Food and Feed Safety and Nutritional Assessment of Dicamba and Glufosinate-Tolerant CottonMON 88701 (OECD Unique Identifier MON-887Ø1-3)

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

April 6, 2012

FDA BNF 135

Monsanto 11-CT-239F

Prepared by:

[redacted]

Contributors:

[redacted]

Submitted by:

[redacted]

Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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). Additionally this submission complies with the Codex Plant Guidelines (CAC/GL 45-2009) insofar as it is within the FDA's jurisdiction.

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) cotton MON 88701 is as safe and nutritious as other commercially-available cotton; and (b) the intended uses of the food and feed derived from MON 88701 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. Upon request, 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 88701.

Signature: Date:

[redacted]
Regulatory Affairs Manager
Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

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.

ABBREVIATIONS AND DEFINITIONS[1]

symbol or abbrev.	definition
~	Approximately
α-Cyano	α-Cyano-4-hydroxycinnamic acid
a.e.	acid equivalent
AA	Amino Acid
AAbA	α-aminobutyric acid
ADF	Acid Detergent Fiber
AD_2011	Allergen, gliadin, and glutenin protein sequence database (Release date February 18, 2011)
APHIS	Animal and Plant Health Inspection Service of the United States Department of Agriculture
bar	Bialaphos Resistance Gene from *Streptomyces hygroscopicus*
BLOCKS	A database of amino acid motifs found in protein families
BLOSUM	Blocks Substitution Matrix, used to score similarities between pairs of distantly related protein or nucleotide sequences
BSA	Bovine Serum Albumin
CFR	Code of Federal Regulations
CHT	Ceramic hydroxyapatite
CoA	Coenzyme A
CTAB	Hexadecyltrimethylammonium bromide
COA	Certificate of Analysis
DAP	Days After Planting
Da	Dalton
dCTP	Deoxycytidine triphosphate
DEAE-	Diethylaminoethyl-
DHB	2,5-dihydroxybenzoic acid
DCSA	3,6-dichlorosalicylic acid
DDI	Daily Dietary Intake
dicamba	3,6-dichloro-2-methoxybenzoic acid
dmo	Mono-oxygenase gene from *Stenotrophomonas maltophilia*
DMO	Dicamba mono-oxygenase
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
DTT	Dithiothreitol
dw	Dry weight
DWCF	Dry weight conversion factor
ECL	Enhanced Chemiluminescence
E. coli	*Escherichia coli*

[1] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

E.coli-produced MON 88701 DMO	DMO protein produced from *E. coli* with the same sequence as MON 88701 DMO
ELISA	Enzyme-linked Immunosorbent Assay
EPA	Environmental Protection Agency
E-Score	Expectation score
FA	Fatty Acid
FARRP	Food Allergy Research and Resource Program
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FDA	Food and Drug Administration (U.S.)
FFDCA	Federal Food, Drug and Cosmetic Act (U.S.)
FT	Flow through
fw	Fresh weight
glufosinate	butanoic acid, 2-amino-4-(hydroxymethylphosphinyl)
GLP	Good Laboratory Practice
g	Gram
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
HU	Hemagglutinating Unit
ILSI	International Life Sciences Institute
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
LB	Laemmli buffer
LOD	Limit of Detection
LOQ	Limit of Quantitation
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry
µg	Microgram
mg	Milligram
MOE	Margin of Exposure
MON 88701 DMO	DMO protein produced in MON 88701
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
N-acetyl glufosinate	2-acetamido-4-methylphosphinico-butanoic acid
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
NDF	Neutral Detergent Fiber
NFDM	Non-fat Dried Milk
NOAEL	No Observable Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame

OSL	Overseason Leaf
p	Probability from PRESS
PAT	Phosphinothricin N-acetyltransferase
PAT (*bar*)	PAT protein produced by the *bar* gene
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline containing Tween-20
PCR	Polymerase Chain Reaction
PI	Prediction Interval
ppm	parts per million
PPT	Phosphinothricin
PRESS	Predicted Residual Sum of Squares
PRT_2011	GenBank protein database, 181.0 (Released December 18, 2010)
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene difluoride
PVP	Polyvinyl pyrrolidone
RBD	Refined, Bleached, and Deodorized
RED	Reregistration Eligibility Decision
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SE	Standard Error
SGF	Simulated Gastric Fluid
S. hygroscopicus	*Streptomyces hygroscopicus*
SIF	Simulated Intestinal Fluid
Sinapinic Acid	3,5-dimethoxy-4-hydroxycinnamic acid
S. maltophilia	*Stenotrophomonas maltophilia*
SOP	Standard Operating Procedure
TBA	Tris-borate buffer with L-ascorbic acid
TBS	Tris Buffered Saline
TCEP	Tris(2-carboxyethyl)phosphine
T-DNA	Transfer DNA
TDF	Total Dietary Fiber
TFA	Trifluoroacetic Acid
TFE	2,2,2,-trifluoroethanol
TIU	Trypsin Inhibitor Unit
Tm	Melting temperature
TNB	5-thio-nitrobenzoate
TOX_2011	Toxin protein sequence database (Release date February 18, 2011)
V	volts
v/v	volume to volume ratio
w/v	weight to volume ratio

NAME AND ADDRESS OF SUBMITTER

The submitter of this safety and nutritional assessment summary for cotton MON 88701 is:

Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

Communications with regard to this submission should be directed to [redacted], M.S., Regulatory Affairs Manager, at the Monsanto address listed above [redacted]

STATUS OF SUBMISSION TO USDA-APHIS

Monsanto will request a Determination of Nonregulated Status for MON 88701, including all progenies derived from crosses between MON 88701 and other cotton, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) in June 2012. Under regulations administered by USDA-APHIS (7 CFR 340), MON 88701 is currently considered a "regulated article." Monsanto will continue to conduct all field tests for MON 88701 in strict compliance with USDA field trial regulations until a Determination of Nonregulated Status is granted for MON 88701. Once MON 88701 is deregulated, authorization for import, interstate movement or environmental release of MON 88701 will no longer be required.

STATUS OF SUBMISSIONS TO U.S. EPA

The safety of dicamba use on many crops, including cotton, was reviewed by the Environmental Protection Agency (EPA) as part of the food, feed, and environmental safety reassessment in 2006. Dicamba can currently be applied to cotton in the U.S. as a pre-plant application, at least 21 days prior to planting. The tolerance of MON 88701 to dicamba facilitates a wider window of application on cotton, allowing pre-emergence application of the herbicide up to the day of crop emergence and post-emergence in-crop applications through seven days pre-harvest. Monsanto will request a registration from U.S. EPA for the expanded use of dicamba on MON 88701, an increase in the dicamba residue tolerance to 3 ppm for cottonseed, the addition of 3,6-dichlorosalicylic acid (DCSA) to the cottonseed residue definition, and the establishment of a tolerance of 70 ppm for cotton gin by-products. No other revisions to dicamba pesticide residue tolerances are needed including animal products such as meat or milk.

The existing 0.2 ppm pesticide residue tolerance for cottonseed supporting the current uses of dicamba on cotton (40 CFR § 180.227) is for the combined residues of parent dicamba and its metabolite 5-hydroxy dicamba. Cotton gin by-products, a ruminant feed supplement, has no established dicamba tolerance. Studies have shown that the proposed use of dicamba on MON 88701 cotton results in total residue concentrations of parent

dicamba and its metabolites, including DCSA and 5-hydroxy dicamba, are less than 3 ppm for cottonseed and less than 70 ppm for gin by-products.

The safety of glufosinate use on many crops, including cotton, was reviewed by the EPA as part of the food, feed, and environmental safety reassessment in 2000 (U.S. EPA, 2003). In addition glufosinate has been used over-the-top of glufosinate-tolerant crops since 1995 with no adverse effects reported. Glufosinate is currently labeled for in-crop application on glufosinate-tolerant cotton from emergence through early bloom growth stage. The use pattern and rate of glufosinate on MON 88701 will follow the existing glufosinate-tolerant cotton uses outlined on the glufosinate herbicide label. Furthermore, glufosinate residues in MON 88701 treated with glufosinate are below the EPA-established pesticide residue tolerances of 4.0 ppm and 15.0 ppm for cottonseed and gin by-products, respectively (40 CFR 180.473). Both of these tolerances include the combined residues of parent glufosinate and its metabolites N-acetyl glufosinate and 3-methylphosphinico-propionic acid. Currently glufosinate is undergoing reregistration at EPA with the Reregistration Eligibility Decision (RED) expected by the end of 2013. It is likely that EPA will affirm the safety and efficacy of glufosinate and approve its continued use in the marketplace upon completion of the registration process. Therefore, Monsanto will not pursue any changes in the glufosinate label or the established tolerances for its use on MON 88701 cotton.

STATUS OF SUBMISSIONS TO OTHER GOVERNMENT AGENCIES

Regulatory submissions will be made to countries that import significant cotton or food and feed products derived from U.S. cotton and have functional regulatory review processes in place. This results in submissions to a number of additional governmental regulatory agencies including, but not limited to: Japan's Ministry of Agriculture, Forestry, and Fisheries and the Ministry of Health, Labour, and Welfare; the Canadian Food Inspection Agency and Health Canada; the Intersectoral Commission for Biosafety of Genetically Modified Organisms, Mexico; the Korea Food and Drug Administration; and the Rural Development Administration of Korea, as well as to regulatory authorities in other cotton importing countries with functioning regulatory systems. As appropriate, notifications will be made to countries that import significant quantities of U.S. cotton and cotton products and do not have a formal regulatory review process for biotechnology-derived crops.

EXECUTIVE SUMMARY

Food and Feed Safety Assessment of MON 88701

MON 88701 Product Description

Monsanto Company has developed dicamba and glufosinate-tolerant cotton, MON 88701, that will permit in-crop applications of dicamba herbicide for the control of broadleaf weeds from pre-emergence to seven days pre-harvest and glufosinate herbicide for broad spectrum weed control from emergence through early bloom growth stage. Both herbicides provide a unique mode-of-action for effective weed management, including the control of glyphosate-resistant weeds. MON 88701 will be combined, through traditional breeding methods, with other approved herbicide-tolerant (i.e. glyphosate) events. The in-crop use of dicamba and glufosinate herbicides, when used in combination with glyphosate herbicide, provides new weed management options in cotton, to control a broad spectrum of grass and broadleaf weed species and effective control of weeds resistant to several herbicide families.

MON 88701 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein to confer tolerance to dicamba herbicide and a bialaphos[2] resistance (*bar*) gene from *Streptomyces hygroscopicus* that expresses the phosphinothricin N-acetyltransferase (PAT) protein to confer tolerance to glufosinate herbicide. DMO protein rapidly demethylates dicamba to the herbicidally inactive metabolite 3,6-dichlorosalicylic acid (DCSA). DCSA has been previously identified as a metabolite of dicamba in cotton, soybean, livestock and soil. PAT (*bar*) protein acetylates the free amino group of glufosinate to produce the herbicidally inactive metabolite 2-acetamido-4-methylphosphinico-butanoic acid (N-acetyl glufosinate).

Monsanto will be requesting an approval from U.S. EPA for the expanded use of dicamba on MON 88701, an increase in the residue tolerance for cottonseed, the addition of DCSA to the dicamba residue definition, and the establishment of a tolerance for cotton gin by-products. No other revisions to the dicamba pesticide residue tolerances are necessary including animal products such as meat and milk. Furthermore, the use of dicamba on MON 88701 does not present any new environmental exposure scenarios not previously evaluated and deemed acceptable by EPA, including estimates of drinking water exposure. The use pattern and rate of glufosinate on MON 88701 will follow the existing glufosinate-tolerant cotton uses outlined on the glufosinate herbicide label and the glufosinate residues in MON 88701 treated with commercial glufosinate rates are

[2] Bialaphos is a bacterial tripeptide composed of L-phosphinothricin (PPT) plus two alanines. *In vivo* the alanines are removed to produce L-PPT, a naturally occurring glutamate analogue with herbicidal activity through the inhibition of glutamine synthetase. Glufosinate is a synthetically produced racemic mixture of D and L-PPT.

below the established pesticide residue tolerances for both cottonseed and gin by-products. Therefore, Monsanto will not pursue any changes in the glufosinate label or the established tolerances for its use on MON 88701 cotton.

Molecular Characterization of MON 88701 Verifies the Integrity and Stability of the Inserted DNA

MON 88701 was developed through *Agrobacterium*-mediated transformation of hypocotyls from cotton variety Coker 130 utilizing vector PV–GHHT6997. PV-GHHT6997 contains one T-DNA that is delineated by Left and Right Border regions. The T-DNA contains the *dmo* and *bar* expression cassettes. The *dmo* expression cassette is regulated by the *PC1SV* promoter, the *TEV* 5′ leader sequence, and the *E6* 3′ untranslated region. The chloroplast transit peptide CTP2 directs transport of the MON 88701 DMO protein to the chloroplast and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene. The *bar* expression cassette is regulated by the *e35S* promoter, the *Hsp70* leader, and the *nos* 3′ untranslated region. After transformation, self pollination and segregation were used to select those plants containing a single homozygous copy of the T-DNA, including both the *dmo* and *bar* expression cassettes, resulting in the selection of MON 88701.

Molecular characterization determined that MON 88701 contains one copy of the T-DNA at a single integration locus and all genetic elements are present. These data also demonstrated that MON 88701 does not contain detectable backbone sequences from the plasmid vector. The complete DNA sequence of the insert and adjacent genomic DNA sequences in MON 88701 confirmed the integrity of the inserted *dmo* and *bar* expression cassettes and identified the 5′ and 3′ insert to flank DNA junctions. Molecular characterization analysis also demonstrated that the insert in MON 88701 has been maintained over five generations of breeding, thereby confirming the stability of the insert. Furthermore, results from segregation analyses showed inheritance and stability of the insert were as expected across multiple generations, which corroborates the molecular insert stability analysis determination that the MON 88701 T-DNA resides at a single chromosomal locus within the cotton genome.

Data Confirm the Safety of Expression Products in MON 88701

MON 88701 contains a *dmo* expression cassette that produces a dicamba mono-oxygenase protein referred to as MON 88701 DMO and a *bar* expression cassette that produces a phosphinothricin N-acetylase transferase protein (PAT) referred to as PAT (*bar*). The safety of PAT protein present in commercial biotechnology-derived crops has been extensively assessed and in 1997 a tolerance exemption was issued for PAT proteins by U.S. EPA. Numerous glufosinate-tolerant products including those in cotton, corn, soy, canola, sugarbeet and rice have been reviewed by the FDA with no concerns identified.

A multistep approach, in accordance with guidelines established by the Codex Alimentarius Commission, OECD, and the principles and guidance of the FDA's 1992

policy on foods from new plant varieties, was used to characterize the MON 88701 DMO and PAT (*bar*) proteins present in MON 88701. These steps include: 1) documentation of the history of safe use of the MON 88701 DMO and PAT (*bar*) proteins and their structural and functional homology with proteins that lack adverse effects on human or animal health; 2) characterization of the physicochemical and functional properties of MON 88701 DMO and PAT (*bar*) proteins; 3) quantification of MON 88701 DMO and PAT (*bar*) expression in plant tissues; 4) examination of the similarity of MON 88701 DMO and PAT (*bar*) proteins to known allergens; 5) evaluation of the digestibility of MON 88701 DMO and PAT (*bar*) in simulated gastrointestinal fluids; 6) evaluation of the stability of the MON 88701 DMO and PAT (*bar*) proteins in response to typical food/feed preparation conditions such as heat treatment; 7) examination of the similarity of MON 88701 DMO and PAT (*bar*) to known toxins or other biologically active proteins known to have adverse effects on mammals; 8) investigation of potential mammalian toxicity through an animal assay and calculating margins of exposure; and 9) examination of the similarity of putative polypeptides encoded by the insert and flanking sequences to known allergens and toxins or other biologically active proteins known to have adverse effects on mammals. The safety assessment supports the conclusion that dietary exposure to MON 88701 DMO and PAT (*bar*) proteins derived from MON 88701 poses no meaningful risk to human or animal health.

A history of safe use has been demonstrated for both MON 88701 DMO and PAT (*bar*) proteins. MON 88701 DMO was fully characterized and the enzymatic activity was found to be specific for dicamba when tested using structurally similar cotton endogenous substrates. The specificity of PAT proteins has been extensively documented in the literature. Neither protein has relevant amino acid sequence similarities to known allergens, gliadins, glutenins, or toxins that may have adverse effects on mammals. MON 88701 DMO and PAT (*bar*) were each rapidly digested in simulated gastric and intestinal fluids. Both proteins lost significant functional activity at temperatures well below those used in cottonseed processing to generate cottonseed meal, oil, and linters. MON 88701 DMO was completely deactivated after heating at temperatures above 55 ºC and PAT (*bar*) lost greater than 90% functional activity at temperatures of 75 ºC and above. Neither MON 88701 DMO nor PAT (*bar*) were acutely toxic and did not cause any observable adverse effects when tested in mouse acute oral toxicity analyses. In addition, the only fractions derived from cottonseed that are used in food applications are oil and linters, which contain undetectable and negligible amounts of protein, respectively. Therefore, MON 88701 DMO and PAT (*bar*) proteins comprise a very low, non-detectable portion of the total protein present in food derived from MON 88701. Based on a history of safe use, an apparent absence of hazard, and lack of dietary exposure a dietary risk assessment for these proteins is considered unnecessary. An open reading frame bioinformatic analysis of the junction site between the cotton genomic DNA and the insert confirms no relevant similarities exist between any putative polypeptides and known toxins or allergens. The safety assessment supports the conclusion that exposure to MON 88701 DMO and PAT (*bar*) from MON 88701 poses no meaningful risk to human and animal health.

Food and Feed Safety Assessments of MON 88701 Demonstrate Equivalence to the Conventional Crop

Detailed compositional analyses, in accordance with OECD guidelines, were conducted to assess whether levels of key nutrients and anti-nutrients in MON 88701 cottonseed were comparable to levels in the conventional cotton Coker 130, with similar background genetics, and several commercial reference cotton varieties.

Cottonseed were harvested from eight sites in which MON 88701 (treated sequentially with glufosinate and dicamba herbicides), the conventional control and a range of commercial reference varieties were grown concurrently in the same field trial. The commercial reference varieties were used to establish a range of natural variability of the key nutrients and anti-nutrients in commercial cotton varieties that have a long history of safe consumption. Nutrients assessed in this analysis included proximates (ash, fat, moisture, protein, and carbohydrates by calculation), calories by calculation, acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber (CF), total dietary fiber (TDF), amino acids (AA, 18 components), fatty acids (FA, C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc) and vitamin E. The key anti-nutrients assessed included gossypol and cyclopropenoid fatty acids (CPFA).

Combined-site analyses were conducted to determine statistically significant differences ($p < 0.05$) between herbicide-treated MON 88701 and the conventional control cottonseed samples. Any significant differences noted from the combined-site statistical comparison were assessed using considerations relevant to the safety and nutritional quality of MON 88701 when compared to the conventional control. Considerations used to assess the relevance of each combined-site statistically significant difference included: 1) the relative magnitude of the difference in the mean values of nutrient and anti-nutrient components between MON 88701 and the conventional control; 2) whether the MON 88701 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of the commercial reference varieties grown concurrently in the same trial; 3) evaluation of the reproducibility of the statistical ($p < 0.05$) combined-site component differences at individual sites; and 4) an assessment of the differences within the context of natural variability of commercial cotton composition published in the scientific literature and in the International Life Sciences Institute (ILSI) Crop Composition Database.

Based on the analyzed nutrient and anti-nutrient levels, herbicide-treated MON 88701 is compositionally equivalent to conventional cotton and therefore the food and feed safety and nutritional quality of this product is comparable to that of the conventional cotton. These results support the overall food and feed safety of MON 88701.

Conclusion

All data and information contained within this document strongly support the conclusion that food and feed derived from MON 88701 and its progeny will be as safe and

nutritious as food and feed derived from conventional cotton. Therefore, the consumption of MON 88701 and its progeny, and the food and feed derived from it will be fully consistent with FDA's Policy (U.S. FDA, 1992) and in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.

I. DESCRIPTION OF MON 88701

This section provides a description of MON 88701 being presented for food and feed safety and nutritional assessment. The description identifies the crop, the transformation event(s) to be reviewed and the type and purpose of the modification, which will aid in understanding the nature of the food and feed products that may be developed from MON 88701. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraph 22 (Codex Alimentarius, 2009).

I.A. MON 88701 Summary

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants" MON 88701 has been assigned the unique identifier MON-887Ø1-3.

Monsanto Company has developed dicamba and glufosinate-tolerant cotton, MON 88701, that will allow in-crop applications of dicamba herbicide for the control of broadleaf weeds from preemergence to seven days preharvest and glufosinate herbicide for broad spectrum weed control from emergence through early bloom growth stage. MON 88701 provides a wider dicamba window of application beyond the current preplant cotton uses and glufosinate tolerance equivalent to current commercial glufosinate-tolerant cotton events. The combination of the two herbicides' unique modes-of-action provides an effective weed management system, as dicamba provides effective control of over 95 annual and biennial weed species, and suppression of over 100 perennial broadleaf and woody plant species and glufosinate is a broad-spectrum contact herbicide that provides nonselective control of about 120 broadleaf and grass weeds. Additionally, dicamba and glufosinate provide control of many herbicide-resistant weeds, including glyphosate-resistant biotypes of palmer pigweed (*Amaranthus palmeri*), marestail (*Conyza canadensis*), common ragweed (*Ambrosia artemisiifolia*), giant ragweed (*Ambrosia trifida*) and waterhemp (*Amaranthus tuberculatus*).

MON 88701 will be combined, through traditional breeding methods, with other approved herbicide-tolerant (i.e. glyphosate) events. The in-crop use of dicamba and glufosinate herbicides, in addition to glyphosate herbicide, provides new weed management options in cotton to control a broad spectrum of grass and broadleaf weed species and effective control of weeds resistant to several herbicide families. Successful integration of MON 88701 into the glyphosate-tolerant cotton system will: 1) provide growers with an opportunity for an efficient, effective weed management system for hard-to-control and herbicide resistant weeds; 2) provide a flexible system for two additional in-crop herbicide modes-of-action in current cotton production practices as recommended by weed science experts to manage future weed resistance development; and 3) provide cotton growers with additional weed management tools to enhance weed management systems necessary to maintain yield and quality to meet the growing needs of the food, feed, and industrial markets.

MON 88701 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein to confer tolerance to dicamba herbicide and a bialaphos resistance (*bar*) gene from *Streptomyces hygroscopicus* that expresses the phosphinothricin N-acetyltransferase (PAT) protein to confer tolerance to glufosinate herbicide. DMO protein rapidly demethylates dicamba to the herbicidally inactive metabolite 3,6-dichlorosalicylic acid (DCSA). DCSA has been previously identified as a metabolite of dicamba in cotton, soybean, livestock and soil. Monsanto will be requesting an approval from U.S. EPA for the expanded use of dicamba on MON 88701, an increase in the residue tolerance for cottonseed, the addition of DCSA to the dicamba residue definition, and the establishment of a tolerance for cotton gin by-products. No other revisions to the dicamba pesticide residue tolerances are necessary including animal products such as meat and milk. Furthermore, the use of dicamba on MON 88701 does not present any new environmental exposure not previously evaluated and deemed acceptable by EPA, including estimates of drinking water exposure.

PAT (*bar*) protein acetylates the free amino group of glufosinate to produce non-herbicidal N-acetyl glufosinate, a well known metabolite in glufosinate tolerant plants (OECD, 2002a). The use pattern and rate of glufosinate on MON 88701 will follow the existing glufosinate-tolerant cotton uses outlined on the glufosinate herbicide label and the glufosinate residues in MON 88701 treated with commercial glufosinate rates are below the established pesticide residue tolerances for both cottonseed and gin by-products. Therefore, Monsanto will not pursue any changes in the glufosinate label or the established tolerances for its use on MON 88701 cotton.

