
Carbohydrates	71.44 (1.22) [70.23 - 73.06]	70.59 (1.39) [67.72 - 72.82]	0.85 (1.34) [-1.78 - 2.51]	-3.42, 5.13	0.571	(60.61 - 77.26) [56.93, 85.88]
Moisture (% FW)	70.47 (0.30) [70.10 - 71.00]	70.85 (0.37) [70.20 - 71.50]	-0.38 (0.48) [-1.40 - 0.80]	-1.91, 1.14	0.482	(66.50 - 80.20) [57.84, 88.56]
Protein	17.17 (0.95) [16.26 - 17.68]	17.62 (1.09) [16.10 - 19.66]	-0.45 (1.06) [-1.98 - 1.46]	-3.83, 2.93	0.700	(12.68 - 22.92) [7.05, 27.27]
Total Fat	5.82 (0.43) [5.28 - 6.82]	5.82 (0.49) [5.13 - 6.74]	-0.0020 (0.48) [0.086 - 0.14]	-1.53, 1.52	0.996	(3.48 - 7.88) [1.11, 9.11]

[1]DW = dry weight; FW = fresh weight; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Table G-10. Statistical Summary of Site NC Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Alanine	1.67 (0.016) [1.66 - 1.68]	1.66 (0.018) [1.66 - 1.68]	0.0055 (0.019) [-0.0051 - 0.0048]	-0.056, 0.067	0.795	(1.66 - 1.93) [1.49, 2.02]
Arginine	2.42 (0.078) [2.36 - 2.51]	2.50 (0.091) [2.53 - 2.54]	-0.085 (0.094) [-0.16 - -0.014]	-0.38, 0.21	0.432	(2.54 - 2.99) [2.22, 3.25]
Aspartic Acid	4.68 (0.073) [4.61 - 4.75]	4.82 (0.085) [4.72 - 4.91]	-0.14 (0.089) [-0.23 - -0.12]	-0.42, 0.14	0.215	(4.74 - 5.50) [4.22, 5.96]
Cystine	0.58 (0.016) [0.57 - 0.59]	0.59 (0.019) [0.57 - 0.61]	-0.016 (0.025) [-0.017 - -0.0080]	-0.096, 0.063	0.561	(0.53 - 0.68) [0.45, 0.77]
Glutamic Acid	7.30 (0.084) [7.25 - 7.34]	7.47 (0.10) [7.39 - 7.58]	-0.18 (0.12) [-0.29 - -0.14]	-0.56, 0.20	0.228	(7.53 - 8.72) [6.60, 9.37]
Glycine	1.67 (0.036) [1.63 - 1.70]	1.66 (0.041) [1.67 - 1.69]	0.0061 (0.037) [-0.0052 - 0.016]	-0.11, 0.12	0.878	(1.67 - 1.99) [1.49, 2.09]
Histidine	1.07 (0.028) [1.05 - 1.11]	1.05 (0.032) [1.06 - 1.08]	0.019 (0.027) [-0.00077 - 0.036]	-0.068, 0.11	0.529	(1.04 - 1.24) [0.94, 1.31]
Isoleucine	1.71 (0.031) [1.68 - 1.75]	1.70 (0.034) [1.72 - 1.73]	0.0084 (0.027) [-0.016 - 0.016]	-0.077, 0.094	0.775	(1.73 - 2.02) [1.54, 2.14]