The data and information presented in this safety summary demonstrate that the food and feed derived from MON 88701 are as safe and nutritious as those derived from conventional cotton varieties for which there is an established history of safe consumption. This safety assessment was conducted utilizing established methods for the evaluation of biotechnology-derived products as articulated in guidelines from the Codex Alimentarius Commission and the OECD (Codex Alimentarius, 2009; OECD, 2003). These established methodologies embody the principles and guidance of the U.S. Food and Drug Administration's (FDA) 1992 policy on foods from new plant varieties (U.S. FDA, 1992). Therefore, the consumption of MON 88701 and the food and feed derived from it will be in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.

I.B. Applications for Which MON 88701 is Not Suitable

Monsanto Company is aware of no food or feed uses of conventional cotton that are not applicable to MON 88701.

II. DESCRIPTION OF THE HOST PLANT AND ITS USES AS FOOD OR FEED

This section includes data and information that provides a comprehensive description of the host plant. It also provides relevant phenotypic information on the host plant and on related species that may have contributed to the genetic background of the host plant. The history of use information provided describes how the plant is typically cultivated, transported and stored, any special processing required to assure the plant is safe to eat, and the plant's usual role in the diet. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraphs 23, 24, and 25 (Codex Alimentarius, 2009).

II.A. Biology of Cotton

The Organisation for Economic Co-operation and Development Consensus Document (OECD, 2008) on the biology of cotton (*Gossypium* sp.) provides key information on:

- general description of cotton biology, including taxonomy and morphology and use of cotton as a crop plant
- agronomic practices in cotton cultivation
- geographic centers of origin
- reproductive biology
- inter-species/genus introgression into relatives and interactions with other organisms
- summary of the ecology of cotton

Additional information on the biology and growth and development of cotton is available in the literature (Kohel and Lewis, 1984; OGTR, 2008; Smith and Cothren, 1999).

II.A.1. History of Cotton Development

Cotton belongs to the genus *Gossypium* that currently has approximately 50 species which are widely cultivated in tropical and subtropical regions around the world (OECD, 2008; Percival et al., 1999). There are four cultivated species that were domesticated independently, two of which account for greater than 95% of world cotton production. *Gossypium hirsutum* (often called upland, American, Mexican, or Acala cotton) covering 90% and *Gossypium barbadense* (often called extra long-staple, pima, and Egyptian cotton) covering 5%. Due to the utility of the fibers for the production of textiles, human selection pressure on cotton has altered the plant from essentially perennial shrubs or trees with small impermeable seeds and sparse hairs to a compact annual row crop yielding large, easily germinating seeds with white, thick, long, and strong fibers (Brubaker et al., 1999).

The four cultivated species, which have widely cultivated across the entire globe, are comprised of two diploid species *G. arboretum* and *G. herbaceum*, which evolved from Africa and the Middle East, and two allotetraploid species *G. barbadense* and *G. hirsutum*, which evolved in the Americas (Brubaker et al., 1999).

Improved modern varieties of *G. hirsutum* and *G. barbadense* are currently cultivated in the southern U.S., with *G. barbadense* grown primarily in the western states of Arizona, California, New Mexico, and Texas; and *G. hirsutum* produced throughout the 17 states comprising the U.S. cotton growing region, commonly referred to as the cotton belt. *G. hirsutum* comprises the vast majority of U.S. cotton production with nearly 11 million acres planted and 18 million bales harvested, whereas *G. barbadense* varieties accounted for approximately 200,000 acres and half a million bales in 2010 (USDA-NASS, 2011). Commercial cotton, including *G. hirsutum* and *G. barbadense*, has a long history of agricultural production (Lee, 1984; USDA-NASS, 2012; USDA, 2001). Extra-long staple lint from *G. barbadense* is segregated and classed separately from *G. hirsutum* and is sold at a premium (USDA, 2001). However, cottonseed and cottonseed byproducts (e.g., oil and meal) are not generally distinguished by species (OECD, 2008; USDA-FAS, 2005).

II.B. Characteristics of the Recipient Plant

The cotton variety used as the recipient for the DNA insertion to create MON 88701 was Coker 130 a non-transgenic conventional upland variety developed by Coker Pedigreed Seed Co., which was released in 1990 in the U.S. ([redacted] et al., 2006). Coker 130 was used as the conventional parental cotton comparator (referred to in this consultation document as the conventional control) in the safety assessment of MON 88701. MON 88701 and the conventional control have similar genetic backgrounds with the exception of the T-DNA, thus, the effect of the T-DNA and the expressed MON 88701 DMO and PAT (*bar*) proteins could be assessed in an objective manner. In addition, commercial, conventional cotton varieties (referred to in this consultation document as commercial reference varieties), were used as reference materials to establish ranges of natural variability representative of commercial cotton varieties. The commercial reference varieties used at each location were selected based on their availability and agronomic fit for the respective geographic region.

II.B.1. Known Toxicity or Allergenicity of Recipient Plant

The primary product of cotton production is lint for textile use. However, cottonseed has a number of industrially important uses including livestock feed in the form of whole or crushed cottonseed and cottonseed meal, as well as human food use in the form of oil and linters. Given the wide range of uses and long history of safe consumption of cottonseed and seed by-products, human and animal consumption has rarely been a concern. Nonetheless, it is noted that cottonseed contains gossypol and cyclopropenoid fatty acids that are considered anti-nutrients. Gossypol is a terpenoid that is present in the secretory structure of most cotton plant tissues including seeds (Abou-Donia, 1976; OGTR, 2008). The levels of gossypol and related terpenoids in cottonseed vary (0.4 to 2.0 %) by different species, variety, fertilizer application, and environmental conditions (OECD, 2008). Gossypol is toxic to non-ruminant animals, and in mammals the toxic effects include reduced appetite, body weight loss and dyspnea (Berardi and Goldblat, 1980). Additionally, gossypol can render lysine metabolically unavailable which impacts the

normal functioning of mitochondria (OECD, 2008). Cyclopropenoid fatty acids, including dihydrosterculic acid, sterculic acid, and malvalic acid, account for 0.5 to 1.0% of the total fat content of cottonseeds (OECD, 2008). Cyclopropenoid fatty acids are anti-nutritional compounds, which interfere with the metabolism of saturated fats (Cao et al., 1993; Rolph et al., 1990) and reportedly have adverse effects on egg yolk discoloration and reduced hatchability in chickens, (Lordelo et al., 2007; OECD, 2004; 2008).

Due to the presence of these anti-nutrients in cottonseed, only highly refined products (refined, bleached, and deodorized (RBD) oil and linters) are suitable for human consumption, because the levels of gossypol and cyclopropenoid fatty acids are drastically reduced during processing (AOCS, 1991; Harris, 1981; NCPA, 1993). Furthermore, highly processed cottonseed oil has been a part of the U.S. diet for well over a century with no reports of allergic reactions (Figley, 1949; Loveless, 1950; OGTR, 2008). Whole cottonseed, cottonseed meal, and processing by-products (hulls and gin by-products) are fed primarily to ruminants, which can tolerate moderate gossypol inclusion in their diets. Highly processed cottonseed meal is also fed to non-ruminant farm animals in limited quantities (OECD, 2009b).

Cotton fiber is most often used in the manufacture of a large number of textiles and processed cotton fibers are used in pharmaceutical and medical applications because of its low capacity to cause irritation (OECD, 2008). Inhalation of cotton dust by mill workers can lead to asthma-like conditions called byssinosis (Salvaggio et al., 1986).

Cotton and cotton by-products have a long history of safe use and human and animal consumption has rarely been a concern.

II.C. Cotton as a Food Source

After ginning to remove fibers for textile manufacturing, cottonseed is processed into four major products: oil, meal, hulls, and linters. Processing of cottonseed typically yields (by weight): 16% oil, 45% meal, 26% hulls, and 9% linters, with 4% lost during processing (Cherry, 1983). Only cottonseed oil and linters are utilized as food sources, both are further discussed below.

Cottonseed is highly processed during the production of oil and meal. After hulling, the cottonseed is flaked by a rolling process to facilitate oil removal. Prior to oil extraction, the flakes are heated at temperatures of 88 ºC to greater than 130 ºC to break down the cell walls, reduce the viscosity of oil, inactivate proteins, and detoxify gossypol (Harris, 1981; NCPA, 1993). After heating, oil is typically removed from the meal by direct solvent extraction with hexane. Crude cottonseed oil is further processed with refining, bleaching and deodorizations steps to produce high purity vegetable oil. Temperatures up to 230 ºC are used in the deodorization process (Harris, 1981; NCPA, 1993).

Further processing (refining) for all the uses of cottonseed oil includes deodorization and bleaching. Deodorization greatly reduces the cyclopropenoid fatty acid content of the oil due to extreme pH and temperature conditions (NCPA, 1993). A winterization step is added to produce cooking oil, whereas for solid shortening a hydrogenation step is added to transform the liquid oil into a solid fat. Previous studies have shown that the resulting oil contains no detectable protein (Reeves and Weihrauch, 1979). Cottonseed oil is traded as premium quality oil that is used for a variety of food uses, including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine, and packing oil.

The material left after the extraction of the crude cottonseed oil is the cottonseed meal. The gossypol levels in the meal after extraction are reduced by approximately half. Cottonseed meal is discussed further in Section II.D.

Linters are the short fibers on American upland cottonseed that remain after the long fibers have been removed at the ginning process for textile manufacturing. Linters consist of nearly pure (*i.e.*, >99%) cellulose (NCPA, 2002a; Nida et al., 1996) and after extensive processing at alkaline pH and temperatures >100 ºC (AOCS, 1991), the linters can be used as a high fiber dietary product. Food uses include fiber supplement, casings for processed meats, binder for solids in the pharmaceutical industry, and to improve viscosity in products such as toothpaste, ice cream, and salad dressings (NCPA, 2002a). The highest grade linters can also be used in the manufacturing of absorbent cotton, medical pads, and gauze (NCPA, 2002a), however as mentioned earlier these would consist of nearly pure cellulose, with negligible amounts of protein.

II.D. Cotton as a Feed Source

Cottonseed meal is primarily sold as feed for livestock, of which the major value is as a protein concentrate (NCPA, 2002a). The presence of gossypol and cyclopropenoid fatty acids in cottonseed does not prevent use in livestock diets but does limit cottonseed use as a protein supplement for ruminants, where most other farm animals (monogastric animals) are not fed cottonseed meal to any appreciable level.

The hull is the tough, protective covering of the cottonseed removed prior to processing the seed for oil and meal. It is used as feed for livestock and can be an economical roughage that provides fiber as well as a good carrier for cottonseed meal and grain (NCPA, 2002a).

Gin by-products, the dried plant material cleaned from the fiber during ginning, is also used as a source of roughage for livestock feeds.

III. DESCRIPTION OF THE DONOR ORGANISMS

This section describes the donor organism(s) for the introduced protein. It contains information describing if the donor organism(s) exhibit characteristics of pathogenicity or toxin production, is a known allergenic source, or has other traits that affect food and feed safety. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraph 26 (Codex Alimentarius, 2009).

III.A. Identity and Sources of the Genetic Material Introduced into MON 88701

III.A.1 Identity and Source of the *dmo* Gene Introduced into MON 88701

The *dmo* gene is derived from the bacterium *Stenotrophomonas maltophilia* strain DI-6, isolated from soil at a dicamba manufacturing plant (Krueger et al., 1989). *S. maltophilia* was originally named *Pseudomonas maltophilia*, and then transferred to the genus *Xanthomonas* before it was given its own genus (Palleroni and Bradbury, 1993). The taxonomy of *S. maltophilia* is (Palleroni and Bradbury, 1993; Ryan et al., 2009):

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Xanthomonadales

Family: Xanthomonadaceae

Genus: Stenotrophomonas

S. maltophilia is an aerobic, environmentally ubiquitous gram negative bacterium commonly present in aquatic environments, soil, and plants. *S. maltophilia* is ubiquitously associated with plants and has been isolated from the rhizosphere of wheat, maize, grasses, beet, cucumber, potato, strawberry, sugarcane, and rapeseed (Berg et al., 1996; Berg et al., 1999; Berg et al., 2002; Denton et al., 1998; Echemendia, 2010; Juhnke and des Jardin, 1989; Juhnke et al., 1987; Lambert et al., 1987). *S. maltophilia* has also been isolated from cottonseed, bean pods, and coffee (Nunes and de Melo, 2006; Swings et al., 1983); thus, *S. maltophilia* can be found in a variety of foods and feeds. *S. maltophilia* is also widespread in the home environment and can be found around sponges, flowers, plants, fruits, vegetables, frozen fish, milk, and poultry (Berg et al., 1999; Denton and Kerr, 1998; Echemendia, 2010). Strains of *S. maltophilia* have been found in the transient flora of hospitalized patients as a commensal organism (Echemendia, 2010). *S. maltophilia* can be found in healthy individuals without causing any harm to human health (Denton et al., 1998) and infections in humans caused by *S. maltophilia* are extremely uncommon (Cunha, 2010). Similar to the indigenous bacteria of the gastrointestinal tract, *S. maltophilia* can be an opportunistic pathogen (Berg, 1996).

As such, *S. maltophilia* is of low virulence in immuno-compromised patients where a series of risk factors (severe debilitation, the presence of indwelling devices such as ventilator tubes or catheters, for prolonged periods of time and prolonged courses of antibiotics) must occur for colonization by *S. maltophilia* in humans (Ryan et al., 2009). Therefore, infections by *S. maltophilia* almost exclusively occur in hospital settings, in which case they are only present in a minimal percentage of infections (Ryan et al., 2009). Finally, *S. maltophilia* has not been reported to be a source of allergens.

The ubiquitous presence of *S. maltophilia* in the environment, the presence in healthy individuals without causing infections, the incidental presence in foods without any adverse safety reports, and the lack of reported allergenicity establishes the safety of the donor organism.

III.A.2 Identity and Source of the *bar* Gene Introduced into MON 88701

The *bar* gene is derived from the bacterium *Streptomyces hygroscopicus* (Thompson et al., 1987). The taxonomy of *S. hygroscopicus* is (Waksman and Henrici, 1943):

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteria

Order: Actinomycetales

Family: Streptomycetaceae

Genus: Streptomyces

S. hygroscopicus is a saprophytic, soil-borne bacterium with no known safety issues. *Streptomyces* species are widespread in the environment and present no known allergenic or toxicity issues (Kämpfer, 2006; Kutzner, 1981), though human exposure is quite common (Goodfellow and Williams, 1983). *S. hygroscopicus* is not considered pathogenic to plants, humans or other animals (Cross, 1989; Goodfellow and Williams, 1983; Locci, 1989). *S. hygroscopicus* history of safe use is discussed in Hérouet et al., (2005) and this organism has been extensively reviewed during the evaluation of several glufosinate-tolerant events with no safety or allergenicity issues identified by FDA or other regulatory agencies.

The ubiquitous presence of *S. hygroscopicus* in the environment, the widespread human exposure without any adverse safety or allergenicity reports, and the successive reviews of several glufosinate-tolerant events by regulators have identified no safety or allergenicity issues further establishes the safety of the donor organism.

III.A.3 Identity and Source of Remaining Genetic Material Introduced into MON 88701

MON 88701 was developed through *Agrobacterium tumefaciens*-mediated transformation of cotton hypocotyl tissues utilizing plasmid vector PV–GHHT6997. PV-GHHT6997 is approximately 9.4 kb and contains the *dmo* and *bar* expression cassettes. The *dmo* expression cassette contains the following genetic elements: *PC1SV* promoter derived from the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (*PC1SV*) (Maiti and Shepherd, 1998); the leader from the RNA of tobacco etch virus (*TEV*) (Niepel and Gallie, 1999); the EPSPS chloroplast transit peptide coding sequence from the *shkG* gene of *Arabidopsis thaliana* (*CTP2*) (Herrmann, 1995; Klee et al., 1987); the codon optimized coding sequence of *dmo* gene from *Stenotrophomonas maltophilia* (*dmo*) (Herman et al., 2005; Wang et al., 1997); and the polyadenylation sequence derived from the 3' untranslated region of the *Gossypium barbadense E6* gene (*E6*) (John, 1996). The *bar* expression cassette contains the following genetic elements: 35S RNA promoter from the cauliflower mosaic virus (Odell et al., 1985) containing a duplicated enhancer region (*e35S*) (Kay et al., 1987); the leader from the *DnaK* gene from *Petunia hybrida* that encodes heat shock protein 70 (*Hsp70*) (Rensing and Maier, 1994; Winter et al., 1988); the coding sequence of *bar* from *Streptomyces hygroscopicus* (*bar*) (Thompson et al., 1987); and the polyadenylation sequence derived from the 3′ untranslated region of the *Agrobacterium tumefaciens* nopaline synthase gene (*nos*) (Bevan et al., 1983; Fraley et al., 1983). Except for the potential of *S. maltophilia* to cause infections in immunocompromised patients (Section III.A.1.), there is no evidence of human or animal pathogenicity, for any of the donor organisms of the coding and noncoding DNA sequences present in MON 88701.

DNA has always been present in food and, upon consumption, is quickly degraded to nucleic acids by nucleases present in the gastrointestinal tract of humans and animals. According to the FDA (U.S. FDA, 1992), nucleic acids are present in the cells of every living organism, do not raise concerns as a component of food, and are generally recognized as safe. Results from an International Life Sciences Institute (ILSI) workshop on safety considerations of DNA in food were reported (Jonas et al., 2001) and confirmed that: 1) all DNA, including recombinant DNA, is composed of the same four nucleotides; 2) there are no changes to the chemical characteristics or the susceptibility to degradation by chemical or enzymatic hydrolysis of recombinant DNA as compared to nonrecombinant DNA; and 3) there is no evidence that DNA from dietary sources has ever been incorporated into the mammalian genome.

IV. DESCRIPTION OF THE GENETIC MODIFICATION

This section provides a description of the transformation process and plasmid vector used in the development of MON 88701. Molecular analyses are an integral part of the characterization of crop products with new traits introduced by methods of biotechnology. Vectors and methods are selected for transformation to achieve high probability of obtaining the trait of interest and integration of the introduced DNA into a single locus in the plant genome. This helps ensure that only the intended DNA encoding the desired trait(s) is integrated into the plant genome and facilitates the molecular characterization of the product. Information provided here allows for the identification of the genetic material present in the transformation vector delivered to the host plant and for an analysis of the data supporting the characterization of the DNA inserted in the plant found in Section V. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraphs 27, 28, and 29 (Codex Alimentarius, 2009).

MON 88701 was developed through *Agrobacterium tumefaciens*-mediated transformation of cotton tissues from Coker 130 variety utilizing plasmid vector PV–GHHT6997. This section describes the plasmid vector, the donor gene, and the regulatory elements used in the development of MON 88701, as well as the deduced amino acid sequence of the MON 88701 DMO protein and PAT (*bar*) protein produced in MON 88701. In this section, transfer DNA (T-DNA) refers to DNA that is transferred to the plant during transformation. An expression cassette is comprised of sequences to be transcribed and the regulatory elements necessary for the expression of those sequences.

IV.A. PV–GHHT6997

PV–GHHT6997 was used in the transformation of cotton to produce MON 88701 and its plasmid map is shown in Figure IV-1. The elements included in this plasmid vector are described in Table IV-1. PV-GHHT6997 is approximately 9.4 kb and contains one T-DNA that is delineated by Left Border and Right Border regions. The T-DNA contains the *dmo* and *bar* expression cassettes. The *dmo* expression cassette is regulated by the peanut chlorotic streak caulimovirus (*PC1SV*) promoter, the tobacco etch virus (*TEV*) 5′ leader sequence, and the 3' untranslated sequence of the *E6* gene from *Gossypium barbadense*. The chloroplast transit peptide CTP2 directs transport of the DMO protein to the chloroplast in MON 88701 and is derived from the *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene (Herrmann, 1995; Klee et al., 1987). The *bar* expression cassette is regulated by the *e35S* promoter from the 35S RNA of cauliflower mosaic virus (CaMV), the heat shock protein 70 (*Hsp70)* leader, and the nopaline synthase (*nos)* 3′ untranslated region.

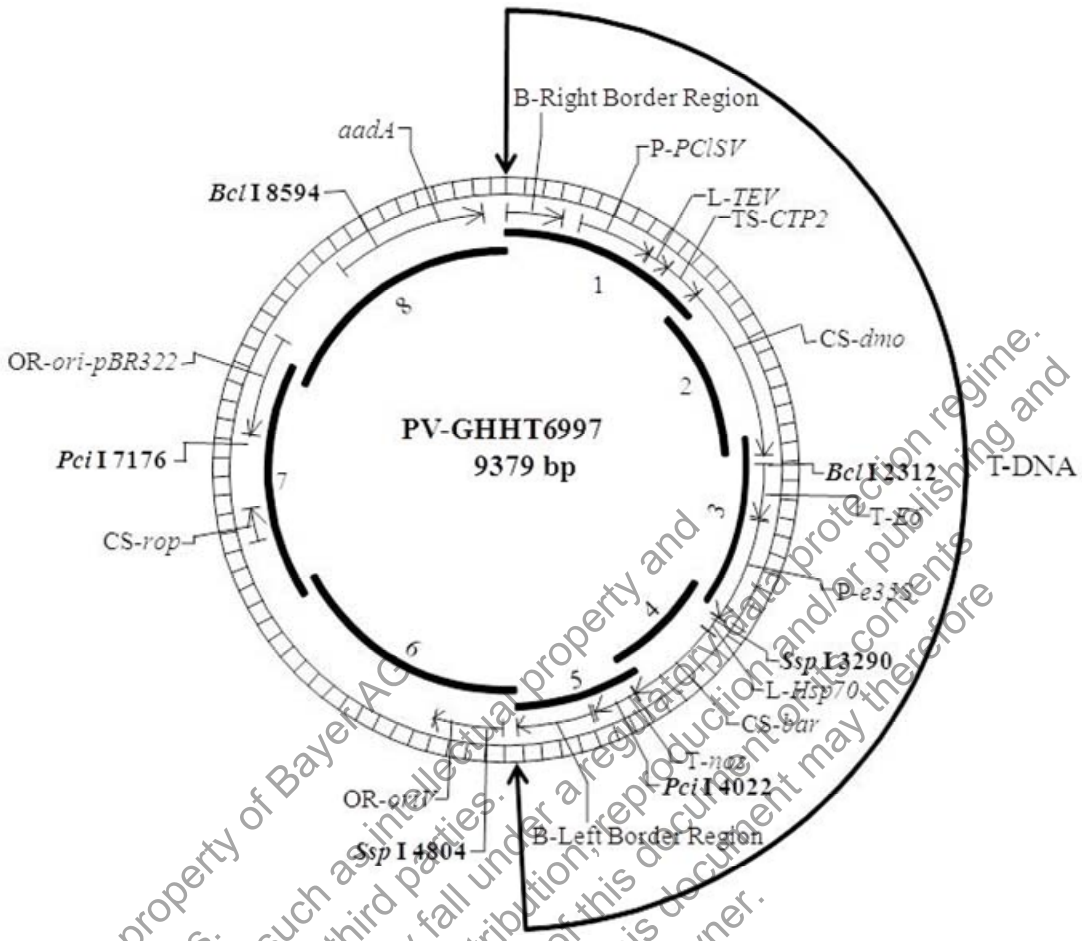
The backbone region of PV–GHHT6997, located outside of the T-DNA, contains two origins of replication for maintenance of plasmid vector in bacteria (*oriV* and *ori-pBR322*), a bacterial selectable marker gene (*aadA*), and a coding sequence for

repressor of primer (*rop*) protein for maintenance of plasmid vector copy number in *Escherichia coli (E. coli)*. A description of the genetic elements and their prefixes (*e.g.*, B-, P-, L-, TS-, CS-, T-, and OR-) in PV–GHHT6997 is provided in Table IV-1.

IV.B. Description of the Transformation System

MON 88701 was developed through *Agrobacterium*-mediated transformation of PV–GHHT6997 (Figure IV-1) into cotton hypocotyls, based on published methods (Duncan, 2010; Duncan and [redacted], 2011). In summary, hypocotyl segments were excised from dark grown seedlings of germinated Coker 130 seed. After co-culturing with the *Agrobacterium* carrying the vector, the hypocotyl segments were placed on a sequence of media for callus growth containing carbenicillin and cefotaxime to inhibit the growth of excess *Agrobacterium* and glufosinate to inhibit growth of untransformed cells. The somatic embryos developing on the culture medium were then placed on medium that contained plant growth regulators conducive to shoot regeneration, but no antibiotics or glufosinate. Rooted plants (R_0) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The R_0 plants generated through the *Agrobacterium*-mediated transformation were self-pollinated to produce R_1 seed. R_0 and R_1 plants were evaluated for tolerance to dicamba and glufosinate and screened for the presence of the T-DNA (*dmo* and *bar* expression cassettes) and absence of plasmid vector backbone (*oriV*). Subsequently, the *dmo* and *bar* homozygous positive R_1 plant was self-pollinated to give rise to R_2 plants. Homozygous positive R_2 plants containing only a single T-DNA insertion, were identified by a combination of analytical techniques including dicamba and glufosinate sprays, polymerase chain reaction (PCR), and Southern blot analysis, resulting in production of dicamba and glufosinate-tolerant cotton MON 88701. MON 88701 was selected as the lead event based on superior phenotypic characteristics and its molecular characteristics. Studies on MON 88701 were initiated to further characterize the genetic insertion and the expressed proteins, and to establish the food, feed, and environmental safety relative to conventional cotton. The major steps involved in the development of MON 88701 are depicted in Figure IV-2.



Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	1	1310	1.3
2	1223	2241	1.0
3	2142	3252	1.1
4	3153	3914	0.8
5	3832	4625	0.8
6	4626	6282	1.7
7	6204	7708	1.5
8	7630	9379	1.8

Figure IV-1. Circular Map of PV–GHHT6997 Showing Probes 1-8
A circular map of PV–GHHT6997 used to develop MON 88701 is shown. PV–GHHT6997 contains a single T-DNA. Genetic elements and restriction sites (in bold) used in Southern analyses (with positions relative to the first base pair of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map and listed in the table.

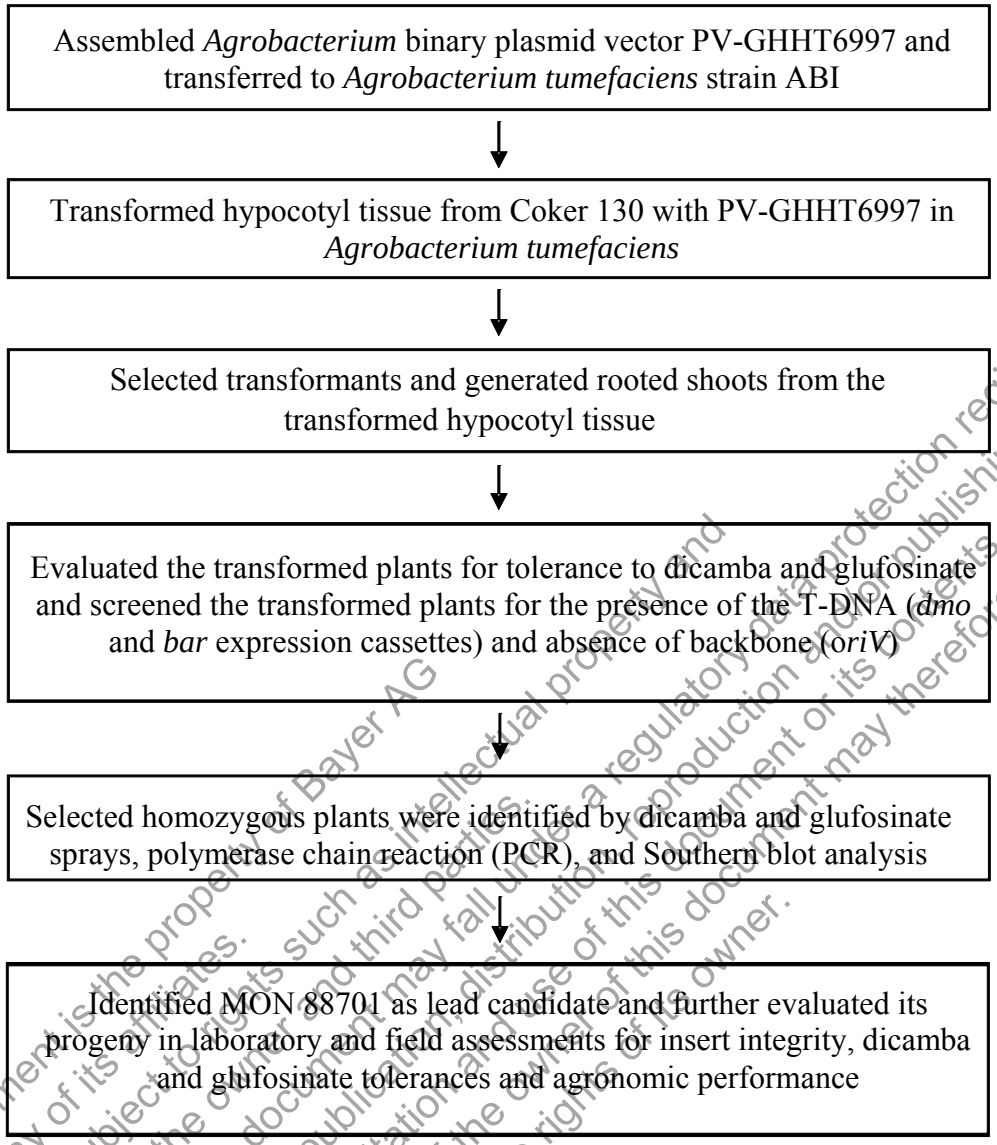


Figure IV-2. Schematic of the Development of MON 88701

IV.C. The *dmo* Coding Sequence and MON 88701 DMO Protein

The *dmo* expression cassette encodes a ~39 kDa MON 88701 DMO precursor protein consisting of a single polypeptide of 416 amino acids (Figure IV-3). The *dmo* coding sequence is the codon optimized coding sequence from *Stenotrophomonas maltophilia* that encodes the DMO protein (Herman et al., 2005; Wang et al., 1997). The presence of MON 88701 DMO protein confers dicamba tolerance.

IV.D. The *bar* Coding Sequence and PAT (*bar*) Protein

The *bar* expression cassette encodes a ~21 kDa PAT (*bar*) protein consisting of a single polypeptide of 183 amino acids (Thompson et al., 1987) (Figure IV-4). The *bar* coding sequence is from *Streptomyces hygroscopicus* and encodes the phosphinothricin N-acetyltransferase (PAT) protein (Thompson et al., 1987). The presence of PAT (*bar*) protein confers glufosinate tolerance.

IV.E. Regulatory Sequences

The *dmo* coding sequence in MON 88701 is under the regulation of the *PC1SV* promoter, the *TEV* 5′ leader, and the *E6* 3′ untranslated region. The *PC1SV* promoter is the promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (Maiti and Shepherd, 1998) that directs transcription in plant cells. The *TEV* leader is the 5′ untranslated region from the tobacco etch virus (Niepel and Gallie, 1999) and is involved in regulating gene expression. The chloroplast transit peptide CTP2 directs transport of the DMO protein to the chloroplast in MON 88701 and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene (Herrmann, 1995; Klee et al., 1987). The *E6 3′* non-translated region is the 3′ untranslated region from the *E6* gene of *Gossypium barbadense* encoding a fiber protein, which functions to direct polyadenylation of the mRNA (John, 1996).

The *bar* coding sequence in MON 88701 is under the regulation of the *e35S* promoter, the *Hsp70* leader, and the *nos 3′* untranslated region. The *e35S* promoter is the promoter for the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells. The *Hsp70* leader is the 5' untranslated region from the *DnaK* gene from *Petunia hybrida* (Rensing and Maier, 1994; Winter et al., 1988) and is involved in regulating gene expression. The *nos 3′* untranslated region is the 3′ untranslated region from the nopaline synthase (*nos*) gene of *Agrobacterium tumefaciens* encoding NOS that directs polyadenylation of the mRNA (Bevan et al., 1983; Fraley et al., 1983).

IV.F. T-DNA Border Regions

PV–GHHT6997 contains Right Border and Left Border regions (Figure IV-1 and Table IV-1) that were derived from *Agrobacterium tumefaciens* plasmids. The border regions each contain a 24-25 bp nick site that is the site of DNA exchange during transformation (Barker et al., 1983; Depicker et al., 1982; Zambryski et al., 1982). The

border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer of T-DNA into the cotton genome.

IV.G. Genetic Elements Outside the T-DNA Border Regions

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV–GHHT6997 in bacteria. The origin of replication, *oriV,* is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host plasmid RK2 (Stalker et al., 1981). The origin of replication, *ori-pBR322,* is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pBR322 (Sutcliffe, 1979). Coding sequence *rop* encodes the repressor of primer (ROP) protein which 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 cotton genome. The absence of detectable backbone sequence in MON 88701 has been confirmed by Southern blot analyses (see Section V.B).

Table IV-1. Summary of Genetic Elements in PV–GHHT6997

Genetic Element	Location in Plasmid Vector	Function (Reference)
T-DNA		
B[1]-Right Border Region	1-331	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening Sequence	332-433	Sequence used in DNA cloning
P[2]-*PC1SV*	434-866	Promoter from the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (*PC1SV*) that directs transcription in plant cells (Maiti and Shepherd, 1998)
Intervening Sequence	867-872	Sequence used in DNA cloning
L[3]-*TEV*	873-1004	5' UTR leader sequence from the RNA of tobacco etch virus (TEV) (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening Sequence	1005-1005	Sequence used in DNA cloning
TS[4]-*CTP2*	1006-1233	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee et al., 1987)
CS[5]-*dmo*	1234-2256	Codon optimized coding sequence for the dicamba mono-oxygenase (DMO) protein of *Stenotrophomonas maltophilia* that confers dicamba tolerance (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	2257-2310	Sequence used in DNA cloning
T[6]-*E6*	2311-2625	3' UTR sequence of the *E6* gene from *Gossypium barbadense* (cotton) encoding a fiber protein involved in early fiber development (John, 1996) that directs polyadenylation of mRNA
Intervening Sequence	2626-2637	Sequence used in DNA cloning
P-*e35S*	2638-3249	Promoter from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells
Intervening Sequence	3250-3252	Sequence used in DNA cloning

Table IV-1 Summary of Genetic Elements in PV–GHHT6997 (continued)

Genetic Element	Location in Plasmid Vector	Function (Reference)
L-*Hsp70*	3253-3348	5' UTR leader sequence of the *DnaK* gene from *Petunia hybrida* that encodes heat shock protein 70 (*HSP70*) (Rensing and Maier, 1994; Winter et al., 1988) that is involved in regulating gene expression
Intervening Sequence	3349-3354	Sequence used in DNA cloning
CS-*bar*	3355-3906	Coding sequence for the phosphinothricin N-acetyltransferase (PAT) protein of *Streptomyces hygroscopicus* that confers glufosinate tolerance (Thompson et al., 1987)
Intervening Sequence	3907-3911	Sequence used in DNA cloning
T-*nos*	3912-4164	3' UTR sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan et al., 1983; Fraley et al., 1983)
Intervening Sequence	4165-4183	Sequence used in DNA cloning
B-Left Border Region	4184-4625	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
		Plasmid Vector Backbone
Intervening Sequence	4626-4711	Sequence used in DNA cloning
OR[7]-*oriV*	4712-5108	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	5109-6616	Sequence used in DNA cloning
CS-*rop*	6617-6808	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	6809-7235	Sequence used in DNA cloning
OR-*ori-pBR322*	7236-7824	Origin of replication from plasmid pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)

Table IV-1 Summary of Genetic Elements in PV–GHHT6997 (continued)

Genetic Element	Location in Plasmid Vector	Function (Reference)
Intervening Sequence	7825-8354	Sequence used in DNA cloning
aadA	8355-9243	Bacterial promoter, coding sequence, and 3' UTR for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase from the transposon *Tn7* (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	9244-9379	Sequence used in DNA cloning

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

```
  1   MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51   LKKSGMTLIG SELRPLKVMS SVSTACMLTF VRNAWYVAAL PEELSEKPLG
101   RTILDTPLAL YRQPDGVVAA LLDICPHRFA PLSDGILVNG HLQCPYHGLE
151   FDGGGQCVHN PHGNGARPAS LNVRSFPVVE RDALIWIWPG DPALADPGAI
201   PDFGCRVDPA YRTVGGYGHV DCNYKLLVDN LMDLGHAQYV HRANAQTDAF
251   DRLEREVIVG DGEIQALMKI PGGTPSVLMA KFLRGANTPV DAWNDIRWNK
301   VSAMLNFIAV APEGTPKEQS IHSRGTHILT PETEASCHYF FGSSRNFGID
351   DPEMDGVLRS WQAQALVKED KVVVEAIERR RAYVEANGIR PAMLSCDEAA
401   VRVSREIEKL EQLEAA
```

Figure IV-3. Deduced Amino Acid Sequence of the MON 88701 DMO Precursor Protein

The amino acid sequence of the MON 88701 DMO precursor protein was deduced from the full-length coding nucleotide sequence present in PV–GHHT6997 (see Table IV-1 for more detail). The chloroplast transit peptide (CTP2) and the first 76 amino acids of the mature protein are underlined. CTP2 targets MON 88701 DMO protein to the chloroplast. The CTP2 is cleaved in the chloroplast producing the mature 349 amino acid MON 88701 DMO protein that begins with the valine at position 68 (See Section VI.C.1). The double underline shows the nine amino acids from CTP2 that are at the N-terminus of the mature MON 88701 protein.

```
  1   MSPERRPADI RRATEADMPA VCTIVNHYIE TSTVNFRTEP QEPQEWTDDL
 51   VRLRERYPWL VAEVDGEVAG IAYAGPWKAR NAYDWTAEST VYVSPRHQRT
101   GLGSTLYTHL LKSLEAQGFK SVVAVIGLPN DPSVRMHEAL GYAPRGMLRA
151   AGFKHGNWHD VGFWQLDFSL PVPPRPVLPV TEI
```

Figure IV-4. Deduced Amino Acid Sequence of the MON 88701-produced PAT (*bar*) Protein

The amino acid sequence of the MON 88701-produced PAT (*bar*) protein was deduced from the full-length coding nucleotide sequence present in PV–GHHT6997 (see Table IV-1 for more detail).

V. CHARACTERIZATION OF THE GENETIC MODIFICATION

This section contains a comprehensive molecular characterization of the genetic modification present in MON 88701. It provides information on the DNA insertion(s) into the plant genome of MON 88701, and additional information relative to the arrangement and stability of the introduced genetic material. The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 30, 31, 32, and 33 (Codex Alimentarius, 2009).

Characterization of the DNA insert in MON 88701 was conducted by Southern blot, PCR and DNA sequence analyses. The results of this characterization demonstrate that MON 88701 contains a single copy of the *dmo* and *bar* expression cassettes, lacks plasmid backbone, the T-DNA is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions were based on several lines of evidence: 1) Southern blot analyses assayed the entire cotton genome for the presence of the T-DNA and absence of the plasmid backbone sequences derived from PV–GHHT6997, and demonstrated that only a single copy of the T-DNA was inserted at a single genomic site and that the insert is stably inherited; 2) DNA sequence analyses to determine the exact sequence of the inserted DNA and the DNA sequences flanking the 5' and 3' ends of the insert, allowing a comparison to the T-DNA sequence in the plasmid vector to confirm that only the expected sequences were integrated; 3) DNA sequences flanking the 5' and 3' ends of the insert were compared to the sequence of the insertion site in conventional cotton to identify any rearrangements that occurred at the insertion site during transformation. Taken together, the characterization of the genetic modification demonstrates that a single copy of the T-DNA was stably integrated at a single locus of the cotton genome and that no plasmid backbone sequences are present in MON 88701.

Southern blot analyses were used to determine the copy number and insertion sites of the integrated DNA as well as the presence or absence of plasmid vector backbone sequences. The Southern blot strategy was designed to ensure that all potential transgenic segments would be identified. The entire cotton genome was assayed with probes that spanned the complete plasmid vector to detect the presence of the insert as well as confirm the absence of any plasmid vector backbone sequences. This was accomplished by using probes that were not more than 2.5 kb in length to ensure a high level of sensitivity. This high level of sensitivity was demonstrated for each blot by detection of a positive control added at 0.1 copies per genome equivalent. Two sets of restriction enzymes were specifically chosen to fully characterize the T-DNA and detect any potential fragments of the T-DNA and backbone sequences. The restriction enzyme sets were chosen such that each enzyme set cleaves once within the inserted T-DNA and at least once within the known DNA flanking the 5' or 3' end of the insert. As a consequence, at least one segment containing a portion of the insert with the adjacent 5' flanking DNA generated by one set of the enzyme(s) is of a predictable size and overlaps with another predictable size segment containing a portion of the insert with the adjacent 3' flanking DNA generated by another set of the enzyme(s). This two-set-enzyme design

ensures that the entire insert is identified in a predictable hybridization pattern. This strategy also maximizes the possibility of detecting an insertion elsewhere in the genome that could be overlooked if that band co-migrated on the gel with an expected band.

To determine the number of copies and insertion sites of the T-DNA, and the presence or absence of the plasmid vector backbone sequences, duplicated samples that consisted of equal amounts of digested DNA were run on the agarose gel. 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 for retaining the small molecular weight DNA on the gel. The molecular weight markers on the left of the figures were used to estimate the sizes of the bands present in the long run lanes of the Southern blots, and the molecular weight markers on the right of the figures were used to estimate the sizes of bands present in the short run lanes of the Southern blots (Figure V-2 through Figure V-5). Southern blot analyses determined that a single copy of the T-DNA was inserted at a single locus of the cotton genome, and no additional genetic elements, including backbone sequences, from PV–GHHT6997 were detected in MON 88701.

The PCR and DNA sequence analyses complement the Southern analyses. PCR and DNA sequence analyses performed on MON 88701 determined the complete DNA sequence of the insert and flanking genomic DNA sequences in MON 88701, confirmed the predicted organization of the genetic elements within the insert, and determined the sequences flanking the insert. In addition, DNA sequence analyses confirmed that each genetic element (except for the border regions) in the insert is intact and the sequence of the insert is identical to the corresponding sequence in PV–GHHT6997 (Figures V-6 and V-7). Furthermore, genomic organization at the MON 88701 insertion site was determined by comparing the sequence flanking the 5' and 3' ends of the insert to the sequence of the insertion site in conventional cotton.

The stability of the T-DNA present in MON 88701 across multiple generations was demonstrated by Southern blot fingerprint analysis (Figure V-9). Genomic DNA from five generations of MON 88701 (Figure V-8) was digested with one of the enzyme sets used for the insert and copy number analyses and was hybridized with two probes that detect restriction segments that encompass the entire insert. This fingerprint strategy consists of two insert segments each containing its adjacent genomic DNA that assesses not only the stability of the insert, but also the stability of the DNA directly adjacent to the insert.

Segregation analysis was conducted to determine the inheritance and stability of the T-DNA insert in MON 88701. Results from this analysis demonstrated the inheritance and stability of the insert was as expected across multiple generations (Figure V-10, Table V-3 and Table V-4), which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA at a single chromosomal locus.

The Southern blot analyses confirmed that the T-DNA reported in Figure V-1 represents the only detectable insert in MON 88701. A circular map of PV–GHHT6997 annotated with the probes used in the Southern blot analysis is presented in Figure IV-1 and the genetic elements within the MON 88701 insert are summarized in Table V-2. A linear map depicting restriction sites within the insert as well as within the DNA immediately flanking the insert in MON 88701 is shown in Figure V-1. Based on the plasmid map and the linear map of the insert, a table summarizing the expected DNA segments for Southern analyses is presented in Table V-1. The results from the Southern blot analyses are presented in Figure V-2 through Figure V-5. PCR amplification of the MON 88701 insert and the insertion site in the conventional control for DNA sequence analysis are shown in Figure V-6 and Figure V-7, respectively. The generations used in the generational stability analysis are depicted in the breeding history shown in Figure V-8 and the results from the generational stability analysis are presented in Figure V-9. The breeding path for generating the segregation data is shown in Figure V-10 and the results for the segregation analysis are presented in Tables V-3 and V-4. Materials and methods used for the characterization of the insert in MON 88701 are found in Appendix A.

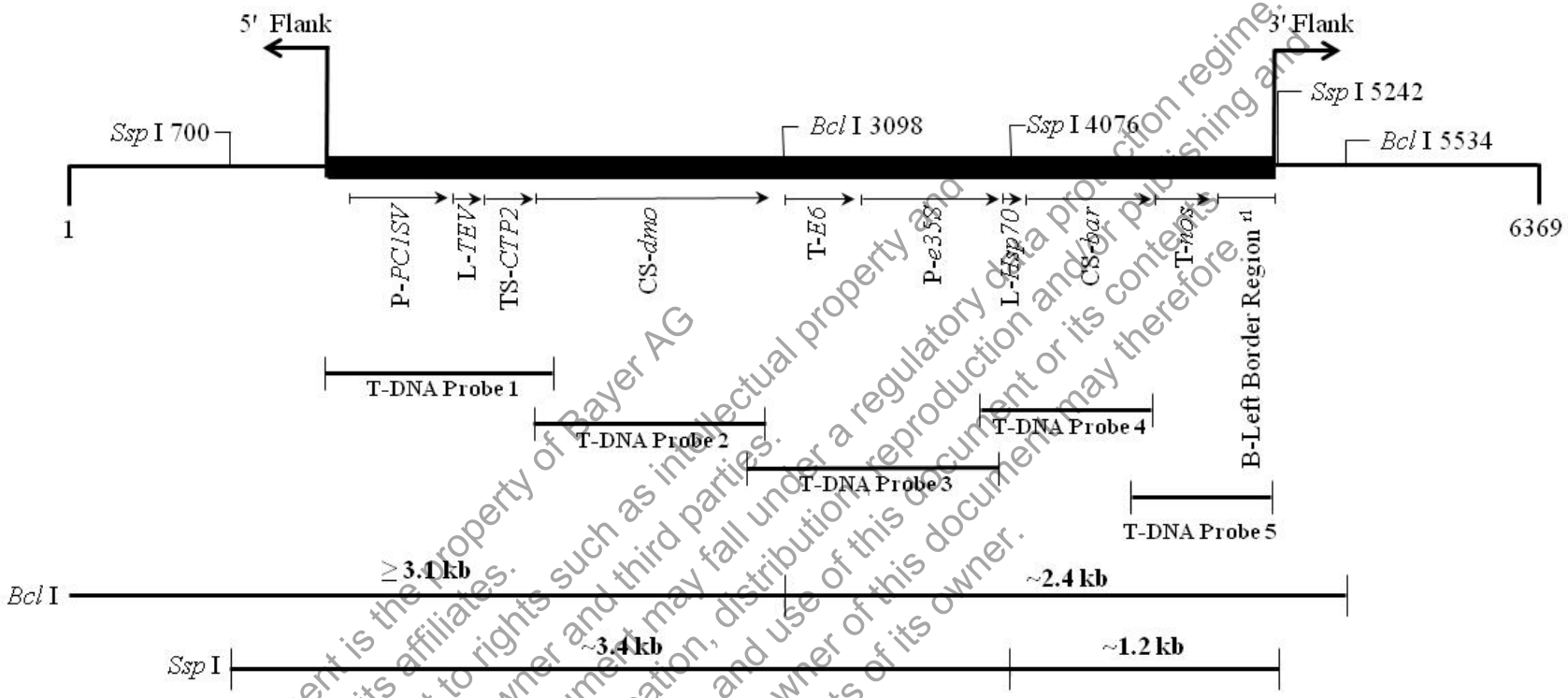


Figure V-1. Schematic Representation of the Insert and Flanking DNA in MON 88701

A linear map of the insert and DNA flanking the insert in MON 88701 is shown. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking DNA. Identified on the linear map are genetic elements within the insert, as well as the sites of the restriction enzymes used in the Southern analyses with positions relative to the first base pair of the DNA sequence represented in this map. The relative sizes and locations of the T-DNA probes and the expected sizes of restriction fragments are indicated in the lower portion of the scheme. This schematic diagram is not drawn to scale. Locations of genetic elements and T-DNA probes are approximate. Probes are also shown in Figure IV-1. [r1]Superscript in Left Border Region indicates that the sequence in MON 88701 was truncated compared to the sequences in PV–GHHT6997.