Table G-10. Statistical Summary of Site NC Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Leucine	2.85 (0.045) [2.82 - 2.88]	2.87 (0.052) [2.89 - 2.91]	-0.024 (0.053) [-0.044 - -0.024]	-0.19, 0.14	0.684	(2.93 - 3.32) [2.64, 3.52]
Lysine	2.55 (0.077) [2.48 - 2.66]	2.42 (0.082) [2.42 - 2.53]	0.14 (0.053) [0.10 - 0.13]	-0.033, 0.31	0.082	(2.35 - 3.15) [2.05, 3.47]
Methionine	0.51 (0.014) [0.49 - 0.53]	0.51 (0.017) [0.48 - 0.53]	0.0014 (0.023) [0.0028 - 0.020]	-0.070, 0.073	0.953	(0.49 - 0.62) [0.42, 0.68]
Phenylalanine	1.96 (0.096) [1.91 - 2.05]	1.96 (0.11) [1.96 - 2.02]	0.0037 (0.11) [-0.036 - 0.027]	-0.36, 0.37	0.975	(1.97 - 2.44) [1.66, 2.64]
Proline	1.89 (0.027) [1.86 - 1.92]	1.92 (0.030) [1.93 - 1.94]	-0.027 (0.028) [-0.058 - -0.0052]	-0.12, 0.063	0.409	(1.92 - 2.25) [1.73, 2.35]
Serine	1.94 (0.031) [1.90 - 1.98]	1.92 (0.035) [1.94 - 1.94]	0.027 (0.031) [0.016 - 0.039]	-0.071, 0.12	0.447	(1.96 - 2.30) [1.75, 2.38]
Threonine	1.52 (0.029) [1.50 - 1.55]	1.52 (0.032) [1.52 - 1.55]	-0.0018 (0.028) [-0.016 - 0.0045]	-0.090, 0.086	0.951	(1.54 - 1.74) [1.40, 1.83]
Tryptophan	0.49 (0.010) [0.47 - 0.51]	0.47 (0.010) [0.47 - 0.49]	0.022 (0.0034) [0.020 - 0.027]	0.012, 0.033	0.006	(0.47 - 0.55) [0.43, 0.59]

Table G-10. Statistical Summary of Site NC Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Amino Acid (% DW)						
Tyrosine	1.03 (0.041) [0.96 - 1.09]	1.07 (0.050) [1.03 - 1.12]	-0.044 (0.063) [-0.088 - 0.057]	-0.24, 0.16	0.534	(1.04 - 1.31) [0.85, 1.48]
Valine	1.82 (0.028) [1.80 - 1.85]	1.82 (0.031) [1.83 - 1.85]	-0.0029 (0.027) [-0.015 - -0.0054]	-0.089, 0.083	0.921	(1.83 - 2.13) [1.64, 2.22]
Fatty Acid (% Total FA)						
10:0 Capric Acid	0.25 (0.0095) [0.24 - 0.25]	0.22 (0.012) [0.21 - 0.24]	0.022 (0.015) [0.017 - 0.037]	-0.025, 0.070	0.231	(0.15 - 0.27) [0.065, 0.34]
14:0 Myristic Acid	0.097 (0.0013) [0.094 - 0.10]	0.095 (0.0015) [0.092 - 0.096]	0.0028 (0.0013) [0.0018 - 0.0025]	-0.0013, 0.0070	0.119	(0.064 - 0.097) [0.052, 0.12]
16:0 Palmitic Acid	12.24 (0.050) [12.19 - 12.30]	12.02 (0.061) [11.91 - 12.13]	0.22 (0.079) [0.057 - 0.40]	-0.036, 0.47	0.072	(9.80 - 12.38) [8.88, 13.53]
16:1 Palmitoleic Acid	0.086 (0.0016) [0.084 - 0.089]	0.10 (0.0020) [0.098 - 0.10]	-0.014 (0.0026) [-0.018 - -0.0093]	-0.022, -0.0056	0.012	(0.073 - 0.14) [0.037, 0.15]
17:0 Heptadecanoic Acid	0.093 (0.0014) [0.090 - 0.095]	0.088 (0.0017) [0.088 - 0.090]	0.0044 (0.0019) [0.0033 - 0.0074]	-0.0017, 0.010	0.104	(0.076 - 0.10) [0.066, 0.11]
17:1 Heptadecenoic Acid	0.038 (0.0032) [0.037 - 0.039]	0.040 (0.0039) [0.040 - 0.040]	-0.0022 (0.0050) [-0.0018 - -0.0011]	-0.018, 0.014	0.690	(0.020 - 0.064) [0.0058, 0.083]

Table G-10. Statistical Summary of Site NC Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fatty Acid (% Total FA)						
18:0 Stearic Acid	4.42 (0.065) [4.34 - 4.49]	4.79 (0.080) [4.66 - 4.92]	-0.36 (0.10) [-0.57 - -0.17]	-0.69, -0.035	0.038	(3.21 - 5.24) [1.88, 6.25]
18:1 Oleic Acid	19.78 (0.35) [19.21 - 20.21]	21.60 (0.43) [20.83 - 22.37]	-1.82 (0.56) [-3.16 - -0.62]	-3.60, -0.036	0.047	(16.69 - 35.16) [5.01, 42.01]
18:2 Linoleic Acid	54.21 (0.31) [53.89 - 54.61]	52.62 (0.38) [51.93 - 53.30]	1.59 (0.49) [0.58 - 2.68]	0.046, 3.14	0.046	(44.17 - 57.72) [38.57, 66.94]
18:3 Linolenic Acid	7.45 (0.087) [7.33 - 7.66]	7.11 (0.11) [6.98 - 7.25]	0.34 (0.14) [0.079 - 0.68]	-0.10, 0.78	0.091	(4.27 - 8.81) [2.69, 10.81]
20:0 Arachidic Acid	0.49 (0.0072) [0.49 - 0.50]	0.51 (0.0088) [0.50 - 0.53]	-0.019 (0.011) [-0.044 - -0.0020]	-0.056, 0.017	0.185	(0.36 - 0.55) [0.23, 0.64]
20:1 Eicosenoic Acid	0.25 (0.0057) [0.24 - 0.26]	0.23 (0.0064) [0.23 - 0.23]	0.014 (0.0059) [0.0054 - 0.014]	-0.0051, 0.033	0.102	(0.21 - 0.30) [0.16, 0.33]
20:2 Eicosadienoic Acid	0.043 (0.0012) [0.041 - 0.044]	0.044 (0.0013) [0.042 - 0.045]	-0.0015 (0.0012) [-0.0013 - -0.00084]	-0.0054, 0.0023	0.296	(0.016 - 0.054) [0.0029, 0.083]
22:0 Behenic Acid	0.55 (0.0059) [0.54 - 0.56]	0.53 (0.0072) [0.52 - 0.54]	0.025 (0.0092) [0.0057 - 0.034]	-0.0038, 0.055	0.069	(0.38 - 0.59) [0.30, 0.67]

Table G-10. Statistical Summary of Site NC Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Fiber (% DW)						
Acid Detergent Fiber	16.49 (0.69) [16.04 - 17.05]	15.85 (0.85) [15.49 - 16.20]	0.64 (1.09) [-0.16 - 1.56]	-2.84, 4.13	0.597	(12.79 - 17.98) [11.13, 20.21]
Neutral Detergent Fiber	20.15 (1.15) [18.97 - 21.80]	17.24 (1.41) [17.16 - 17.32]	2.91 (1.82) [2.53 - 4.47]	-2.87, 8.69	0.207	(13.32 - 23.57) [7.24, 28.70]
Proximate (% DW, unless noted)						
Ash	4.99 (0.039) [4.92 - 5.04]	5.05 (0.048) [4.98 - 5.12]	-0.063 (0.062) [-0.12 - -0.061]	-0.26, 0.13	0.383	(4.32 - 5.62) [3.74, 6.45]
Carbohydrates	31.82 (3.28) [21.58 - 37.50]	36.64 (4.00) [36.15 - 36.58]	-4.82 (4.94) [-15.00 - 0.23]	-20.55, 10.91	0.401	(31.97 - 38.00) [28.17, 40.99]
Moisture (% FW)	7.21 (0.34) [7.08 - 7.33]	6.92 (0.41) [6.77 - 7.06]	0.29 (0.47) [0.27 - 0.31]	-1.22, 1.79	0.585	(5.48 - 11.70) [1.45, 12.81]
Protein	37.02 (1.11) [36.49 - 37.46]	35.25 (1.36) [32.37 - 38.18]	1.77 (1.72) [-0.72 - 4.74]	-3.71, 7.25	0.378	(38.14 - 42.66) [35.30, 45.38]
Total Fat	21.11 (0.65) [21.02 - 21.20]	20.63 (0.65) [20.59 - 20.66]	0.48 (0.92) [0.61 - 0.61]	-3.47, 4.43	0.651	(17.90 - 23.56) [14.74, 25.18]
Vitamin (mg/100g DW)						
Vitamin E	7.83 (0.29) [7.59 - 8.19]	6.14 (0.34) [5.47 - 6.55]	1.69 (0.35) [1.03 - 2.25]	0.56, 2.82	0.017	(1.65 - 8.08) [0, 11.09]