Table V-1. Summary Chart of the Expected DNA Segments Based on Hybridizing Probes and Restriction Enzymes Used in MON 88701 Analysis

Southern Blot Analysis		T-DNA			Backbone
Figure		**V-2**	**V-3**	**V-4**	**V-5**
Probe(s) Used		1,5	2,4	3	6, 7, 8
Probing Target	**Digestion enzyme**	**Expected Band Sizes on each Southern Blot**			
PV–GHHT6997	*Pci* I	~6.2 kb ~3.2 kb	~6.2 kb	~6.2 kb	~6.2 kb ~3.2 kb
Probe Templates[1]	N/A	~1.3 kb ~0.8 kb	~1.0 kb ~0.8 kb	~~[2]	~1.5 kb ~1.7 kb ~1.8 kb
MON 88701	*Bcl* I	≥3.1 kb ~2.4 kb	≥3.1 kb ~2.4 kb	≥3.1 kb ~2.4 kb	None
	Ssp I	~3.4 kb ~1.2 kb	~3.4 kb ~1.2 kb	~3.4 kb	None

[1]Probe template spikes were used as positive hybridization controls in Southern blot analyses when multiple probes were hybridized to the blot simultaneously.
[2]' ~~' indicates that probe template was not used.

Table V-2. Summary of Genetic Elements in MON 88701

Genetic Element	Location in Sequence	Function (Reference)
5′ Flank	1-1126	Cotton genomic DNA
Intervening Sequence	1127-1219	Sequence used in DNA cloning
P[1]-*PC1SV*	1220-1652	Promoter from the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (*PC1SV*) that directs transcription in plant cells (Maiti and Shepherd, 1998)
Intervening Sequence	1653-1658	Sequence used in DNA cloning
L[2]-*TEV*	1659-1790	5' UTR leader sequence from the RNA of tobacco etch virus (TEV) (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening Sequence	1791-1791	Sequence used in DNA cloning
TS[3]-*CTP2*	1792-2019	Targeting sequence of the *ShkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Herrmann, 1995; Klee et al., 1987)
CS[4]-*dmo*	2020-3042	Codon optimized coding sequence for the dicamba mono-oxygenase (DMO) protein of *Stenotrophomonas maltophilia* that confers dicamba tolerance (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	3043-3096	Sequence used in DNA cloning
T[5]-*E6*	3097-3411	3' UTR sequence of the *E6* gene from *Gossypium barbadense* (cotton) encoding a fiber protein involved in early fiber development (John, 1996) that directs polyadenylation of mRNA
Intervening Sequence	3412-3423	Sequence used in DNA cloning
P-*e35S*	3424-4035	Promoter from the 35S RNA of cauliflower mosaic virus (CaMV) (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells

Table V-2 Summary of Genetic Elements in MON 88701 (continued)

Genetic Element	Location in Sequence	Function (Reference)
Intervening Sequence	4036-4038	Sequence used in DNA cloning
L-*Hsp70*	4039-4134	5' UTR leader sequence of the *DnaK* gene from *Petunia hybrida* that encodes heat shock protein 70 (*HSP70*) (Rensing and Maier, 1994; Winter et al., 1988) that is involved in regulating gene expression
Intervening Sequence	4135-4140	Sequence used in DNA cloning
CS-*bar*	4141-4692	Coding sequence for the phosphinothricin N-acetyltransferase (PAT) protein of *Streptomyces hygroscopicus* that confers glufosinate tolerance (Thompson et al., 1987)
Intervening Sequence	4693-4697	Sequence used in DNA cloning
T-*nos*	4698-4950	3' UTR sequence of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan et al., 1983; Fraley et al., 1983)
Intervening Sequence	4951-4969	Sequence used in DNA cloning
B[6]-Left Border Region [r1]	4970-5231	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
3' Flank	5232-6369	Cotton genomic DNA

[1]P, Promoter
[2]L, Leader
[3]TS, Targeting Sequence
[4]CS, Coding Sequence
[5]T, Transcription Termination Sequence
[6]B, Border
[r1]Superscript in Left Border Region indicates that the sequence in MON 88701 was truncated compared to the sequences in PV–GHHT6997.

V.A. Insert and Copy Number of T-DNA in MON 88701

The numbers of copies and insertion sites of the T-DNA sequences in the cotton genome were evaluated by digesting MON 88701 and conventional control genomic DNA samples with the restriction enzyme *Bcl* I or the restriction enzyme *Ssp* I and hybridizing Southern blots with probes that span the T-DNA (Figure IV-1). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table V-1). Any additional copies and/or integration sites would be detected as additional bands on the blots.

The restriction enzyme *Bcl* I cleaves once within the inserted T-DNA and within the known genomic DNA flanking the 3' end of the insert (Figure V-1). Therefore, if T-DNA sequences were present as a single copy at a single integration site in MON 88701, the digestion with *Bcl* I was expected to generate two border segments with expected sizes of ≥3.1 kb and ~2.4 kb (Figure V-1 and Table V-1). The restriction enzyme *Ssp* I cleaves once within the inserted T-DNA and within the known genomic DNA flanking the 5' and 3' ends of the insert (Figure V-1). If T-DNA sequences were present as a single copy at a single integration site in MON 88701, the digestion with *Ssp* I was expected to generate two border segments with expected sizes of ~3.4 kb and ~1.2 kb (Figure V-1 and Table V-1).

The Southern blots were hybridized with T-DNA probes that collectively span the entire inserted DNA sequence (Figures IV-1 and V-1, Probe 1, Probe 2, Probe 3, Probe 4, and Probe 5). Conventional control genomic DNA digested with the restriction enzyme *Bcl* I and spiked with either probe templates and/or digested PV–GHHT6997 DNA served as positive hybridization controls. The positive hybridization control was spiked at approximately 0.1 and 1.0 copies of genome equivalents to demonstrate sufficient sensitivity of the Southern blot. Conventional control genomic DNA digested with the appropriate restriction enzymes was used as a negative control. The results of these analyses are shown in Figure V-2 through Figure V-4.

V.A.1. T-DNA Probes 1 and 5

Conventional control genomic DNA digested with *Bcl* I (Figure V-2, Lane 1 and Lane 8) or with *Ssp* I (Figure V-2, Lane 3 and Lane 10) and simultaneously hybridized with Probe 1 and Probe 5 (Figures IV-1 and V-1) produced no detectable hybridization bands as expected for the negative control in the reported exposure shown in Figure V-2. In a longer exposure of the blot, faint endogenous hybridization bands were present in both the *Bcl* I digest and the *Ssp* I digest in the conventional control genomic DNA (data not shown). Conventional control genomic DNA digested with *Bcl* I and spiked with probe templates of Probe 1 and Probe 5 (Figure IV-1) produced the expected bands at ~1.3 kb and ~0.8 kb (Figure V-2, Lane 5 and Lane 6). Conventional control genomic DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure IV-1), produced two bands at ~6.2 kb and ~3.2 kb

(Figure V-2, Lane 7), as expected. Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 88701 DNA digested with *Bcl* I and simultaneously hybridized with Probe 1 and Probe 5 (Figures IV-1 and V-1) produced the expected bands at ~3.5 kb and ~2.4 kb (Figure V-2, Lane 2 and Lane 9) which is consistent with the expected ≥3.1 kb and ~2.4 kb bands (Figure V-1 and Table V-1), respectively. MON 88701 DNA digested with the restriction enzyme *Ssp* I and hybridized with Probe 1 and Probe 5 (Figures IV-1 and V-1) produced two bands at ~3.4 kb and ~1.2 kb (Figure V-2, Lane 4 and Lane 11), as expected.

The results presented in Figure V-2 indicate that the sequences covered by Probe 1 and Probe 5 reside at a single detectable locus of integration in MON 88701.

V.A.2. T-DNA Probes 2 and 4

Conventional control genomic DNA digested with *Bcl* I (Figure V-3, Lane 1 and Lane 8) or with *Ssp* I (Figure V-3, Lane 3 and Lane 10) and simultaneously hybridized with Probe 2 and Probe 4 (Figures IV-1 and V-1) produced no detectable hybridization bands as expected for the negative control. Conventional control genomic DNA digested with *Bcl* I and spiked with probe templates of Probe 2 and Probe 4 (Figure IV-1) produced the expected bands at ~1.0 kb and ~0.8 kb (Figure V-3, Lane 5 and Lane 6). Conventional control genomic DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure IV-1), produced one band at ~6.2 kb (Figure V-3, Lane 7), as expected. Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 88701 DNA digested with *Bcl* I and simultaneously hybridized with Probe 2 and Probe 4 (Figures IV-1 and V-1) produced the expected bands at ~3.5 kb and ~2.4 kb (Figure V-3, Lane 2 and Lane 9) which is consistent with the expected ≥3.1 kb and ~2.4 kb bands (Figure V-1 and Table V-1), respectively. MON 88701 DNA digested with the restriction enzyme *Ssp* I and hybridized with Probe 2 and Probe 4 (Figures IV-1 and V-1) produced two bands at ~3.4 kb and ~1.2 kb (Figure V-3, Lane 4 and Lane 11), as expected.

The results presented in Figure V-3 indicate that the sequences covered by Probe 2 and Probe 4 reside at a single detectable locus of integration in MON 88701.

V.A.3. T-DNA Probe 3

Conventional control DNA digested with *Bcl* I (Figure V-4, Lane 1 and Lane 7) or with *Ssp* I (Figure V-4, Lane 3 and Lane 9) and hybridized with Probe 3 (Figures IV-1 and V-1) produced endogenous hybridization signals that were present in all lanes (Figure V-4, Lane 1 through Lane 10). The same hybridization band was produced in conventional control and MON 88701 DNA lanes when digested with the same enzyme.

When digested with *Bcl* I and hybridized with Probe 3 hybridization bands of ~1.9 kb and ~1.7 kb were produced with conventional control genomic DNA and MON 88701 DNA (Figure V-4, Lane 1, Lane 2, and Lanes 5–8). When digested with *Ssp* I and hybridized with Probe 3, a hybridization band of ~2.5 kb was produced with conventional control genomic DNA and MON 88701 DNA (Figure V-4, Lane 3, Lane 4, Lane 9, and Lane 10). Since these bands are present in both control and test substances, these signals are considered to be weak hybridization of probes to endogenous *E6* sequences and are not specific to the inserted DNA in MON 88701.

Conventional control genomic DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure IV-1), produced one band at ~6.2 kb (Figure V-4, Lane 5 and Lane 6), as expected. Detection of the spiked controls indicates that the probe hybridized to its target sequence.

MON 88701 DNA digested with *Bcl* I and hybridized with Probe 3 (Figures IV-1 and V-1) produced two expected bands at ~3.5 kb and ~2.4 kb, which is consistent with the expected ≥3.1 kb and ~2.4 kb bands (Figure V-1 and Table V-1), and is in addition to the endogenous hybridization bands discussed above (Figure V-4, Lane 2 and Lane 8). The ~3.5 kb band is less intense than the ~2.4 kb band. The difference in band intensity is likely due to hybridization of a smaller portion of Probe 3 to the ~3.5 kb fragment. The ~3.5 kb band represents the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert; this correlates with the expected border fragment size of ≥ 3.1 kb. The ~2.4 kb band represents the 3′ end of the inserted DNA and the adjacent DNA flanking the 3′ end of the insert. MON 88701 DNA digested with *Ssp* I (Figure V-4, Lane 4 and Lane 10, Figure V-1, and Table V-1) and hybridized with Probe 3 produced one expected band at ~3.4 kb in addition to the endogenous hybridization bands discussed above. The ~3.4 kb band represents the 5′ end of the inserted DNA and the adjacent DNA flanking the 5′ end of the insert.

The results presented in Figure V-4 indicate that the sequence covered by Probe 3 resides at a single detectable locus of integration in MON 88701.

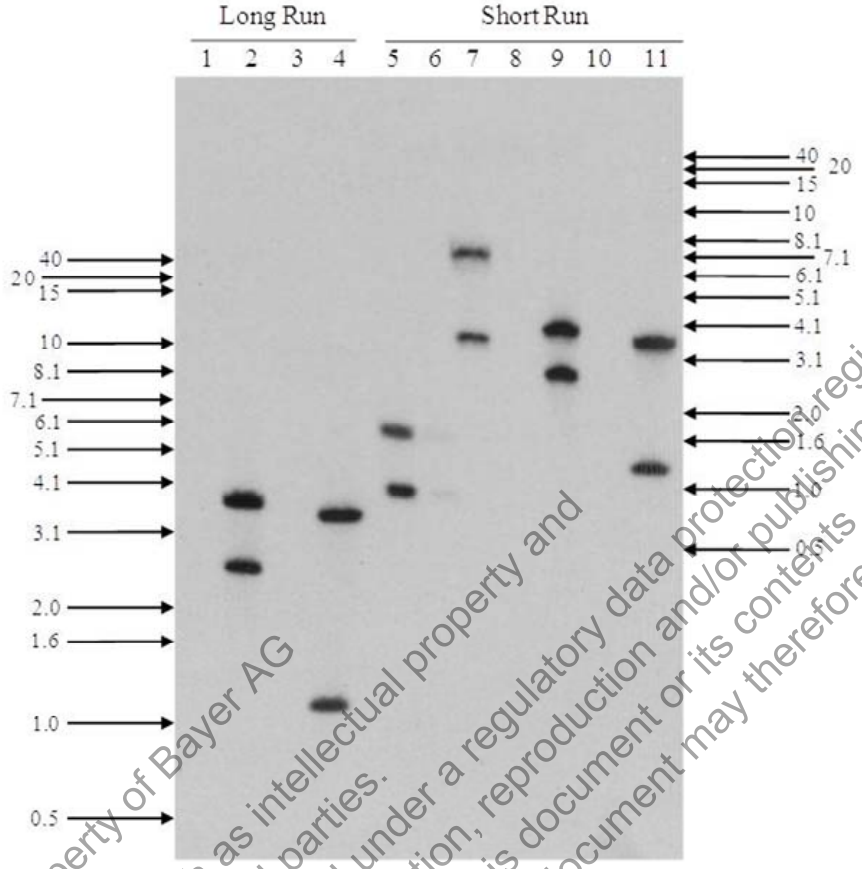


Figure V-2. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88701: Probes 1 and 5

The blot was simultaneously hybridized with two ^{32}P-labeled probes that span a portion of the T-DNA sequence (Figure IV-1, Probe 1 and Probe 5). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional Control (*Bcl* I)
2	MON 88701 (*Bcl* I)
3	Conventional Control (*Ssp* I)
4	MON 88701 (*Ssp* I)
5	Conventional Control (*Bcl* I) spiked with Probe 1 and Probe 5 template [~1.0 genome equivalent]
6	Conventional Control (*Bcl* I) spiked with Probe 1 and Probe 5 template [~0.1 genome equivalent]
7	Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
8	Conventional Control (*Bcl* I)
9	MON 88701 (*Bcl* I)
10	Conventional Control (*Ssp* I)
11	MON 88701 (*Ssp* I)

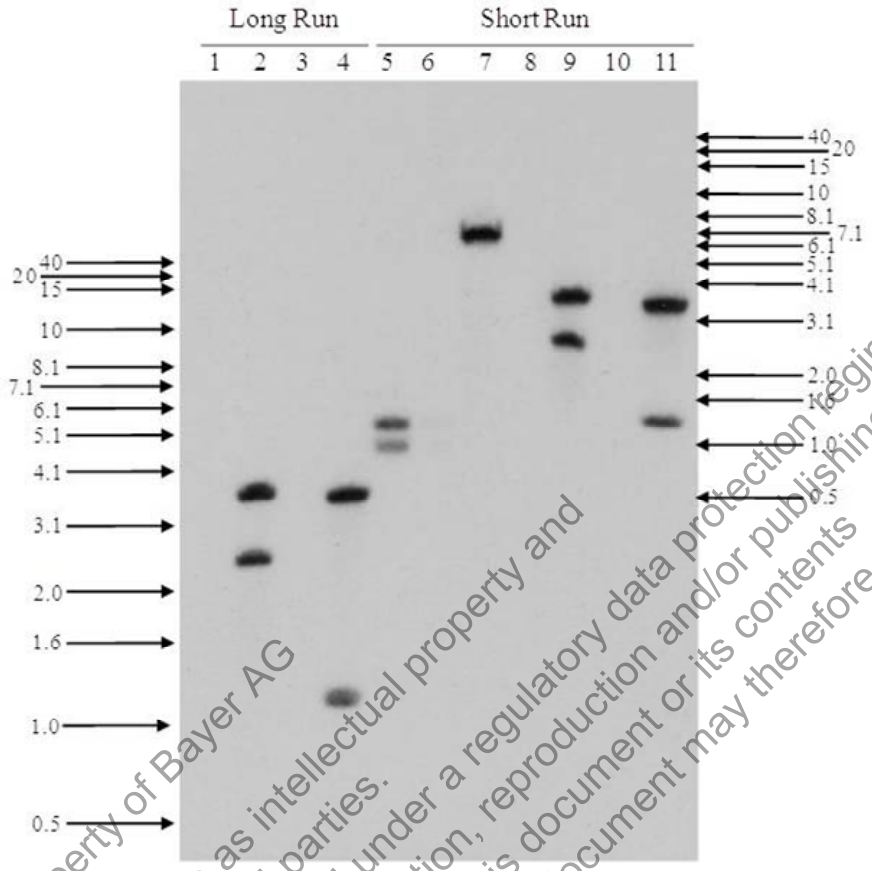


Figure V-3. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88701: Probes 2 and 4

The blot was simultaneously hybridized with two ^{32}P-labeled probes that span a portion of the T-DNA sequence (Figure IV-1, Probe 2 and Probe 4). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane

1 Conventional Control (*Bcl* I)
2 MON 88701 (*Bcl* I)
3 Conventional Control (*Ssp* I)
4 MON 88701 (*Ssp* I)
5 Conventional Control (*Bcl* I) spiked with Probe 2 and Probe 4 template [~1.0 genome equivalent]
6 Conventional Control (*Bcl* I) spiked with Probe 2 and Probe 4 template [~0.1 genome equivalent]
7 Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
8 Conventional Control (*Bcl* I)
9 MON 88701 (*Bcl* I)
10 Conventional Control (*Ssp* I)
11 MON 88701 (*Ssp* I)

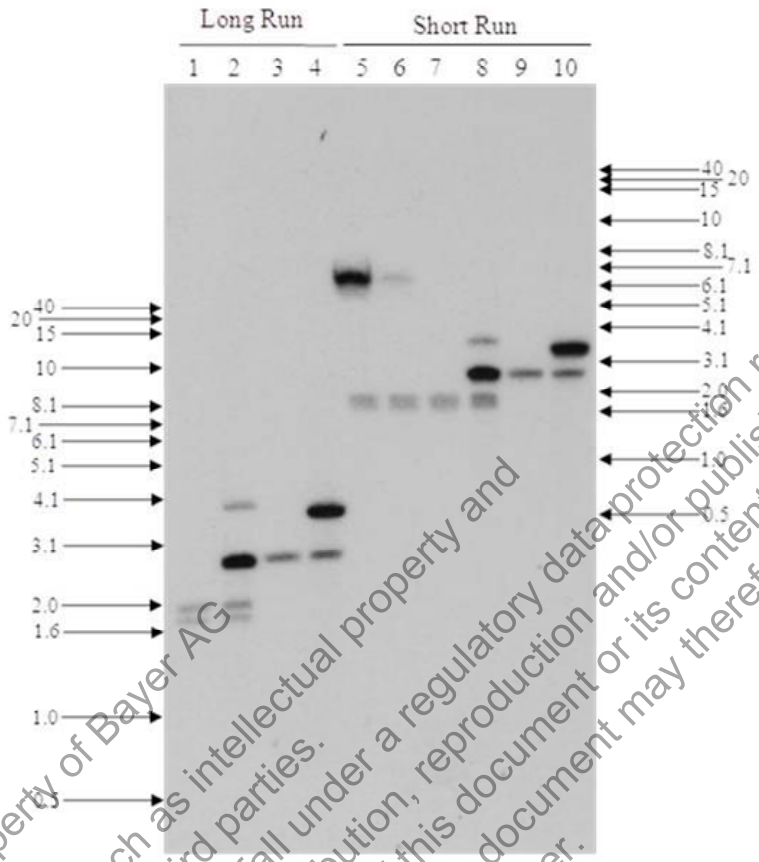


Figure V-4. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88701: Probe 3

The blot was hybridized with a ^{32}P-labeled probe that spans a portion of the T-DNA sequence (Figure IV-1, Probe 3). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional Control (*Bcl* I)
2	MON 88701 (*Bcl* I)
3	Conventional Control (*Ssp* I)
4	MON 88701 (*Ssp* I)
5	Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
6	Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~0.1 genome equivalent]
7	Conventional Control (*Bcl* I)
8	MON 88701 (*Bcl* I)
9	Conventional Control (*Ssp* I)
10	MON 88701 (*Ssp* I)

V.B. Southern Blot Analysis to Determine the Presence or Absence of PV–GHHT6997 Backbone Sequences in MON 88701

To determine the presence or absence of the PV–GHHT6997 backbone sequences, MON 88701 and conventional control genomic DNA were digested with the restriction enzyme *Bcl* I or restriction enzyme *Ssp* I, and hybridized with the three backbone probes that collectively span the entire backbone sequences (Figure IV-1, Probe 6, Probe 7, and Probe 8). If backbone sequences are present in MON 88701, then probing with backbone probes should result in hybridizing bands. Conventional control genomic DNA digested with the restriction enzyme *Bcl* I and spiked with probe templates and with digested PV–GHHT6997 DNA served as positive hybridization controls. The positive hybridization control was spiked at approximately 0.1 and 1.0 copies of genome equivalents to demonstrate sufficient sensitivity of the Southern blot. Conventional control genomic DNA digested with the appropriate restriction enzymes was used as a negative control. The results of these analyses are shown in Figure V-5.

V.B.1. Backbone Probes 6, 7, and 8

Conventional control DNA digested with *Bcl* I (Figure V-5, Lane 1 and Lane 10) or the restriction enzyme *Ssp* I (Figure V-5, Lane 3 and Lane 12) and hybridized with Probe 6, Probe 7, and Probe 8 (Figure IV-1) produced no detectable hybridization bands as expected for the negative control.

Conventional control genomic DNA digested with *Bcl* I and spiked with probe templates of Probe 7 and Probe 8 (Figure IV-1) produced the expected bands at ~1.5 kb and ~1.8 kb (Figure V-5, Lane 5 and Lane 6). Conventional control genomic DNA digested with *Bcl* I and spiked with probe template of Probe 6 (Figure IV-1) produced the one expected band at ~1.7 kb (Figure V-5, Lane 7 and Lane 8). Conventional control DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure IV-1), produced two bands at ~6.2 kb and ~3.2 kb (Figure V-5, Lane 9), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88701 DNA digested with *Bcl* I (Figure V-5, Lane 2 and Lane 11) or the restriction enzyme *Ssp* I (Figure V-5, Lane 4 and Lane 13) and hybridized with Probes 6, 7, and 8 produced no detectable bands.