Table G-10. Statistical Summary of Site NC Soybean Seed Amino Acid, Fatty Acid, Fiber, Proximate, Vitamin, Antinutrient and Isoflavone Content for MON 87701 vs. the Conventional Control (A5547) (cont'd.)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control) Mean (S.E.) [Range]	Difference (Test minus Control) 95% CI (Lower, Upper)	Difference (Test minus Control) p-Value	Commercial (Range) [99% Tolerance Interval[2]]
Antinutrient (% DW, unless noted)						
Lectin (H.U./mg FW)	0.96 (0.39) [0.53 - 1.74]	0.54 (0.47) [0.32 - 0.85]	0.42 (0.57) [-0.23 - 1.42]	-1.39, 2.24	0.511	(0.090 - 2.47) [0, 3.40]
Phytic Acid	1.76 (0.039) [1.69 - 1.83]	1.85 (0.047) [1.86 - 1.88]	-0.088 (0.051) [-0.11 - -0.026]	-0.25, 0.074	0.181	(1.10 - 2.32) [0.54, 3.05]
Raffinose	1.38 (0.067) [1.23 - 1.47]	1.41 (0.082) [1.39 - 1.43]	-0.033 (0.11) [0.0042 - 0.080]	-0.37, 0.31	0.774	(0.52 - 1.62) [0.038, 2.24]
Stachyose	4.56 (0.11) [4.32 - 4.72]	5.50 (0.13) [5.36 - 5.73]	-0.94 (0.14) [-1.00 - -0.72]	-1.37, -0.51	0.006	(1.97 - 5.55) [0.99, 7.93]
Trypsin Inhibitor (TIU/mg DW)	24.82 (1.64) [24.57 - 25.04]	25.72 (2.01) [22.49 - 28.96]	-0.90 (2.60) [-4.10 - 2.55]	-9.17, 7.37	0.751	(20.84 - 37.24) [13.58, 46.02]
Isoflavone (mg/kg DW)						
Daidzein	661.66 (45.55) [617.52 - 710.29]	589.26 (55.79) [583.50 - 595.01]	72.41 (72.02) [62.16 - 126.79]	-156.79, 301.60	0.388	(213.98 - 1273.94) [0, 1585.14]
Genistein	715.20 (41.48) [668.18 - 746.88]	598.93 (50.80) [591.01 - 606.84]	116.27 (65.58) [123.71 - 155.87]	-92.43, 324.98	0.174	(148.06 - 1024.50) [0, 1352.86]
Glycitein	187.13 (17.29) [147.65 - 228.15]	145.09 (20.56) [139.44 - 165.70]	42.05 (22.80) [19.91 - 88.71]	-30.52, 114.62	0.162	(32.42 - 208.45) [0, 272.12]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

Appendix H. Supplementary Compositional Analysis Tables

1. Data Description

The purpose of this report is to provide statistical summaries of re-expressed soybean seed amino acid and fatty acid composition data. The data used were from a SAS® data set (datag457v2.sas7bdat, created 9/11/2008) containing all of the composition data sent to Certus International for statistical analysis of Monsanto study REG-08-065.