The results presented in Figure V-5 indicate that MON 88701 contains no detectable backbone sequences covered by Probes 6, 7, and 8.

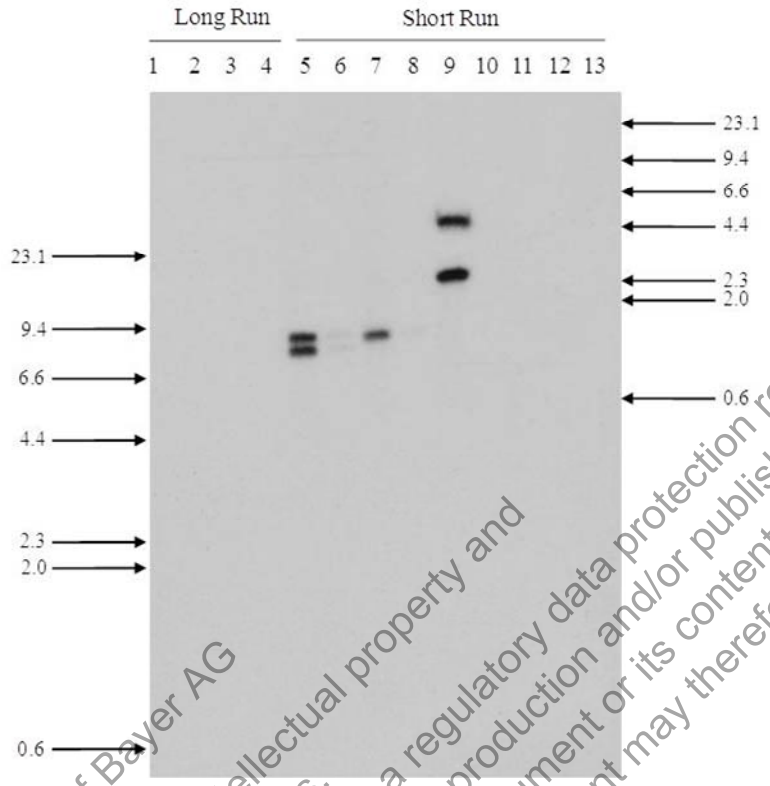


Figure V-5. Southern Blot Analysis to Determine the Presence or Absence of PV–GHHT6997 Backbone Sequences in MON 88701: Probes 6, 7, and 8

The blot was hybridized with three ^{32}P-labeled probes that spans the plasmid vector backbone sequences (Figure IV-1, Probe 6, 7, and 8). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from λ DNA/*Hind* III fragments on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional Control (*Bcl* I)
2	MON 88701 (*Bcl* I)
3	Conventional Control (*Ssp* I)
4	MON 88701 (*Ssp* I)
5	Conventional Control (*Bcl* I) spiked with Probe 7 and Probe 8 template [~1.0 genome equivalent]
6	Conventional Control (*Bcl* I) spiked with Probe 7 and Probe 8 template [~0.1 genome equivalent]
7	Conventional Control (*Bcl* I) spiked with Probe 6 template [~1.0 genome equivalent]
8	Conventional Control (*Bcl* I) spiked with Probe 6 template [~0.1 genome equivalent]
9	Conventional Control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
10	Conventional Control (*Bcl* I)
11	MON 88701 (*Bcl* I)
12	Conventional Control (*Ssp* I)
13	MON 88701 (*Ssp* I)

V.C. Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 88701

The organization and sequence of the elements within the MON 88701 insert was confirmed by DNA sequence analysis. PCR primers were designed with the intent to amplify three overlapping DNA amplicons that span the entire length of the insert and the associated DNA flanking the 5' and 3' ends of the insert (Figure V-6). The amplified PCR products were subjected to DNA sequence analyses. This analysis determined that the DNA sequence of the MON 88701 insert is 4105 bp long (Table V-2) and is identical to the corresponding T-DNA sequence of PV–GHHT6997 as described in Table IV-1.

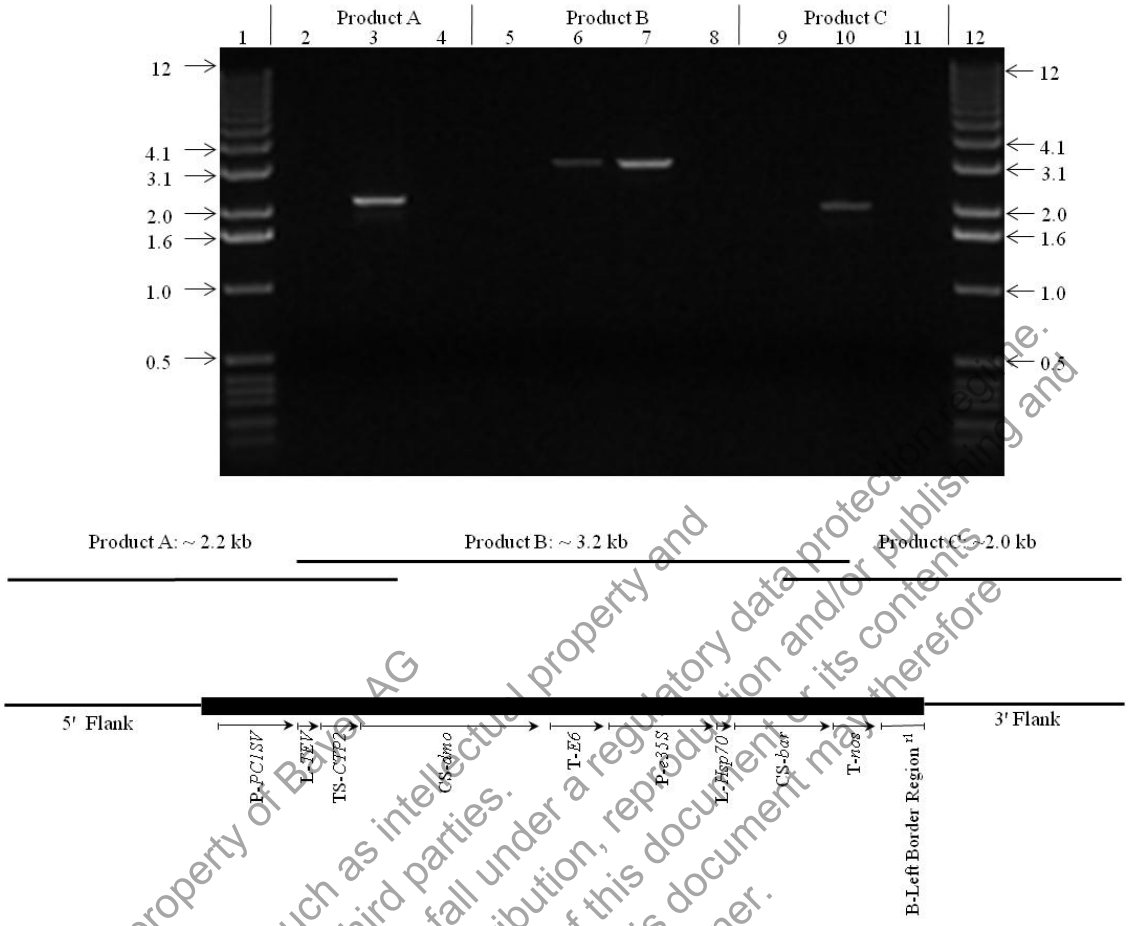


Figure V-6. Overlapping PCR Analysis across the Insert in MON 88701

PCR was performed on both conventional control genomic DNA and MON 88701 genomic DNA using three pairs of primers to generate overlapping PCR fragments from MON 88701 for sequence analysis. Approximately five microliters of each of the PCR reactions was loaded on the gel. The expected product size for each amplicon and an illustration of the insert in MON 88701 is provided at the bottom of the figure. Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	1 Kb DNA Ladder
2	Conventional Control
3	MON 88701
4	No template DNA control
5	Conventional Control
6	MON 88701
7	PV–GHHT6997
8	No template DNA control
9	Conventional Control
10	MON 88701
11	No template DNA control
12	1 Kb DNA Ladder

V.D. PCR and DNA Sequence Analyses to Examine the MON 88701 Insertion Site

PCR and sequence analyses were performed on genomic DNA extracted from MON 88701 and the conventional control to examine the MON 88701 insertion site. The PCR was performed with a forward primer specific to the genomic DNA sequence flanking the 5' end of the insert paired with a reverse primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure V-7). The amplified PCR product from the conventional control was subjected to DNA sequence analysis. Alignments between the conventional control sequence obtained from this analysis and the sequences immediately flanking the 5' and 3' end of the MON 88701 insert were separately performed to determine the integrity and genomic organization of the insertion site in MON 88701. The alignment analyses indicated a 123 base pair deletion from the conventional genomic DNA occurred upon T-DNA insertion in MON 88701. Minor deletions and/or insertions of DNA due to double-strand break repair mechanisms in the plant during *Agrobacterium*-mediated transformation process are not uncommon (Salomon and Puchta, 1998).

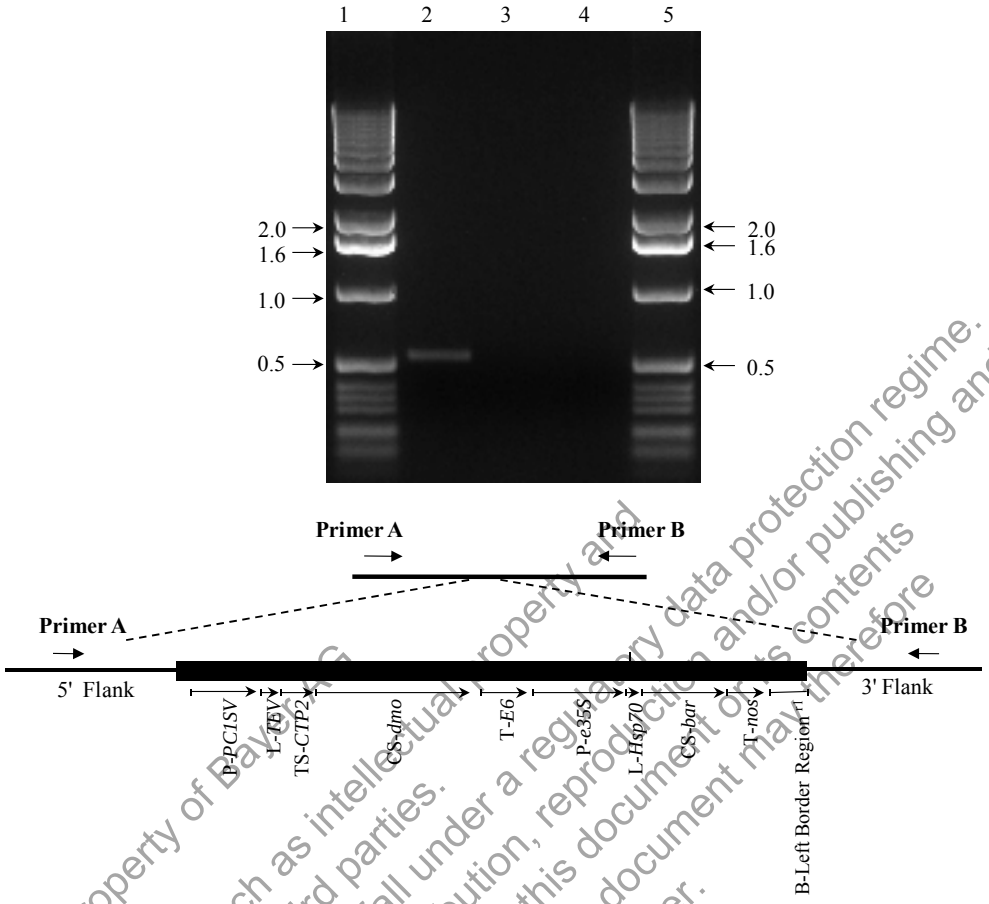


Figure V-7. PCR Amplification of the MON 88701 Insertion Site in Conventional Control

PCR was performed on both conventional control genomic DNA and MON 88701 genomic DNA, using Primer A specific to the 5' flanking sequence and Primer B specific to the 3' flanking sequence of the insert in MON 88701, to generate DNA fragments for sequence analysis. The insertion site in the conventional control (top) and MON 88701 (bottom) are illustrated at the bottom of the figure. Approximately five microliters of each of the PCR reactions were loaded on the gel. Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	1 Kb DNA Ladder
2	Conventional Control
3	MON 88701
4	No template DNA control
5	1 Kb DNA Ladder

V.E. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 88701

In order to demonstrate the stability of the insert in MON 88701, Southern blot analysis was performed using genomic DNA extracted from leaf tissues from five breeding generations of MON 88701. For reference, the breeding history of MON 88701 is presented in Figure V-8. The specific generations tested are indicated in the legend of Figure V-8. The R_3 generation was used for the molecular characterization analyses shown in Figure V-2 through Figure V-5. To analyze insert stability, four samples from four additional generations of MON 88701 were evaluated by Southern blot analysis and compared to the R_3 generation. Genomic DNA, isolated from each of the selected generations of MON 88701, was digested with the restriction enzyme *Bcl* I and simultaneously hybridized with Probe 2 and Probe 4 (Figures IV-1 and V-1), which was designed to detect both fragments generated by the *Bcl* I digest. Any instability associated with the insert would be detected as extra bands within the fingerprint on the Southern blot. The Southern blot has the same controls as described in Section V.A.2.

V.E.1. T-DNA Probes 2 and 4

Conventional control genomic DNA digested with restriction enzyme *Bcl* I and simultaneously hybridized with Probe 2 and Probe 4 (Figures IV-1 and V-1) produced no hybridization signals (Figure V-9, Lane 1) as expected for the negative control. Conventional control genomic DNA digested with *Bcl* I and spiked with the PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I (Figure IV-1 and Table V-1), produced one expected band at ~6.2 kb (Figure V-9, Lane 2). Conventional control genomic DNA digested with *Bcl* I and spiked with probe templates of Probe 2 and Probe 4 produced the expected bands at ~1.0 kb and ~0.8 kb (Figure V-9, Lane 3 and Lane 4). Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 88701 genomic DNA digested with *Bcl* I and hybridized with Probe 2 and Probe 4 (Figures IV-1 and V-1) is expected to produce a Southern fingerprint with two bands at ~3.5 kb and ~2.4 kb (Figure V-1 and Table V-1). Southern fingerprints produced from multiple generations (Figure V-9, Lane 5 and Lanes 7-9) of MON 88701 are consistent with the one produced from the fully characterized generation R_3 (Figure V-3, Lane 2 and Lane 9, and Figure V-9, Lane 6), indicating that MON 88701 contains one copy of the T-DNA insert that is stable across multiple generations.

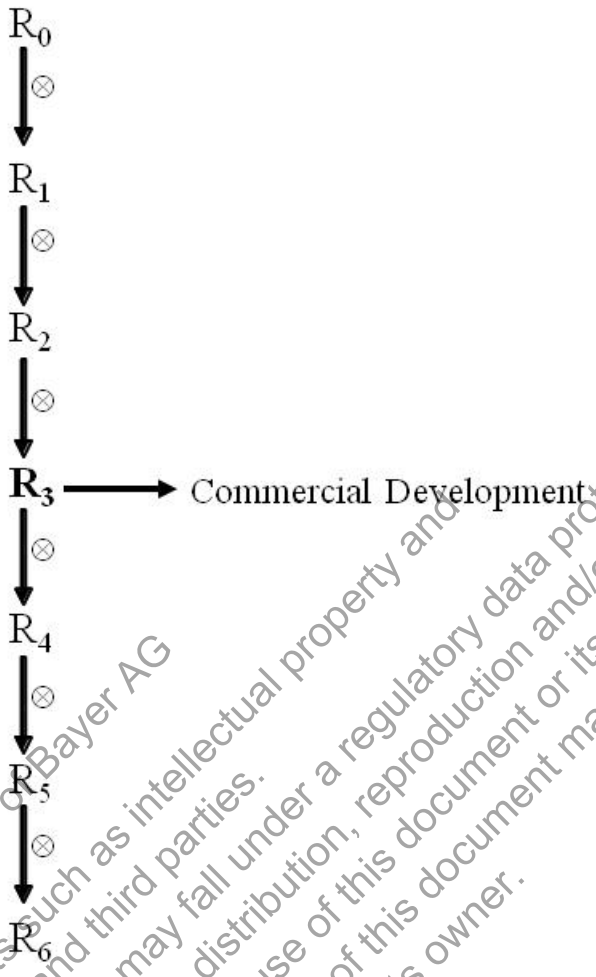


Figure V-8. Breeding History of MON 88701

R_0 corresponds to the original transformed cotton plant. ⊗ designates self-pollination. The R_3 generation was used for the molecular characterization and commercial development of MON 88701. The R_2, R_3, R_4, R_5, and R_6 generations of MON 88701 were used to analyze the stability of the insert across generations.

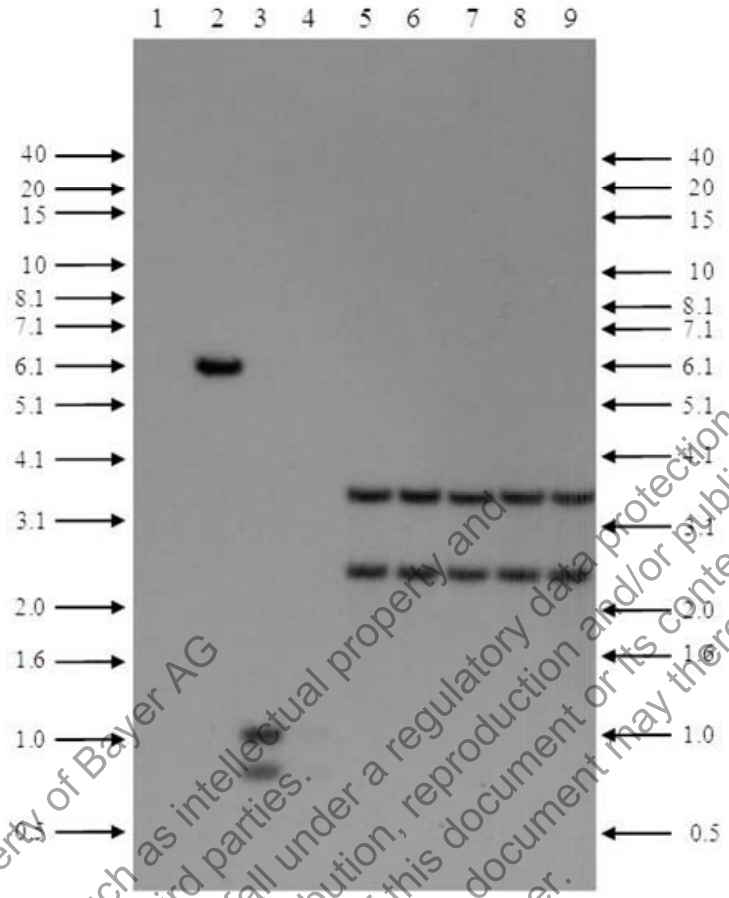


Figure V-9. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 88701: Probes 2 and 4

The blot was simultaneously hybridized with two ^{32}P-labeled probes that span a portion of the T-DNA sequence (Figure IV-1, Probe 2 and Probe 4). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional control (*Bcl* I)
2	Conventional control (*Bcl* I) spiked with PV–GHHT6997 (*Pci* I) [~1.0 genome equivalent]
3	Conventional control (*Bcl* I) spiked with Probe 2 and Probe 4 template [~1.0 genome equivalent]
4	Conventional control (*Bcl* I) spiked with Probe 2 and Probe 4 template [~0.1 genome equivalent]
5	MON 88701 (R_2) (*Bcl* I)
6	MON 88701 (R_3) (*Bcl* I)
7	MON 88701 (R_4) (*Bcl* I)
8	MON 88701 (R_5) (*Bcl* I))
9	MON 88701 (R_6) (*Bcl* I)

V.F. Inheritance of the Genetic Insert in MON 88701

The MON 88701 T-DNA resides at a single locus within the cotton genome and is inherited according to Mendelian principles of inheritance. During development of MON 88701, phenotypic and genotypic segregation data were recorded to assess the inheritance and stability of the MON 88701 T-DNA using Chi-square (χ^2) analysis over several generations. The χ^2 analysis is based on comparing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 88701 breeding path for generating pollinated segregation data is described in Figure V-10. The transformed R_0 plant was self-pollinated to generate R_1 seed. The segregating R_1 generation was assessed using Real-Time TaqMan analysis for the *dmo* coding region. A single homozygous positive R_1 plant was selected and self-pollinated to give rise to R_2 plants that were self-pollinated to produce R_3 seed. Phenotypic and genotypic assays confirmed the lack of segregation in these self-pollinated generations.

Homozygous positive R_3 plants were crossed to a Monsanto proprietary cotton inbred, which does not contain the *dmo* or *bar* coding sequence, via traditional breeding techniques to produce hemizygous F_1 seed. The F_1 plants, hemizygous for the dicamba and glufosinate tolerant trait, were crossed with a Monsanto proprietary cotton inbred, which does not contain the *dmo* or *bar* coding sequence, to produce $BC1F_1$ seed. The $BC1F_1$ generation was assessed using a glufosinate herbicide application to select for plants containing the MON 88701 T-DNA. The plants that survived the herbicide application were confirmed to be hemizygous for the MON 88701 T-DNA by End-Point TaqMan analysis. The hemizygous $BC1F_1$ plants were self-pollinated to produce the $BC1F_2$ plants. For the $BC1F_2$ generation, the plants were assessed using a glufosinate herbicide application and the surviving plants were assessed by End-Point TaqMan analysis for the MON 88701 T-DNA.

The inheritance of the MON 88701 T-DNA was assessed in the R_1, $BC1F_1$, and $BC1F_2$ generations. At the $BC1F_1$ generation, the MON 88701 T-DNA was predicted to segregate at a 1:1 ratio (hemizygous: homozygous negative) according to Mendelian inheritance principles. At the R_1 and $BC1F_2$ generations, the MON 88701 T-DNA was predicted to segregate at a 1:2:1 ratio (homozygous positive: hemizygous: homozygous negative) according to Mendelian inheritance principles.

A Chi-square (χ^2) analysis was used to compare the observed segregation ratios of the MON 88701 T-DNA to the expected ratios. The Chi-square (χ^2) analysis used the statistical program R Version 2.12.0 (2010-10-15).

The Chi-square was calculated as:

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

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

The results of the χ^2 analysis of the MON 88701 segregating progeny are presented in Table V-3 and Table V-4. The χ^2 value in the $BC1F_1$ generation indicated no statistically significant difference between the observed and expected 1:1 segregation ratio (hemizygous: homozygous negative) of the MON 88701 T-DNA. The χ^2 value for the R_1 and $BC1F_2$ generations indicated no statistically significant difference between the observed and expected 1:2:1 segregation ratio (homozygous positive: hemizygous: homozygous negative) of MON 88701 T-DNA. These results support the conclusion that the MON 88701 T-DNA resides at a single locus within the cotton genome and is inherited according to Mendelian principles of inheritance. These results are also consistent with the molecular characterization data indicating that MON 88701 contains a single intact copy of the *dmo* and *bar* expression cassettes inserted at a single locus in the cotton genome.

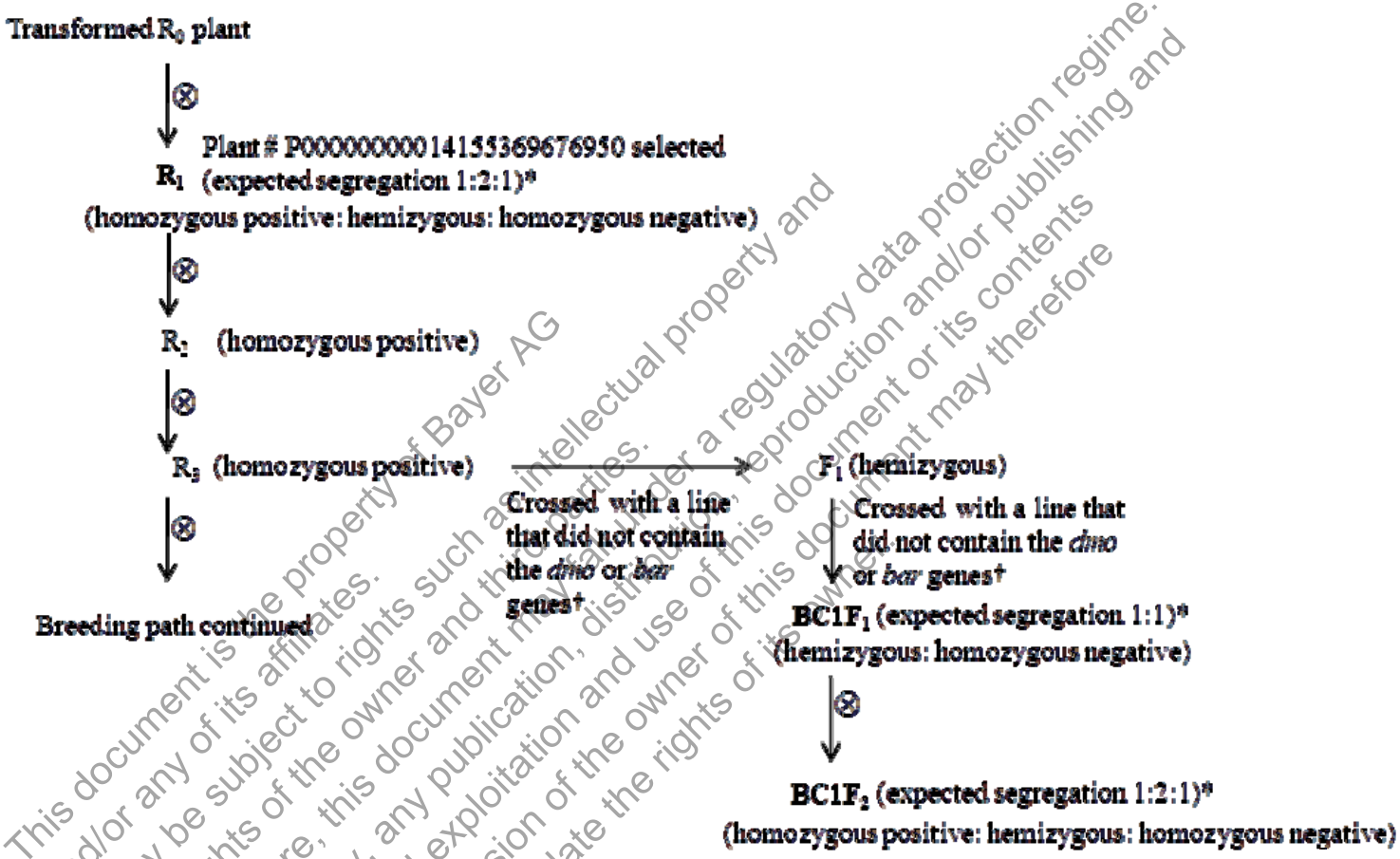


Figure V-10. Breeding Path for Generating Segregation Data for MON 88701

*Chi-square analysis was conducted on segregation data from the R_1, $BC1F_1$, and $BC1F_2$ generations (bolded text).

†The cotton line used in the cross that did not contain the *dmo* or *bar* genes is a Monsanto proprietary cotton inbred.

⊗=Self- Pollinated

Table V-3. Segregation of the T-DNA During the Development of MON 88701: 1:1 Segregation

				1:1 Segregation			
Generation	**Total Plants**	**Observed # Plants Hemizygous**	**Observed # Plants Homozygous Negative**	**Expected # Plants Hemizygous**	**Expected # Plants Homozygous Negative**	**χ^2**	**Probability[2]**
$BC1F_1$[1]	261	123	138	130.5	130.5	0.862	0.3532

[1] Segregation was evaluated using a glufosinate herbicide application followed by End-Point TaqMan analysis for the MON 88701 insert.
[2] Chi-square analysis was performed to analyze the segregation ratios ($p \leq 0.05$).

Table V-4. Segregation of the T-DNA During the Development of MON 88701: 1:2:1 Segregation

					1:2:1 Segregation				
Generation	**Total Plants**	**Observed # Plants Homozygous Positive**	**Observed # Plants Hemizygous**	**Observed # Plants Homozygous Negative**	**Expected # Plants Homozygous Positive**	**Expected # Plants Hemizygous**	**Expected # Plants Homozygous Negative**	**χ^2**	**Probability[3]**
R_1[1]	173	33	99	41	43.25	86.50	43.25	4.353	0.1135
$BC1F_2$[2]	118	36	56	26	29.50	59.00	29.50	2.000	0.3679

[1] Segregation was evaluated using Real-Time TaqMan analysis for the *dmo* coding region.
[2] Segregation was evaluated using a glufosinate herbicide application followed by End-Point TaqMan analysis for the MON 88701 insert.
[3] Chi-square analysis was performed to analyze the segregation ratios ($p \leq 0.05$).

V.G. Characterization of the Genetic Modification Summary and Conclusion

Molecular characterization of MON 88701 by Southern blot analyses confirmed that the T-DNA was inserted into the cotton genome at a single locus containing one copy of the *dmo* and *bar* expression cassettes. No backbone DNA sequences from PV–GHHT6997 were detected in MON 88701.

PCR and DNA sequence analyses performed on MON 88701 and the conventional control determined the following: the complete DNA sequence of the insert and the DNA sequences flanking the 5′ and 3′ ends of the insert in MON 88701; the organization of the genetic elements within the insert; and the 5′ and 3′ insert-to-genomic DNA junctions. The PCR and DNA sequence analysis also determined the DNA sequence at the insertion site in the conventional control and identified a rearrangement (123 base pair deletion) that occurred at the insertion site in MON 88701. Minor deletions and/or insertions of DNA due to double-strand break repair mechanisms in the plant during *Agrobacterium*-mediated transformation process are not uncommon (Salomon and Puchta, 1998).

Southern blot analysis of multiple MON 88701 generations demonstrated that the inserted DNA has been stably maintained through five generations of breeding, thereby, confirming the stability of the insert. Results from segregation analyses show inheritance and stability of the insert was as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behaviour of the T-DNA in MON 88701 at a single chromosomal locus.

VI. SAFETY ASSESSMENT OF EXPRESSED PRODUCTS

The safety of the PAT proteins present in biotechnology-derived crops has been extensively assessed (ILSI-CERA, 2011) and in 1997 a tolerance exemption was issued for PAT proteins by U.S. EPA (U.S. EPA, 1997). Numerous glufosinate-tolerant products including those in cotton, corn, soy, canola, sugarbeet, and rice have been reviewed by the FDA with no concerns identified (U.S. FDA, 1995a; 1995c; 1995b; 1997; 1998b; 1998a; 1999; 2002). Further, a comprehensive study on the safety of PAT proteins present in biotechnology-derived crops (Hérouet et al., 2005) demonstrated the history of safe use, lack of sequence homology to know allergens and toxins, lack of glycosylation sites, rapid degradation in gastric and intestinal fluids, loss of functional activity following heat treatment, and no adverse effects in mice treated with high doses of PAT proteins. Hérouet et al. (2005) concluded that there is a reasonable certainty of no harm resulting from the inclusion of PAT proteins in human food or animal feed. The data below were generated to confirm the previously documented safety assessments.

A multistep approach to the safety assessment was conducted according to guidelines established by the Codex Alimentarius Commission and OECD and which embody the principles and guidance of the FDA's 1992 policy on foods from new plant varieties. This approach was used to characterize the MON 88701 DMO and PAT (*bar*) proteins present in MON 88701 as a result of the genetic modification. These steps include: 1) documentation of the history of safe use of the MON 88701 DMO and PAT (*bar*) proteins and their homology with proteins that lack adverse effects on human or animal health; 2) characterization of the physicochemical and functional properties of MON 88701 DMO and PAT (*bar*) proteins; 3) quantification of MON 88701 DMO and PAT (*bar*) expression in plant tissues; 4) examination of the similarity of MON 88701 DMO and PAT (*bar*) to known allergens; 5) evaluation of the digestibility of MON 88701 DMO and PAT (*bar*) in simulated gastrointestinal fluids; 6) evaluation of the stability of the MON 88701 DMO and PAT (*bar*) proteins in response to typical food/feed preparation conditions such as heat treatment; 7) examination of the similarity of MON 88701 DMO and PAT (*bar*) proteins to known toxins or other biologically active proteins known to have adverse effects on mammals; 8) investigation of potential mammalian toxicity through an animal assay and calculating margins of exposure (MOE); and 9) examination of the similarity of putative polypeptides encoded by the insert and flanking sequences to known allergens, toxins, or other biologically active proteins known to have adverse effects on mammals. Additionally, this section includes a stepwise approach to assess the potential allergenicity for the newly expressed proteins (Codex Alimentarius, 2009). The safety assessment supports the conclusion that dietary exposure to MON 88701 DMO and PAT (*bar*) proteins derived from MON 88701 poses no meaningful risk to human or animal health.

The purified MON 88701 DMO and PAT (*bar*) proteins produced in MON 88701 were characterized to demonstrate the equivalence between MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins, and between MON 88701- and *E. coli*-produced PAT (*bar*) proteins. The purified MON 88701 DMO and

E. coli-produced MON 88701 DMO proteins were shown to be biochemically, structurally, and functionally equivalent. Similarly, the purified MON 88701- and *E. coli*-produced PAT (*bar*) proteins were shown to be biochemically, structurally, and functionally equivalent. Demonstration of protein equivalence between MON 88701- and *E. coli*-produced MON 88701 DMO and PAT (*bar*) proteins, respectively, allows utilization of the *E. coli*-produced MON 88701 DMO and PAT (*bar*) proteins in the safety assessment of the respective MON 88701 DMO and PAT (*bar*) proteins produced in MON 88701.

The information provided in this section also addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 34, 35, 36, 37, 38, 39, and 40 for assessment of possible toxicity and paragraphs 41, 42, and 43 and Annex 1 for assessment of possible allergenicity (Codex Alimentarius, 2009).

VI.A. Description, Mode-of-Action, and Specificity of MON 88701 DMO and PAT (*bar*) Proteins

VI.A.1. Description of MON 88701 DMO Protein

In MON 88701, the introduced DMO protein is active in the chloroplast, a plastid organelle, where it can interact with other proteins needed for its function (Section VI.A.2.) (Behrens et al., 2007). In the construction of the PV–GHHT6997 plasmid vector used in the development of MON 88701 a transit peptide coding sequence (*CTP2,* Table V-2) was joined to the *dmo* coding sequence. This coding sequence results in the production of a precursor protein consisting of the DMO protein and an additional 76 amino acids at the N-terminus of the protein. These additional amino acids correspond to the chloroplast transit peptide (CTP) from *Arabidopsis thaliana* EPSPS (CTP2), which is incorporated to improve the targeting of the precursor protein to the chloroplast (Herrmann, 1995; Klee et al., 1987). Typically, transit peptides are precisely removed from the precursor protein following delivery to the targeted plastid (Della-Cioppa et al., 1986) resulting in the full-length protein. However, there are examples in the literature of alternatively processed forms of a protein targeted to a plant's chloroplast, where part of the transit peptide remains (Behrens et al., 2007; Clark and Lamppa, 1992). Such alternative processing is observed with the MON 88701 DMO protein produced in MON 88701.

Analysis of cottonseed extracts from MON 88701 determined that the expressed protein had an apparent molecular weight of 39.5 kDa and corresponded to the DMO protein with nine amino acids on the N-terminus originating from the EPSPS chloroplast transit peptide. The resulting 349 amino acid polypeptide is referred to as MON 88701 DMO. Alternative processing of DMO precursor proteins has been observed in other dicamba-tolerant plants containing the *dmo* gene (Behrens et al., 2007).

Except for the nine amino acids derived from the CTP2 and an additional leucine at position two, the MON 88701 DMO protein has an identical sequence to the wild-type DMO protein from the DI-6 strain of *S. maltophilia* (Herman et al., 2005) (See Appendix

B.1., Figure B-1). The differences in the amino acid sequence between the wild-type DMO protein and MON 88701 DMO protein are not expected to have an effect on structure, activity, or specificity because the N-terminus and position two are sterically distant from the catalytic site (D'Ordine et al., 2009; Dumitru et al., 2009). The DMO protein produced in MON 88701 is hereinafter referred to as MON 88701 DMO protein. Accordingly, the DMO protein produced from *E. coli* with the same sequence as MON 88701 DMO is referred to as *E. coli*-produced MON 88701 DMO protein.

MON 88701 DMO was purified from cottonseed of MON 88701 and its activity was confirmed during characterization (Section VI.C).

VI.A.2. MON 88701 DMO Mode-of-Action

DMO is an enzyme classified as a mono-oxygenase. Mono-oxygenases are enzymes that incorporate a single oxygen atom as a hydroxyl group with the concomitant production of water and oxidation of nicotinamide adenine dinucleotide (NADH) (Harayama et al., 1992) and are found in diverse phyla ranging from bacteria to plants (Ferraro et al., 2005; Schmidt and Shaw, 2001). The active form of DMO, necessary to confer dicamba tolerance, is a trimer comprised of three DMO monomers (Chakraborty et al., 2005; D'Ordine et al., 2009; Herman et al., 2005). The formation of a trimer is required because the electron transport that culminates in the demethylation of dicamba occurs from one monomer to another in the native conformation of the enzyme (D'Ordine et al., 2009).

Wild-type DMO was initially purified from the *S. maltophilia* strain DI-6 that was isolated from soil at a dicamba manufacturing plant (Krueger et al., 1989). DMO is an enzyme that catalyzes the demethylation of dicamba to the non-herbicidal compound DCSA and formaldehyde (Chakraborty et al., 2005). DCSA is a known cotton, soy, livestock, and soil metabolite of dicamba whose safety has been evaluated and deemed safe (reasonable certainty of no harm as defined by FFDCA) by the EPA (U.S. EPA, 2009). Formaldehyde is routinely produced in plants and is present at levels up to several hundred ppm across those different plants (Adrian-Romero et al., 1999). Thus, neither DCSA nor formaldehyde generated by the action of DMO on dicamba pose a significant food or feed safety risk.

DMO is a Rieske-type non-heme iron oxygenase, that forms part of a three component system comprised of a reductase, a ferredoxin, and a terminal oxygenase, in this case a DMO. These three proteins work together in a redox system similar to many other oxygenases to transport electrons from NADH to oxygen and catalyze the demethylation of an electron acceptor substrate, in this case dicamba (Behrens et al., 2007). This three component redox system is presented in Figure VI-1.

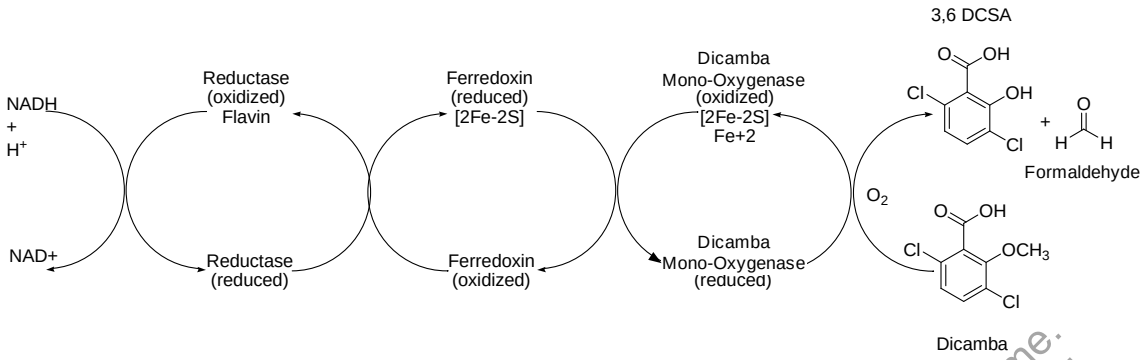


Figure VI-1. Three Components of the DMO Redox System
Depicted is the electron transport chain that starts with NADH and ends with DMO resulting in the demethylation of dicamba to form DCSA.

The crystal structure of DMO has been solved using a C-terminal histidine tagged DMO (D'Ordine et al., 2009; Dumitru et al., 2009), which is identical to wild-type DMO, except for containing an additional alanine at position two, which was added for cloning purposes, and a histidine tag at the C terminus. The addition of a polyhistidine tag fused to the N or C terminus of a protein of interest is a common tool used to aid in protein purification (Hochuli et al., 1988). The crystal structure of DMO was determined to be a trimer comprised of three identical DMO monomers (D'Ordine et al., 2009; Dumitru et al., 2009). Each monomer contains a Rieske [2Fe-2S] cluster domain and a non-heme iron center domain (D'Ordine et al., 2009; Dumitru et al., 2009) that are typical of all Rieske-type mono-oxygenases and are the key domains involved in electron transport (Ferraro et al., 2005). The catalytic site in each monomer was characterized to determine the fit of dicamba in the site and hypothesize the reaction mechanism of dicamba demethylation (D'Ordine et al., 2009; Dumitru et al., 2009).

The trimeric quaternary structure of DMO was the native form of the enzyme observed during crystallization and is required for electron transport and catalysis (D'Ordine et al., 2009; Dumitru et al., 2009). To catalyze the demethylation of dicamba, electrons transferred from NADH are shuttled through an endogenous reductase and ferredoxin to the terminal DMO (Figure VI-1). The electrons are received from ferredoxin by the Rieske [2Fe-2S] cluster on one of the DMO monomers of the trimer and transferred to the non-heme iron center at the catalytic site of an adjacent monomer (D'Ordine et al., 2009; Dumitru et al., 2009), where it reductively activates oxygen to catalyze the final demethylation of dicamba. For this electron transfer to occur between adjacent monomers of DMO, a trimeric structure is required with precise spacing and orientation between the three monomers (D'Ordine et al., 2009). Electron transport from the Rieske [2Fe-2S] cluster domain to the non-heme iron center domain cannot occur within a monomer since the distance it too vast (D'Ordine et al., 2009; Dumitru et al., 2009).

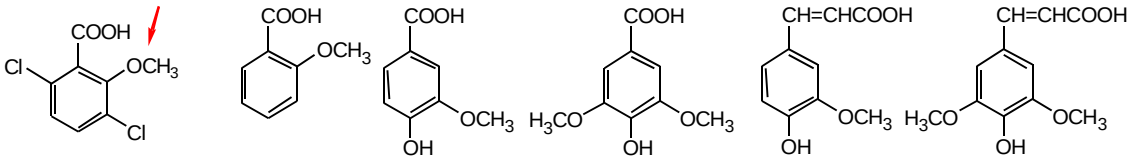
Therefore, in order for MON 88701 to be tolerant to dicamba, a functional trimeric MON 88701 DMO must be formed. The active trimeric form of MON 88701 DMO, as

purified from MON 88701, confers dicamba tolerance to MON 88701, and its demethylase activity on dicamba was confirmed during characterization (Section VI.C. and Appendix B) supporting the conclusion that the trimer required for functional activity was formed in MON 88701.

VI.A.3. MON 88701 DMO Specificity

The substrate specificity of MON 88701 DMO was evaluated to understand potential interactions DMO may have with potential substrates present in MON 88701 cotton. The literature indicates the specificity of DMO for dicamba is due to the specific interactions that occur at the catalytic site (D'Ordine et al., 2009; Dumitru et al., 2009). Dicamba interacts with amino acids in the catalytic site of DMO through both the carboxylate moiety and the chlorine atoms of dicamba, which are primarily involved in orienting the substrate in the catalytic site. These chlorine atoms are required for catalysis (D'Ordine et al., 2009; Dumitru et al., 2009). Given the limited existence of chlorinated compounds with structures similar to dicamba in plants and other eukaryotes (Wishart, 2010; Wishart et al., 2009), it is unlikely that MON 88701 DMO will catalyze the conversion of other endogenous substrates.

The potential for MON 88701 DMO to metabolize endogenous plant substrates was evaluated in *in vitro* experiments using a purified N-terminal histidine tagged DMO that was identical to wild-type DMO, except for a histidine tag at the N-terminus added to aid in protein purification. A comparison of DMO versions is shown in Appendix B, Figure B-1. A set of potential endogenous substrates was selected for evaluation based on structural similarity of the compounds to dicamba and their presence in cotton, corn and soybean (Buchanan et al., 2000; Janas et al., 2000; Lege et al., 1995; Schmelz et al., 2003). The potential substrates tested were *o*-anisic acid (2-methoxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid), ferulic acid [3-(4-hydroxy-3-methoxy-phenyl) prop-2-enoic acid] and sinapic acid [3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid] (Figure VI-2). The assay mixture included NADH, reductase, ferredoxin and DMO. Dicamba was first used as a positive control to demonstrate that the assay system was functional. The disappearance of potential substrates and the formation of potential oxidation products were monitored using LC-UV and LC-MS. None of the tested substrates, except dicamba, were metabolized by the histidine tagged DMO in these *in vitro* experiments. To assess whether MON 88701 DMO protein has the same specificity as the histidine tagged DMO used in the *in vitro* experiments, the *E. coli*-produced MON 88701 DMO protein, shown to be equivalent to the plant produced MON 88701 DMO protein (Section V1.C.), was incubated with *o*-anisic acid, the endogenous compound that has the greatest structural similarity to dicamba. Again dicamba was used as a positive control to demonstrate the assay system was functional. This analysis demonstrated that *o*-anisic acid was not metabolized by the *E. coli*-produced MON 88701 DMO protein, but dicamba was. These results indicate that DMO, including the MON 88701 DMO protein, is specific for dicamba as a substrate.



Dicamba o-Anisic Acid Vanillic Acid Syringic Acid Ferulic Acid Sinapic Acid

Figure VI-2. Dicamba and Potential Endogenous Substrates Tested in *In Vitro* Experiments with DMO
The arrow indicates methyl group removed by DMO.

VI.A.4. Description of PAT (*bar*) Protein

Phosphinothricin N-acetyltransferase (PAT) proteins conferring tolerance to glufosinate herbicide (2-amino-4-(hydroxymethylphosphinyl) butanoic acid) have been isolated from two separate species of *Streptomyces*, *S. hygroscopicus* (Thompson et al., 1987) and *S. viridochromogenes* (Wohllenben et al., 1988). The PAT protein isolated from *S. hygroscopicus* is encoded by the *bar* gene, and the PAT protein isolated from *S. viridochromogenes* is encoded by the *pat* gene. These PAT proteins are made up of 183 amino acids with 85% identity at the amino acid level. Based on previous studies (Wehrmann et al., 1996) that have extensively characterized PAT proteins produced from *bar* and *pat* genes, OECD recognizes both proteins to be equivalent with regard to function and safety (OECD, 1999). In addition, EPA has issued a tolerance exemption for PAT protein regardless of the encoding gene (U.S. EPA, 1997). The safety of PAT proteins present in biotechnology-derived crops has been extensively assessed (Hérouet et al., 2005; ILSI-CERA, 2011).

The PAT protein produced in MON 88701 is from the *bar* gene, and for clarity, the PAT protein produced in MON 88701 will be referred to as PAT (*bar*). Analysis of cottonseed extracts from MON 88701 determined that the expressed protein corresponded to the 183 amino acid polypeptide, resulting in a 24.1 kDa PAT (*bar*) protein. The activity of the PAT (*bar*) protein purified from MON 88701 cottonseed was confirmed during characterization (Section VI.C).