The seed fatty acid, total fat, amino acid, protein and moisture data from test substance MON 87701, conventional control substance A5547, and twenty commercial conventional reference substances were selected from the data set.

Soybean forage and seed of the test, control, and reference substances were collected from replicated plots at five U.S. sites during 2007. Test and control substances were planted in a randomized complete block design. Samples were unavailable for one replicate of MON 87701 at Site AL and one replicate of A5547 at Site NC. Reference substances were distributed as follows across sites:

Site AL	Site AR	Site GA	Site IL	Site NC
A5843, A5959, CMA 5804AOC, H6686	Anand, Ozark, Hornbeck C5894, UA 4805	A5403, A5560, CMC 5901COC, LEE 74	A4922, A5427, H4994, H5218	DP 5989, Fowler, Hutcheson, USG 5601T

Analytes with greater than fifty percent of observations below the assay's limit of quantitation (LOQ) were excluded from analysis. Otherwise, results below the LOQ were assigned a value equal to half the LOQ. The following analytes were assigned values:

		Obs. Below LOQ				
Analyte	**Units**	**N**	**(%)**	**Total N**	**LOQ**	**Value Assigned**
Seed Fatty Acid						
17:1 Heptadecenoic Acid	% FW	8	12.7	63	0.0051	0.0026
20:2 Eicosadienoic Acid	% FW	9	14.3	63	0.0052	0.0026

The following formulas were used for re-expression of amino acid and fatty acid data:

Analyte	From (X)	To	Formula[1]
Amino Acids (AA)	% FW	% Total Protein	X/(fp)
		% Total AA	$(100)X_j/\Sigma X$, for each AA_j where ΣX is over all the AA

(Cont.)

Analyte	From (X)	To	Formula[1]
Fatty Acid (FA)	% FW	% Total Fat	X/(ff)
		% DW	X/(d)

[1]'X' is the individual sample value; fp is the protein fraction of fresh weight obtained by proximate analysis = (% protein /100); ff is the total fat fraction of fresh weight obtained by proximate analysis = (% total fat / 100); d is the fraction of the sample that is dry matter.

2. Statistical Methods

Summary statistics for the analytes of interest were generated using SAS®[1] software.

3. Results Discussion

Statistical results of re-expression of amino acid and fatty acid analytes of test substance MON 87701, conventional control substance A5547, and commercial conventional reference substances are summarized across sites in Table H-1. For each amino acid and fatty acid analyte re-expression, the overall mean, standard error of the mean (S.E.), and the range of observed values are presented. In addition, the overall range of observed values for commercial reference substances is presented in the desired unit.

4. References

1. SAS Software Release 9.1 (TS1M3). Copyright (c) 2002-2003 by SAS Institute Inc., Cary, NC, USA.

Table H-1. Statistical Summary of Combined Site Soybean Amino Acid and Fatty Acid Content for MON 87701, Conventional Control and Commercial Conventional Soybean Varieties