VI.A.5. PAT (*bar*) Mode-of-Action

The mode-of-action for PAT protein has been extensively assessed, as numerous glufosinate-tolerant products including those in cotton, corn, soy, canola, sugarbeet and rice have been reviewed by the FDA (U.S. FDA, 1995a; 1995c; 1995b; 1997; 1998b; 1998a; 1999; 2002) and several other regulatory agencies (ILSI-CERA, 2011; OECD, 1999; 2002a). PAT, including the PAT (*bar*) protein produced in MON 88701, is an enzyme classified as an acetyltransferase which acetylates glufosinate to produce non-herbicidal N-acetyl glufosinate. Glufosinate is a racemic mixture of the D- and L- forms of phosphinothricin. The herbicidal activity of glufosinate results from the binding of L-phosphinothricin to glutamine synthetase (OECD, 1999; 2002a). Glutamine synthetase is

lookresponsible for the assimilation of ammonia generated during photorespiration. The binding of L-phosphinothricin to glutamine synthetase results in the inactivation of glutamine synthetase and a subsequent toxic build-up of ammonia within the plant, resulting in death of the plant (Manderscheid and Wild, 1986; OECD, 1999; 2002a; Wild and Manderscheid, 1984).

The PAT (*bar*) protein produced in MON 88701 acetylates the free amine group of L-phosphinothricin form of glufosinate to produce non-herbicidal N-acetyl glufosinate. The acetylated glufosinate is unable to bind to glutamine synthetase and therefore does not disrupt photorespiration and avoids the build-up of ammonia. MON 88701 confers glufosinate herbicide via the detoxification of phosphinothricin acetyltransferase, as described above (Appendix V1.C.3.6.).

VI.A.6. PAT (*bar*) Specificity

The PAT proteins, including PAT (*bar*), are highly specific for glufosinate in the presence of acetyl-CoA (Thompson et al., 1987; Wehrmann et al., 1996). While the herbicidal activity of glufosinate comes from the L-amino acid form, other L-amino acids are unable to be acetylated by PAT protein, and competition assays containing glufosinate, high concentrations of other amino acids and PAT showed no inhibition of glufosinate acetylation (Wehrmann et al., 1996). Furthermore, L-glutamate, an analogue of glufosinate, also showed no inhibition of glufosinate acetylation in competition assays (Wehrmann et al., 1996). In addition, the PAT (*bar*) protein has more than 30-fold higher affinity towards L-phosphinothricin over other plant analogues (Thompson et al., 1987). Thus, the PAT (*bar*) protein has high substrate specificity for L-phosphinothricin, the herbicidal component of glufosinate, and it is unlikely to affect the metabolic system of MON 88701 cotton. Numerous glufosinate-tolerant products including those in cotton, corn, soy, canola, sugarbeet and rice have been reviewed with no concerns identified (ILSI-CERA, 2011).

VI.B. History of Safe Use of MON 88701 DMO and PAT (*bar*) Proteins

VI.B.1 History of Safe Use of MON 88701 DMO Protein

As described below, MON 88701 DMO is homologous to proteins that are common in the environment and in the diets of animals and humans. Given the extensive exposure of humans and animals to these homologous oxygenase proteins, it can be concluded that the oxygenase proteins have a history of safe use. When determining the homology among proteins both the linear amino acid sequence of the protein, as well as the higher order structure of the proteins should be taken into account. Higher order structures are a relevant measure of homology since structure is more conserved than amino acid sequence. Changes in amino acid sequence are, evolutionarily, mostly conservative, meaning that the changes do not affect the structure which also determines function (Caetano-Anollés et al., 2009; Illergård et al., 2009). This conservation of structure is predominant within important functional and structural domains of proteins in similar classes (Illergård et al., 2009). Therefore, it is necessary to understand the different

levels of protein structure to properly assess homology and determine if homologues of MON 88701 DMO are widely distributed in nature or are present in sources that have been consumed by humans and animals.

As noted earlier, DMO is classified as an oxygenase. Oxygenases are enzymes that incorporate one or two oxygen atoms into substrates, and are widely distributed in many universal metabolic pathways (Harayama et al., 1992). Within this large enzymatic class are mono-oxygenases that incorporate a single oxygen atom as a hydroxyl group with the concomitant production of water and oxidation of NADH (Harayama et al., 1992). Non-heme iron oxygenases, where iron is involved in the catalytic site, are an important class of oxygenases. Within this class are Rieske non-heme iron oxygenases, which contain a Rieske iron-sulfur [2Fe-2S] cluster. All Rieske non-heme iron oxygenases contain two catalytic domains, a non-heme iron domain (nh-Fe) that is a site of oxygen activation, and a Rieske [2Fe-2S] domain which functions by transporting electrons from ferredoxin to the non-heme iron domain (Ferraro et al., 2005). MON 88701 DMO belongs to this class of oxygenases which are found in diverse phyla ranging from bacteria to plants consumed by humans and animals (Ferraro et al., 2005; Schmidt and Shaw, 2001).

The crystallography results demonstrated that the quaternary structure of DMO is a trimer, where each individual monomer is in a precise orientation that allows for electron transport between two conserved domains; the Rieske and the non-heme iron domain. Similar to all Rieske non-heme iron oxygenases, DMO monomers contain these two catalytically important and highly conserved domains (D'Ordine et al., 2009; Dumitru et al., 2009; Ferraro et al., 2005). The primary structure of these domains are highly conserved, leading to secondary and tertiary structural domains that result in the correct spatial orientation of the non-heme iron and the Rieske [2Fe-2S] domains in DMO monomers to ensure electron transport from ferredoxin and between the monomers of DMO (D'Ordine et al., 2009; Ferraro et al., 2005).

Rieske domains are ubiquitous in numerous bacterial and plant proteins such as the iron-sulfur protein of the cytochrome bc_1 complex, chloroplast cytochrome b_6-f complex in spinach, and choline mono-oxygenases (Breyton, 2000; Darrouzet et al., 2004; Gray et al., 2004; Hibino et al., 2002; Rathinasabapathi et al., 1997; Russell et al., 1998). The presence of two conserved domains, a Rieske [2Fe-2S] domain and a non-heme iron domain, suggests that all Rieske type non-heme iron oxygenases share the same reaction mechanism, by which the Rieske domain transfers electrons from the ferredoxin to the non-heme iron to allow catalysis (Chakraborty et al., 2005; Dumitru et al., 2009; Ferraro et al., 2005). The conservation of these important structural domains required for enzymatic activity is further evidence of the evolutionary relatedness of all Rieske non-heme iron oxygenases to each other (Nam et al., 2001; Rosche et al., 1997; Werlen et al., 1996). Therefore, enzymes with structural and functional homologies to MON 88701 DMO have been described in plants and bacteria and have been extensively consumed.

Additionally, a FASTA alignment search of publicly-available databases using the MON 88701 DMO protein sequence as a query yielded homologous sequences from many different species, predominantly bacteria, with amino acid sequence identity ranging up to approximately 42%. Alignments of MON 88701 DMO with plant proteins revealed homologous oxygenases present in crops, such as canola (*Brassica napus*), corn (*Zea mays*), pea (*Pisum sativum*), rice (*Orysa sativa*), and soy (*Glycine max*), which were determined to have sequence identities up to approximately 27% (Table V1-1). The highest homology was observed to proteins that are involved in chlorophyll metabolism. Chlorophyllide A oxygenase (Accession number: ACG42449) is a Rieske-type oxygenase that is required for the formation of chlorophyll *b,* which is present in all plants (Tanaka et al., 1998). Pheophorbide A oxygenase (Accession number: ABD60316) is also a Rieske-type oxygenase that plays a key role in the overall regulation of chlorophyll degradation in plants (Rodoni et al., 1997). Pheophorbide A oxygenase is constitutively present in all green tissues and, at slightly lower levels, in etiolated and non-photosynthetic tissues including seeds (Yang et al., 2004). As a Rieske-type oxygenase, Pheophorbide A oxygenase is expected to have high degree of secondary and tertiary structure homology to similar structural elements in DMO as described above. The presence of these conserved structural domains in these plant proteins is further evidence that exposure to a structural homolog of MON 88701 DMO has occurred through consumption of these crops.

Therefore, MON 88701 DMO shares homologies across all levels of protein structure (*i.e.*, primary, secondary, tertiary) with a wide variety of oxygenases present in bacteria and plants widely prevalent in the environment and consumed, establishing that animals and humans are extensively exposed to these structural homologs without any reports of adverse effects due to the protein.

Table VI-1. Amino Acid Sequence Identity between MON 88701 DMO and Other Proteins Present in Plants

Protein	Accession Number[1]	Scientific Name	Common Name	Sequence Identity (%)[2]
Chlorophyllide A oxygenase	ACG42449	*Zea mays*	Corn	27.3
Pheophorbide A oxygenase	ABD60316	*Brassica napus*	Canola/Oilseed Rape	26.0
Lethal leaf spot-1 like protein*	ABA40832	*Glycine max*	Soybean	25.7
Rieske iron-sulfur protein Tic55	CAA04157	*Pisum sativum*	Pea	25.4
Pheophorbide A oxygenase	CAR82238	*Pisum sativum*	Pea	24.6
Pheophorbide A oxygenase	ACG28057	*Zea mays*	Corn	24.3
Rieske domain containing protein	ABF99438	*Oryza sativa*	Rice	23.7
Flavonoid-3-hydroxylase	AAV74195	*Sorghum bicolor*	Sorghum	21.1
Sparse infloroscence1	ACI43576	*Zea mays*	Corn	17.8
Choline mono-oxygenase	AAB52509	*Spinacia oleracea*	Spinach	17.6
Beta-carotene hydroxylase	AAX45523	*Zea mays*	Corn	15.8
Rieske domain containing protein	ACG43734	*Zea mays*	Corn	14.5
Choline mono-oxygenase	CAE17617	*Oryza sativa*	Rice	12.6

*Later identified as Pheophorbide A Oxygenase (Yang et al., 2004).
[1]The accession numbers shown are from the GenBank database.
[2]Protein sequences were utilized from publicly available databases. Each sequence was aligned to the MON 88701 DMO protein by Clustal W method and sequence identity was calculated using the MegAlign function of the Lasergene suite of sequence analysis software [version 8.0.2 (13)] (DNASTAR, Inc. Madison, Wisconsin).

VI.B.2. History of Safe Use of PAT (*bar*) Protein

The PAT (*bar*) protein expressed in MON 88701 is identical to the wild type protein produced in *S. hygroscopicus* and is analogous to the PAT proteins in commercially-available glufosinate-tolerant products in several crops including cotton, corn, soybean and canola. Based on studies characterizing the kinetic and chemical mechanisms of PAT proteins (Wehrmann et al., 1996), OECD recognizes PAT proteins produced from different genes to be equivalent with regard to function and safety (OECD, 1999).

The safety of PAT protein present in biotechnology-derived crops has been extensively assessed (ILSI-CERA, 2011) and in 1997 a tolerance exemption was issued for PAT proteins by U.S. EPA (1997). This exemption was based on a safety assessment that included rapid digestion in simulated gastric fluids, lack of significant homology to known toxins and known allergens, and lack of toxicity in an acute oral mouse gavage study. Numerous glufosinate-tolerant products including those in cotton, corn, soy, canola, sugarbeet and rice have been reviewed by the FDA (U.S. FDA, 1995a; 1995c; 1995b; 1997; 1998b; 1998a; 1999; 2002) with no concerns identified. Further, a comprehensive study on the safety of PAT proteins present in biotechnology-derived crops (Hérouet et al., 2005) demonstrated structural similarity only with other

acetyltransferase known not to cause adverse effects after consumption, lack of sequence homology to know allergens and toxins, lack of glycosylation sites, rapid degradation in gastric and intestinal fluids and no adverse effects in mice treated with high doses of PAT proteins. Hérouet et al. (2005) concluded that there is a reasonable certainty of no harm resulting from the inclusion of PAT proteins in human food or animal feed.

The history of safe use of PAT is supported by the lack of any documented reports of adverse effects related to this protein since the introduction of glufosinate-tolerant crops in 1995 (Duke and Powles, 2009). Since then, approvals have been issued by regulatory agencies of 11 different countries for the environmental release of greater than 38 transformation events, including 8 different species of plants expressing the PAT protein (ILSI-CERA, 2011).

VI.C. Characterization of the MON 88701 DMO and PAT (*bar*) Proteins from MON 88701

VI.C.1. MON 88701 DMO Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of and confirmation of the safety of the introduced protein(s). For the safety data generated using *E. coli*-produced MON 88701 DMO to be applied to MON 88701 DMO protein produced in MON 88701, the equivalence of the plant- and *E. coli*-produced proteins must be assessed. To assess the equivalence between MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins, a small quantity of the MON 88701 DMO protein was purified from MON 88701 cottonseed. The MON 88701 DMO protein was characterized and the equivalence of the physicochemical characteristics and functional activity between the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins was assessed using a panel of six analytical tests as shown in Table VI-2. Taken together, these data provide a detailed characterization of the MON 88701 DMO protein and establish the equivalence of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins.

Table VI-2. Summary of MON 88701 DMO Protein Identity and Equivalence

Analytical Test Assessment	Section Cross Reference	Analytical Test Outcome
1. N-terminal sequence analysis of the MON 88701 DMO protein to assess identity	VI.C.1.1. and VI.C.1.2.	• The identity could not be confirmed by N-terminal sequence analysis • MALDI-TOF MS[1] analysis of peptides derived from tryptic digested MON 88701 DMO established the N-terminal sequence of MON 88701 DMO
2. MALDI-TOF MS[1] analysis of peptides derived from tryptic digested MON 88701 DMO protein to assess identity	VI.C.1.2.	• MALDI-TOF MS[1] analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 88701 DMO sequence
3. Western blot analysis using anti-DMO polyclonal antibodies to assess identity and immunoreactive equivalence between MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins	VI.C.1.3.	• MON 88701 DMO protein identity was confirmed using a western blot probed with antibodies specific for DMO protein • Immunoreactive properties of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent
4. SDS-PAGE[2] to assess equivalence of the apparent molecular weight between MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins	VI.C.1.4.	• Electrophoretic mobility and apparent molecular weight of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent
5. Glycosylation analysis of the MON 88701 DMO protein to assess equivalence between the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins	VI.C.1.5.	• Glycosylation status of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent
6. DMO enzymatic activity analysis to assess functional equivalence between MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins	VI.C.1.6.	• Functional activity of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent

[1] MALDI-TOF MS = Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

[2] SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

The details of the materials and methods for the panel of analytical tests used to evaluate and compare the properties of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins are described in Appendix B. A summary of the data obtained to support a conclusion of protein equivalence is below.

VI.C.1.1. Results of the N-Terminal Sequencing Analysis

N-terminal sequencing reaction was performed on MON 88701 DMO protein. The reaction did not yield any observable sequence, presumably because the N-terminus was blocked. Although this analysis did not yield N-terminal sequence data, the N-terminus of the MON 88701 DMO protein was determined using MALDI-TOF tryptic mass map analysis (see Section VI.C.1.2).

VI.C.1.2. Results of MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 88701 DMO protein was confirmed by MALDI-TOF MS analysis of peptide fragments produced from tryptic digestion of the MON 88701 DMO protein. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by peptide mapping is considered to be reliable if ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments (Biron et al., 2006; Krause et al., 1999).

There were 19 unique peptides identified that corresponded to the masses expected to be produced by tryptic digestion of the MON 88701 DMO protein (Table VI-3). The identified masses were used to assemble a coverage map of the entire MON 88701 DMO protein (Figure VI-3). The experimentally-determined mass coverage of the MON 88701 DMO protein was 66.5% (232 out of 349 amino acids). This analysis serves as identity confirmation for the MON 88701 DMO protein.

To identify the N-terminus, the experimentally-determined masses of the peptides produced from tryptic digestion of the MON 88701 DMO protein were examined for the presence of a mass that matched the theoretical mass expected from the MON 88701 DMO protein deduced from the *dmo* gene present in MON 88701. A mass was identified that corresponded to the predicted mass of an acetylated peptide with nine amino acids from CTP2 followed by the MON 88701 DMO protein deduced from the *dmo* gene present in MON 88701. The additional nine amino acids of CTP2 resulted from the alternative processing of CTP2. Alternative processing of DMO precursor proteins has been observed in other dicamba-tolerant plants containing the *dmo* gene (Behrens et al., 2007). Hence, the MON 88701 DMO protein was designated to have an N-terminal end as shown in Figure VI-3.

Table VI-3. Summary of the Tryptic Masses[1] Identified for the MON 88701 DMO Protein Using MALDI-TOF MS

α-cyano	DHB	Sinapinic acid	Expected Mass	Diff.[2]	Fragment[3]	Sequence
720.40			720.37	0.03	140-145	VDPAYR
833.51	833.45		833.45	0.06	108-114	SFPVVER
856.49			856.43	0.06	251-257	EQSIHSR
914.60			914.53	0.07	305-312	VVVEAIER
	1030.58		1030.57	0.01	293-301	SWQAQALVK
1108.61	1108.59		1108.50	0.11	176-185	ANAQTDAFDR
1275.87	1275.83		1275.73	0.14	35-45	TILDTPLALYR
1286.83			1286.70	0.13	302-312	EDKVVVEAIER
1428.84	1428.83		1428.69	0.15	218-230	GANTPVDAWNDIR
	1470.74		1470.63	0.11	146-158	TVGGYGHVDCNYK
	1501.91		1501.79	0.12	189-202	EVIVGDGEIQAALMK
	1506.86		1506.73	0.13	176-188	ANAQTDAFDRLER
	1577.89	1577.80	1577.73	0.16	279-292	NFGIDDPEMDGVLR
		1731.92	1731.80	0.12	1-15	VMSSVSTACMLTFVR +42 Da (N-acetylation)
	1745.09	1744.99	1744.93	0.16	234-250	VSAMLNFIAVAPEGTPK
	1994.30	1994.23	1994.03	0.27	159-175	LLVDNLMDLGHAQYVHR
		2143.35	2143.12	0.23	16-34	NAWYVAALPEELSEKPLGR
	2398.37	2398.35	2398.09	0.28	258-278	GTHILTPETEASCHYFFGSSR
		2724.72	2724.31	0.41	115-139	DALIWIWPGDPALADPGAIPGCR

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The difference between the expected mass and the first column mass. Other masses shown within a row are also within 1 Da of the expected mass.
[3]Position refers to amino acid residues within the predicted MON 88701 DMO sequence as depicted in Figure VI-3.
DHB = 5-dihydroxybenzoic acid matrix, α-cyano = α-cyano-4-hydroxycinnamic acid matrix, Sinapinic acid = 3, 5-dimethoxy-4-hydroxycinnamic acid matrix.

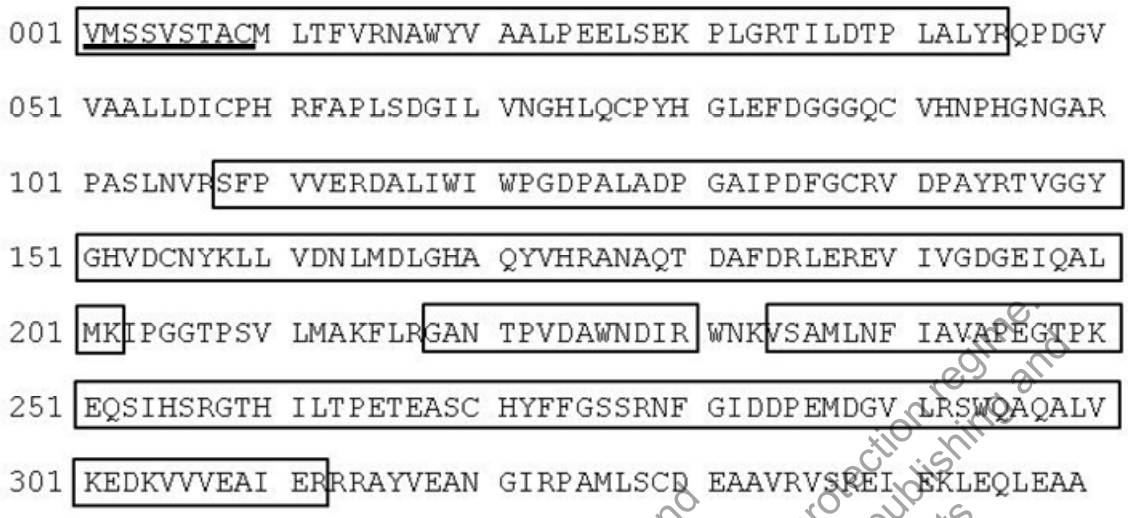


Figure VI-3. MALDI-TOF MS Coverage Map of the MON 88701 DMO Protein
The amino acid sequence of the MON 88701 DMO protein was deduced from the *dmo* gene present in MON 88701. Boxed regions correspond to regions covered by tryptic peptides that were identified from the MON 88701 DMO protein sample using MALDI-TOF MS. Underlined region corresponds to the nine amino acids from CTP2 retained at the N-terminus of the MON 88701 DMO. In total, 66.5% (232 of 349 total amino acids) of the expected protein sequence was covered by the identified peptides.

VI.C.1.3. Results of Western Blot Analysis of the MON 88701 DMO Protein Isolated from the Cottonseed of MON 88701 and Immunoreactivity Comparison to *E. coli*-produced MON 88701 DMO

Western blot analysis was conducted using goat anti-DMO polyclonal antibodies to 1) assess the identity of the MON 88701 DMO protein isolated from the cottonseed of MON 88701; and 2) to determine the relative immunoreactivity of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins. The results demonstrated that the anti-DMO antibodies recognized the MON 88701 DMO protein that migrated to the same position on the blot as the *E. coli*-produced MON 88701 DMO protein (Figure VI-4). Furthermore, the immunoreactive signal increased with increasing amounts of MON 88701 DMO protein loaded. Two other bands, one migrating at ~75 kDa and the other at ~17 kDa were also observed. These bands were prominent in lanes with higher load amounts (Figure VI-4, Lanes 3-6), and may represent products of aggregation and degradation of DMO, respectively.

Densitometric analysis was conducted to compare the immunoreactivity of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins. The mean signal intensity ($OD \times mm^2$) from the MON 88701 DMO bands and from the *E. coli*-produced MON 88701 DMO bands at each amount of protein analyzed was calculated and then overall mean signal intensity was calculated (Table VI-4). The immunoreactivity was considered equivalent if the overall mean signal intensity of all MON 88701 DMO protein bands was within ±35% of the overall mean signal intensity of *E. coli*-produced MON 88701 DMO protein bands across all loading levels (Appendix B.5.3.).

The overall mean signal intensity of the *E. coli*-produced MON 88701 DMO bands was 6.500 $OD \times mm^2$ and the overall mean signal intensity of the MON 88701 DMO bands was 4.440 $OD \times mm^2$. Because overall mean signal intensity of the MON 88701 DMO protein bands was between 4.225 and 8.775 (between -35% and +35% of the *E. coli*-produced MON 88701 DMO bands), the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined to have equivalent immunoreactivity.