Analyte (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Commercial [Range]
Combined Site Seed Amino Acid (% Total AA)			
Alanine (% Total AA)	4.32 (0.024) [4.19 - 4.49]	4.33 (0.0099) [4.26 - 4.38]	[4.16 - 4.38]
Arginine (% Total AA)	6.70 (0.064) [6.30 - 7.17]	6.65 (0.031) [6.50 - 6.87]	[6.53 - 6.98]
Aspartic Acid (% Total AA)	12.27 (0.068) [11.86 - 12.77]	12.46 (0.073) [11.90 - 12.85]	[11.87 - 13.02]
Cystine (% Total AA)	1.56 (0.020) [1.41 - 1.70]	1.58 (0.033) [1.38 - 1.83]	[1.32 - 1.63]
Glutamic Acid (% Total AA)	19.15 (0.078) [18.67 - 19.71]	19.37 (0.093) [18.70 - 19.88]	[18.95 - 20.31]
Glycine (% Total AA)	4.39 (0.015) [4.31 - 4.49]	4.38 (0.024) [4.25 - 4.56]	[4.20 - 4.81]
Histidine (% Total AA)	2.82 (0.018) [2.70 - 2.93]	2.78 (0.021) [2.63 - 2.91]	[2.61 - 2.89]
Isoleucine (% Total AA)	4.54 (0.011) [4.46 - 4.60]	4.53 (0.014) [4.45 - 4.64]	[4.41 - 4.61]
Leucine (% Total AA)	7.60 (0.023) [7.49 - 7.77]	7.56 (0.026) [7.43 - 7.79]	[7.37 - 7.69]
Lysine (% Total AA)	6.87 (0.043) [6.65 - 7.07]	6.74 (0.062) [6.32 - 6.98]	[6.14 - 7.36]
Methionine (% Total AA)	1.35 (0.021) [1.19 - 1.46]	1.37 (0.028) [1.21 - 1.58]	[1.21 - 1.51]
Phenylalanine (% Total AA)	5.37 (0.071) [5.05 - 5.79]	5.25 (0.053) [5.00 - 5.70]	[5.03 - 5.92]
Proline (% Total AA)	5.03 (0.0095) [4.99 - 5.08]	5.05 (0.0057) [5.02 - 5.09]	[4.90 - 5.07]
Serine (% Total AA)	5.08 (0.017) [4.99 - 5.25]	5.06 (0.017) [4.95 - 5.16]	[4.91 - 5.16]
Threonine (% Total AA)	4.01 (0.015) [3.92 - 4.09]	3.99 (0.024) [3.81 - 4.12]	[3.85 - 4.05]

Table H-1. Statistical Summary of Combined Site Soybean Amino Acid and Fatty Acid Content for MON 87701, Conventional Control and Commercial Conventional Soybean Varieties (cont'd.)

Analyte (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Commercial [Range]
Combined Site Seed Amino Acid (% Total AA)			
Tryptophan (% Total AA)	1.29 (0.017) [1.16 - 1.39]	1.29 (0.019) [1.19 - 1.40]	[1.15 - 1.34]
Tyrosine (% Total AA)	2.84 (0.047) [2.58 - 3.13]	2.82 (0.033) [2.65 - 3.02]	[2.59 - 3.13]
Valine (% Total AA)	4.81 (0.0084) [4.73 - 4.86]	4.79 (0.012) [4.71 - 4.87]	[4.61 - 4.84]
Combined Site Seed Amino Acid (% Total Protein)			
Alanine (% Total Protein)	4.39 (0.025) [4.22 - 4.58]	4.46 (0.071) [4.25 - 5.12]	[4.20 - 4.58]
Arginine (% Total Protein)	6.81 (0.072) [6.35 - 7.38]	6.85 (0.12) [6.41 - 7.92]	[6.49 - 7.28]
Aspartic Acid (% Total Protein)	12.47 (0.063) [12.18 - 13.02]	12.84 (0.21) [12.02 - 14.59]	[12.03 - 13.27]
Cystine (% Total Protein)	1.58 (0.020) [1.43 - 1.73]	1.63 (0.038) [1.39 - 1.83]	[1.32 - 1.68]
Glutamic Acid (% Total Protein)	19.46 (0.071) [18.95 - 20.11]	19.95 (0.34) [18.78 - 22.83]	[18.96 - 20.71]
Glycine (% Total Protein)	4.46 (0.019) [4.34 - 4.59]	4.51 (0.080) [4.24 - 5.22]	[4.23 - 4.97]
Histidine (% Total Protein)	2.86 (0.019) [2.75 - 3.00]	2.86 (0.057) [2.62 - 3.33]	[2.59 - 2.97]
Isoleucine (% Total Protein)	4.61 (0.021) [4.46 - 4.73]	4.67 (0.077) [4.42 - 5.35]	[4.31 - 4.81]
Leucine (% Total Protein)	7.73 (0.037) [7.51 - 8.00]	7.80 (0.13) [7.37 - 8.97]	[7.31 - 8.02]
Lysine (% Total Protein)	6.98 (0.046) [6.70 - 7.19]	6.94 (0.11) [6.37 - 8.02]	[6.14 - 7.56]
Methionine (% Total Protein)	1.37 (0.020) [1.22 - 1.48]	1.41 (0.032) [1.22 - 1.60]	[1.21 - 1.51]
Phenylalanine (% Total Protein)	5.46 (0.081) [5.14 - 5.95]	5.41 (0.11) [4.99 - 6.25]	[4.92 - 6.18]

Table H-1. Statistical Summary of Combined Site Soybean Amino Acid and Fatty Acid Content for MON 87701, Conventional Control and Commercial Conventional Soybean Varieties (cont'd.)

Analyte (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Commercial [Range]
Combined Site Seed Amino Acid (% Total Protein)			
Proline (% Total Protein)	5.11 (0.012) [5.02 - 5.18]	5.21 (0.087) [4.97 - 5.97]	[4.92 - 5.34]
Serine (% Total Protein)	5.17 (0.017) [5.08 - 5.29]	5.21 (0.091) [4.94 - 5.98]	[4.92 - 5.45]
Threonine (% Total Protein)	4.07 (0.019) [3.93 - 4.19]	4.11 (0.075) [3.80 - 4.79]	[3.83 - 4.23]
Tryptophan (% Total Protein)	1.31 (0.016) [1.18 - 1.41]	1.33 (0.031) [1.20 - 1.64]	[1.17 - 1.36]
Tyrosine (% Total Protein)	2.88 (0.051) [2.63 - 3.22]	2.91 (0.060) [2.65 - 3.47]	[2.62 - 3.15]
Valine (% Total Protein)	4.88 (0.017) [4.73 - 4.97]	4.94 (0.083) [4.69 - 5.72]	[4.55 - 5.05]
Combined Site Seed Fatty Acid (% DW)			
10:0 Capric Acid (% DW)	0.029 (0.0015) [0.023 - 0.038]	0.031 (0.0014) [0.023 - 0.036]	[0.022 - 0.037]
14:0 Myristic Acid (% DW)	0.013 (0.00042) [0.0099 - 0.016]	0.014 (0.00046) [0.012 - 0.017]	[0.0087 - 0.017]
16:0 Palmitic Acid (% DW)	1.71 (0.041) [1.39 - 1.93]	1.74 (0.029) [1.61 - 1.92]	[1.30 - 2.19]
16:1 Palmitoleic Acid (% DW)	0.013 (0.00048) [0.0088 - 0.016]	0.014 (0.00039) [0.011 - 0.017]	[0.010 - 0.021]
17:0 Heptadecanoic Acid (% DW)	0.014 (0.00032) [0.011 - 0.016]	0.014 (0.00030) [0.013 - 0.016]	[0.011 - 0.017]
17:1 Heptadecenoic Acid (% DW)	0.0059 (0.00030) [0.0027 - 0.0076]	0.0061 (0.00030) [0.0027 - 0.0075]	[0.0027 - 0.0096]
18:0 Stearic Acid (% DW)	0.67 (0.024) [0.47 - 0.80]	0.69 (0.019) [0.57 - 0.82]	[0.42 - 0.78]
18:1 Oleic Acid (% DW)	3.20 (0.11) [2.38 - 3.87]	3.37 (0.15) [2.89 - 4.59]	[2.32 - 6.13]
18:2 Linoleic Acid (% DW)	7.60 (0.17) [6.54 - 8.87]	7.57 (0.061) [7.19 - 7.89]	[5.75 - 9.90]

Table H-1. Statistical Summary of Combined Site Soybean Amino Acid and Fatty Acid Content for MON 87701, Conventional Control and Commercial Conventional Soybean Varieties (cont'd.)

Analyte (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Commercial [Range]
18:3 Linolenic Acid (% DW)	1.07 (0.037) [0.76 - 1.35]	1.04 (0.029) [0.82 - 1.18]	[0.71 - 1.56]
20:0 Arachidic Acid (% DW)	0.073 (0.0028) [0.050 - 0.086]	0.075 (0.0028) [0.059 - 0.090]	[0.046 - 0.084]
20:1 Eicosenoic Acid (% DW)	0.034 (0.0013) [0.023 - 0.042]	0.034 (0.0015) [0.025 - 0.044]	[0.028 - 0.053]
20:2 Eicosadienoic Acid (% DW)	0.0059 (0.00048) [0.0027 - 0.0081]	0.0062 (0.00028) [0.0027 - 0.0072]	[0.0028 - 0.0081]
22:0 Behenic Acid (% DW)	0.081 (0.0029) [0.056 - 0.094]	0.079 (0.0035) [0.062 - 0.10]	[0.051 - 0.088]
Combined Site Seed Fatty Acid (% Total Fat)			
10:0 Capric Acid (% Total Fat)	0.14 (0.0060) [0.10 - 0.18]	0.15 (0.0067) [0.12 - 0.20]	[0.12 - 0.19]
14:0 Myristic Acid (% Total Fat)	0.065 (0.0023) [0.039 - 0.075]	0.068 (0.0010) [0.061 - 0.075]	[0.044 - 0.087]
16:0 Palmitic Acid (% Total Fat)	8.26 (0.32) [5.07 - 10.44]	8.69 (0.085) [8.27 - 9.33]	[6.98 - 12.07]
16:1 Palmitoleic Acid (% Total Fat)	0.065 (0.0030) [0.037 - 0.079]	0.070 (0.0016) [0.063 - 0.080]	[0.050 - 0.094]
17:0 Heptadecanoic Acid (% Total Fat)	0.065 (0.0028) [0.039 - 0.085]	0.068 (0.0014) [0.058 - 0.077]	[0.056 - 0.093]
17:1 Heptadecenoic Acid (% Total Fat)	0.029 (0.0017) [0.016 - 0.041]	0.030 (0.0014) [0.014 - 0.037]	[0.014 - 0.045]
18:0 Stearic Acid (% Total Fat)	3.23 (0.15) [1.87 - 3.85]	3.45 (0.096) [2.96 - 3.95]	[2.27 - 3.66]
18:1 Oleic Acid (% Total Fat)	15.46 (0.68) [8.41 - 18.79]	16.72 (0.47) [14.22 - 20.61]	[11.60 - 26.04]
18:2 Linoleic Acid (% Total Fat)	36.79 (1.60) [22.43 - 48.02]	37.92 (0.76) [33.57 - 43.37]	[31.46 - 53.62]
18:3 Linolenic Acid (% Total Fat)	5.21 (0.31) [3.05 - 7.30]	5.23 (0.24) [3.66 - 6.60]	[3.17 - 8.60]
Phenylalanine (% Total Protein)	5.46 (0.081) [5.14 - 5.95]	5.41 (0.11) [4.99 - 6.25]	[4.92 - 6.18]

Table H-1. Statistical Summary of Combined Site Soybean Amino Acid and Fatty Acid Content for MON 87701, Conventional Control and Commercial Conventional Soybean Varieties (cont'd.)

Analyte (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Commercial [Range]
Combined Site Seed Fatty Acid (% Total Fat)			
20:0 Arachidic Acid (% Total Fat)	0.35 (0.015) [0.21 - 0.41]	0.37 (0.010) [0.30 - 0.42]	[0.24 - 0.38]
20:1 Eicosenoic Acid (% Total Fat)	0.17 (0.0078) [0.10 - 0.20]	0.17 (0.0042) [0.15 - 0.20]	[0.15 - 0.23]
20:2 Eicosadienoic Acid (% Total Fat)	0.028 (0.0026) [0.012 - 0.043]	0.031 (0.0013) [0.016 - 0.036]	[0.012 - 0.042]
22:0 Behenic Acid (% Total Fat)	0.39 (0.015) [0.23 - 0.43]	0.39 (0.011) [0.34 - 0.47]	[0.27 - 0.44]

[1]AA = amino acid; DW = dry weight; S.E. = standard error.