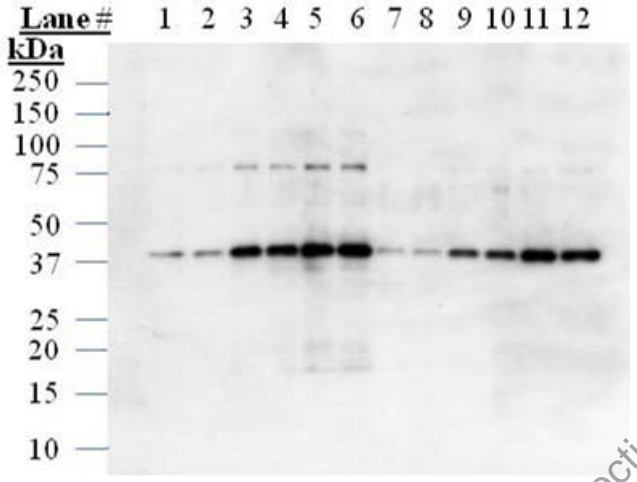


Figure VI-4. Western Blot Analysis of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO Proteins

Aliquots of the MON 88701 DMO protein and the *E. coli*-produced MON 88701 DMO protein were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was incubated with anti-DMO antibodies and immunoreactive bands were visualized using an ECL system (GE Healthcare, Piscataway, NJ). Approximate molecular weights (kDa) are shown on the left. Lanes loaded with molecular weight markers were cropped, and lanes were renumbered relative to the original gel loading. The 6 min exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	*E. coli*-produced MON 88701 DMO protein	0.5
2	*E. coli*-produced MON 88701 DMO protein	0.5
3	*E. coli*-produced MON 88701 DMO protein	2
4	*E. coli*-produced MON 88701 DMO protein	2
5	*E. coli*-produced MON 88701 DMO protein	6
6	*E. coli*-produced MON 88701 DMO protein	6
7	MON 88701 DMO protein	0.5
8	MON 88701 DMO protein	0.5
9	MON 88701 DMO protein	2
10	MON 88701 DMO protein	2
11	MON 88701 DMO protein	6
12	MON 88701 DMO protein	6

Table VI-4. Comparison of Immunoreactive Signals between MON 88701 DMO and *E. coli*-produced MON 88701 DMO Proteins

Mean Signal intensity from MON 88701 DMO[1] (OD × mm^2)	Mean Signal intensity from *E. coli*-produced MON 88701 DMO (OD × mm^2)	Preset Acceptance limits for MON 88701 DMO[1] (OD × mm^2)
4.440	6.500	4.225 – 8.775

[1]The acceptance limits for MON 88701 DMO are based on the interval between +35% (6.500 × 1.35) and -35% (6.500 × 0.65) of the overall mean of the *E. coli*-produced MON 88701 DMO signal intensity across six loads (Appendix B.5.3.).

VI.C.1.4. Results of the MON 88701 DMO Protein Apparent Molecular Weight and Purity Analysis

The molecular weight and purity of the MON 88701 DMO protein was determined to be 39.5 kDa and 97%, respectively. To assess molecular weight (MW) and purity, the MON 88701 DMO protein was subjected to SDS-PAGE. The gel was stained with Brilliant Blue G Colloidal stain and analyzed by densitometry (Figure VI-5). *E. coli*-produced MON 88701 DMO protein was loaded in a single lane for reference (Figure VI-5, Lane 2). The MON 88701 DMO protein (Figure VI-5, Lanes 3-8) had an apparent molecular weight of 39.5 kDa (Table VI-5). The apparent molecular weight of the *E. coli*-produced MON 88701 DMO protein as reported on its Certificate of Analysis was 38.7 kDa (Table VI-5). Because the apparent MW of MON 88701 DMO protein was within the preset acceptance limits for equivalence (Appendix B.5.7; Table VI-5), the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined to have equivalent apparent MWs.

The purity of the MON 88701 DMO protein was calculated based on the six loads on the gel (Appendix B.5.4.1.; Figure VI-5, Lanes 3-8). The average purity was determined to be 97%.

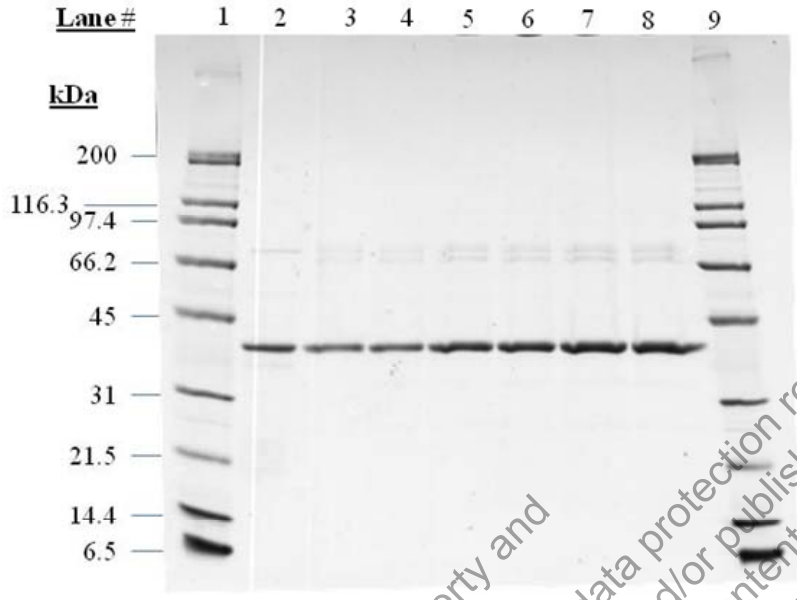


Figure VI-5. Molecular Weight and Purity Analysis of the MON 88701 DMO Protein

Aliquots of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were separated by SDS-PAGE and then stained with Brilliant Blue G Colloidal stain. Approximate molecular weights are shown on the left and correspond to the markers loaded in Lanes 1 and 9. Empty lane was partially cropped. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range Molecular Weight Markers	4.5
2	*E. coli*-produced MON 88701 DMO protein	0.5
3	MON 88701 DMO protein	0.5
4	MON 88701 DMO protein	0.5
5	MON 88701 DMO protein	1
6	MON 88701 DMO protein	1
7	MON 88701 DMO protein	1.5
8	MON 88701 DMO protein	1.5
9	Broad Range Molecular Weight markers	4.5

Table VI-5. Molecular Weight Comparison Between the MON 88701 DMO and *E. coli*-produced MON 88701 DMO Proteins Based on SDS-PAGE

Apparent MW of MON 88701 DMO[1] (kDa)	Apparent MW of *E. coli*-produced MON 88701 DMO[2] (kDa)	Preset Acceptance Limits for MON 88701 DMO[3] (kDa)
39. 5	38.7	38.5-39.7

[1] The reported value is the mean molecular weight across all six loads.
[2]The molecular weight of the *E. coli*-produced MON 88701 DMO protein as reported on its Certificate of Analysis.
[3]See Appendix B.5.7.

VI.C.1.5. MON 88701 DMO Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). To test whether DMO protein was glycosylated when expressed in the cottonseed of MON 88701, the MON 88701 DMO protein was analyzed using an ECL Glycoprotein Detection Module (GE, Healthcare, Piscataway, NJ). Transferrin, a glycosylated protein, was used as a positive control in the assay. To assess equivalence of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins, the *E. coli*-produced MON 88701 DMO protein was also analyzed. The positive control was clearly detected at expected molecular weight (~80 kDa) and the band intensity increased with increasing concentration (Figure VI-6, Panel A, Lanes 1-2). In contrast, signals were not observed in the lanes containing the MON 88701- or *E. coli*-produced protein at the expected molecular weight for the MON 88701 protein (Figure VI-6 Panel A, Lanes 7-8 and Lanes 4-5, respectively). To assess that sufficient MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were present for glycosylation analysis, a second membrane (with identical loadings and transfer times) was stained with Coomassie Blue R250 for protein detection (Figure VI-6, Panel B). Both the MON 88701- and *E. coli*-produced MON 88701 DMO proteins were clearly detected (Figure VI-6 Panel B, Lanes 7-8 and Lanes 4-5, respectively). These data indicate that the glycosylation status of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins are equivalent and that neither is glycosylated.

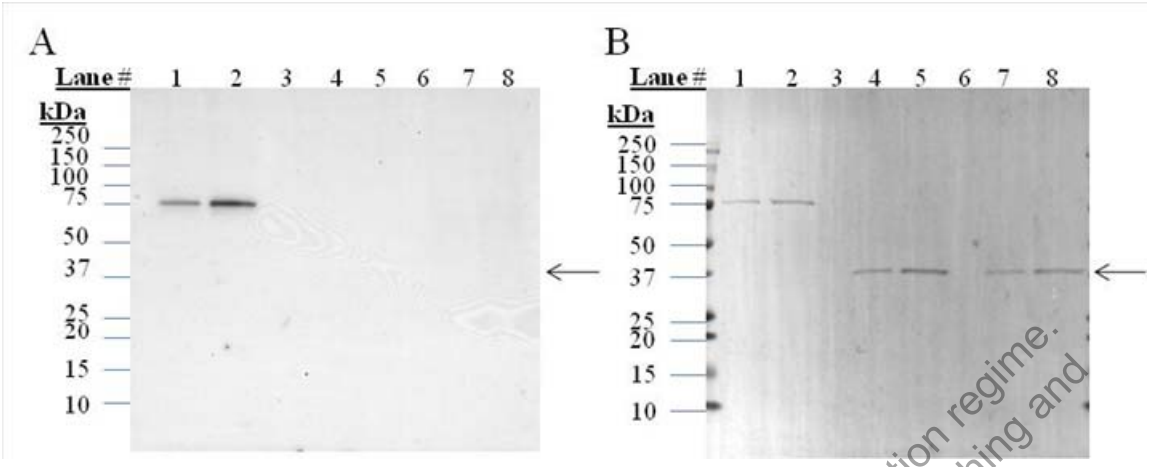


Figure VI-6. Glycosylation Analysis of the MON 88701 DMO Protein
Aliquots of the transferrin (positive control), *E. coli*-produced MON 88701 DMO protein and MON 88701 DMO protein were subjected to SDS-PAGE and electrotransferred to PVDF membranes. Panel A corresponds to detection of the labeled carbohydrate moieties, where present, using the ECL-based system with exposure to Hyperfilm. A 6 min exposure is shown. Panel B corresponds to Coomassie Blue R250 staining of an equivalent blot to confirm the presence of proteins. The signal was captured using a Bio-Rad GS-800 with Quantity One software (version 4.4.0). Approximate molecular weights (kDa) correspond to the Precision Plus, dual color markers (used to verify transfer and MW). Lanes loaded with molecular weight markers were partially cropped, and lanes were renumbered relative to the original gel loading. Arrows indicate the expected migration MON 88701 DMO protein. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Transferrin (positive control)	50
2	Transferrin (positive control)	100
3	Empty	-
4	*E. coli*-produced MON 88701 DMO (negative control)	50
5	*E. coli*-produced MON 88701 DMO (negative control)	100
6	Empty	-
7	MON 88701 DMO	50
8	MON 88701 DMO	100

VI.C.1.6. MON 88701 DMO Functional Activity

The functional activities of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined by quantifying the conversion of dicamba to DCSA using HPLC separation and fluorescence detection. In this assay, protein-specific activity is expressed as nmol DCSA × minute^{-1} × mg^{-1} of DMO.

The experimentally-determined specific activities for the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins are presented in Table VI-6. The specific activities of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were 5.48 and 7.23 nmol DCSA × minute^{-1} × mg^{-1} of DMO, respectively. Because the mean specific activities of the MON 88701-produced and *E. coli*-produced MON 88701 DMO proteins fall within the preset acceptance criterion (Appendix B.5.7., Table VI-6), the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined to have equivalent functional activity.

Table VI-6. MON 88701 DMO Functional Activity

MON 88701 DMO[1] (nmol DCSA × minute^{-1} × mg^{-1})	***E. coli*-produced MON 88701 DMO[1]** (nmol DCSA × minute^{-1} × mg^{-1})	**Preset Acceptance Limits for MON 88701 DMO[2]** (nmol DCSA × minute^{-1} × mg^{-1})
5.48 ± 1.3	7.23 ± 2.1	1.69-20.74

[1]Value refers to mean and standard deviation calculated based on n = 5.
[2]See Appendix B.5.7.

VI.C.2. MON 88701 DMO Protein Identity and Equivalence Conclusion

The MON 88701 DMO protein purified from cottonseed of MON 88701 was characterized and the equivalence of the physicochemical and functional properties between the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins was established using a panel of analytical tests: 1) the identity could not be confirmed by N-terminal sequence analysis; however, MALDI-TOF MS analysis of peptides derived from tryptic digested MON 88701 DMO established the N-terminal sequence of MON 88701 DMO; 2) MALDI-TOF MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 88701 DMO sequence; 3) MON 88701 DMO protein was detected on a western blot probed with antibodies specific for DMO protein and the immunoreactive and physiochemical properties of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent; 4) the electrophoretic mobility and apparent molecular weight of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent; 5) glycosylation status of MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were determined to be equivalent; and 6) functional activity of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO proteins were demonstrated to be equivalent.

Taken together, these data provide a detailed characterization of the MON 88701 DMO protein and establish the equivalence of the MON 88701 DMO and the *E. coli*-produced MON 88701 DMO protein. This equivalence justifies the use of the *E. coli*-produced MON 88701 DMO as a test subtance in the protein safety studies.

VI.C.3. MON 88701-produced PAT (*bar*) Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of and confirmation of the safety of the introduced protein(s). For the safety data generated using *E. coli*-produced PAT (*bar*) protein to be applied to PAT (*bar*) protein produced in MON 88701, the equivalence of the plant- and *E. coli*-produced PAT (*bar*) proteins was assessed. To assess the equivalence between MON 88701- and *E. coli*-produced PAT (*bar*) proteins, a small quantity of the PAT (*bar*) protein was purified from MON 88701 cottonseed. The MON 88701-produced PAT (*bar*) protein was characterized and the equivalence of the immunoreactive and physicochemical characteristics and functional activity between the MON 88701-produced PAT (*bar*) and the *E. coli*-produced PAT (*bar*) proteins was assessed using a panel of six analytical tests as shown in Table VI-7. Taken together, these data provide a detailed characterization of the MON 88701-produced PAT (*bar*) protein and establish the equivalence of MON 88701- and *E. coli*-produced PAT (*bar*) proteins.

Table VI-7. Summary of MON 88701-produced PAT (*bar*) Protein Identity and Equivalence

Analytical Test Assessment	Section Cross Reference	Analytical Test Outcome
1. N-terminal sequence analysis of the MON 88701-produced PAT (*bar*) protein to assess identity	VI.C.3.1.	• The identity was confirmed by N-terminal sequence analysis
2. MALDI-TOF MS[1] analysis of peptides derived from tryptic digested MON 88701-produced PAT (*bar*) protein to assess identity	VI.C.3.2.	• MALDI-TOF MS[1] analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 88701 PAT (*bar*) sequence
3. Western blot analysis using anti-PAT (*bar*) polyclonal antibodies to assess identity and immunoreactive equivalence between MON 88701- and the *E. coli*-produced PAT (*bar*) proteins	VI.C.3.3.	• MON 88701-produced PAT (*bar*) protein identity was confirmed using a western blot probed with antibodies specific for PAT protein • Immunoreactive properties of the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent
4. SDS-PAGE[2] to assess equivalence of the apparent molecular weight between MON 88701- and the *E. coli*-produced PAT (*bar*) proteins	VI.C.3.4.	• Electrophoretic mobility and apparent molecular weight of the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent
5. Glycosylation analysis of the PAT (*bar*) protein to assess equivalence between the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins	VI.C.3.5.	• Glycosylation status of MON 88701- and the *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent
6. PAT (*bar*) enzymatic activity analysis to assess functional equivalence between MON 88701- and the *E. coli*-produced PAT (*bar*) proteins	VI.C.3.6.	• Functional activity of the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent

[1] MALDI-TOF MS = Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

[2] SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

The details of the materials and methods for the panel of analytical tests used to evaluate and compare the properties of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins

are described in Appendix B. A summary of the data obtained to support a conclusion of protein equivalence is below.

VI.C.3.1. Results of the N-Terminal Sequencing Analysis

N-terminal sequencing of the first 15 amino acids was performed on MON 88701-produced PAT (*bar*). The expected sequence for the PAT (*bar*) protein deduced from the *bar* gene present in MON 88701 was observed. The data obtained correspond to the deduced PAT (*bar*) protein beginning at amino acid positions 2 and 3 (Figure VI-7, Experimental Sequence 1 and 2, respectively). The N-terminal methionine residue in the PAT (*bar*) protein was not observed, indicating that it was removed during post-translational processing of the precursor protein. This result is expected as removal of the N-terminal methionine, catalyzed by methionine aminopeptidase, is common in many organisms and has no effect on protein structure or activity (Arfin and Bradshaw, 1988; Bradshaw et al., 1998; Polevoda and Sherman, 2000). Hence, the sequence information confirms the identity of the PAT (*bar*) protein isolated from the cottonseed of MON 88701.

Amino acid residue # from the N-terminus	→	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Expected Sequence	→	M	S	P	E	R	R	P	A	D	I	R	R	A	T	E	A
Experimental Sequence 1	→	-	S	P	E	R	R	P	A	D	I	R	R	A	T	E	A
Experimental Sequence 2	→	-	-	P	E	R	X	X	A	D	I	X	X	X	T	E	-

Figure VI-7. N-Terminal Sequence of the MON 88701-produced PAT (*bar*) Protein
The expected amino acid sequence of the N-terminus of PAT (*bar*) protein was deduced from the *bar* coding region present in MON 88701. The experimental sequences obtained from the MON 88701-produced PAT (*bar*) protein were compared to the expected sequence. The single letter IUPAC-IUB amino acid code is M, methionine; S, serine; P, proline; E, glutamic acid; R, arginine; A, alanine; D, aspatic acid; I, isoleucine; and T, threonine. X indicates that the residue was not identifiable; (-) indicates the residue was not observed.

VI.C.3.2. Results of MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 88701-produced PAT (*bar*) protein was also confirmed by MALDI-TOF MS analysis of peptide fragments produced from tryptic digestion of the MON 88701-produced PAT (*bar*) protein. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by peptide mapping is considered to be reliable if ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments (Biron et al., 2006; Krause et al., 1999).

There were 10 unique peptides identified that corresponded to the masses expected to be produced by tryptic digestion of the PAT (*bar*) protein (Table VI-8). The identified masses were used to assemble a coverage map of the entire MON 88701-produced PAT (*bar*) protein (Figure VI-8). The experimentally determined mass coverage of the MON 88701-produced PAT (*bar*) protein was 84.7% (155 out of 183 amino acids). This analysis serves as additional identity confirmation for the MON 88701-produced PAT (*bar*) protein.

Table VI-8. Summary of the Tryptic Masses[1] Identified for the MON 88701-produced PAT (*bar*) Protein Using MALDI-TOF MS

α-cyano		DHB		Sinapinic acid		Expected Mass	Diff.[2]	Fragment[3]	Sequence
Extract 1	Extract 2	Extract 1	Extract 2	Extract 1	Extract 2				
		879.65				879.46	0.19	113-120	SLEAQGFK
1144.65	1144.75	1144.84				1144.56	0.09	136-145	MHEALGYAPR
1403.93	1404.03	1404.12	1404.18			1403.79	0.14	100-112	TGLGSTLYTHLLK
1523.02	1523.13	1523.14	1523.19	1522.93		1522.86	0.16	121-135	SVVAVIGLPNDPSVR
1843.07	1843.18	1843.27		1842.98	1843.19	1842.85	0.22	38-52	TEPQEPQEWTDDLVR
1859.06	1859.22	1859.22		1858.98	1859.18	1858.86	0.20	81-96	NAYDWTAESTVYVSPR
				2391.45	2391.64	2391.20	0.25	57-78	YPWLVAEVDGEVAGIAYAGPWK
2676.67				2676.64	2676.88	2676.35	0.32	55-78	ERYPWLVAEVDGEVAGIAYAGPWK
				2840.62		2840.32	0.30	13-37	ATEADMPAVCTIVNHYIETSTVNFR
3353.14	3353.36			3353.17	3353.48	3352.73	0.41	155-183	HGNWHDVGFWQLDFSLPVPPRPVLPVTEI

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The difference between the expected mass and the first column mass. Other masses shown within a row are also within 1 Da of the expected mass.
[3]Position refers to amino acid residues within the predicted PAT (*bar*) sequence as depicted in Figure VI-8.
DHB = 5-dihydroxybenzoic acid matrix, α-cyano = α-cyano-4-hydroxycinnamic acid matrix, Sinapinic acid = 3, 5-dimethoxy-4-hydroxycinnamic acid matrix.

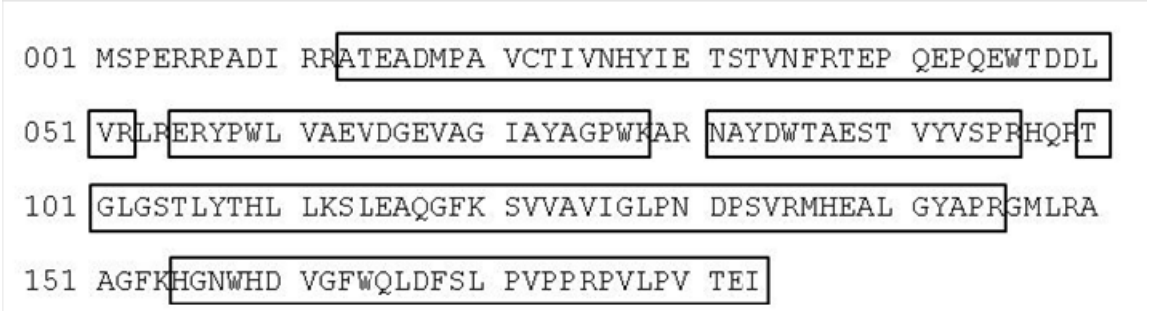


Figure VI-8. MALDI-TOF MS Coverage Map of the MON 88701-produced PAT (*bar*) Protein

The amino acid sequence of the PAT (*bar*) protein was deduced from the *bar* gene present in MON 88701. Boxed regions correspond to regions covered by tryptic peptides that were identified from the MON 88701-produced PAT (*bar*) protein sample using MALDI-TOF MS. In total, 84.7% (155 out of 183 amino acids) of the expected protein sequence was covered by the identified peptides.

VI.C.3.3. Results of Western Blot Analysis of the PAT (*bar*) Protein Isolated from the Cottonseed of MON 88701 and Immunoreactivity Comparison to *E. coli*-produced PAT (*bar*) Protein

Western blot analysis was conducted using goat anti- PAT (*bar*) polyclonal antibodies to 1) assess the identity of the PAT (*bar*) protein isolated from the cottonseed of MON 88701; and 2) to determine the relative immunoreactivity of the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins. The results demonstrated that the anti-PAT (*bar*) antibodies recognized the MON 88701-produced PAT (*bar*) protein that migrated to an identical position on the blot as the *E. coli*-produced PAT (*bar*) protein (Figure VI-9). Furthermore, the immunoreactive signal increased with increasing amounts of PAT (*bar*) protein loaded.

Densitometric analysis was conducted to compare the immunoreactivity of MON 88701- and the *E. coli*-produced PAT (*bar*) proteins. The mean signal intensity (OD × mm^2) from the MON 88701-produced PAT (*bar*) bands and from the *E. coli*-produced PAT (*bar*) bands at each amount of protein analyzed was calculated and then overall mean signal intensity was calculated (Table VI-9). The immunoreactivity was considered equivalent if the overall mean signal intensity of all MON 88701-produced PAT (*bar*) protein bands was within ±35% of the overall mean signal intensity of all *E. coli*-produced PAT (*bar*) protein bands (Appendix B.5.3.).

The overall mean signal intensity of the *E. coli*-produced PAT (*bar*) bands was 4.669 OD × mm^2 and the overall mean signal intensity of the MON 88701-produced PAT (*bar*) bands was 4.167 OD × mm^2. Because overall mean signal intensity of the MON 88701-produced PAT (*bar*) protein bands was between 3.035 and 6.303 OD × mm^2 (between -35% and +35% of the *E. coli*-produced PAT (*bar*) bands), the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins were determined to have equivalent immunoreactivity.

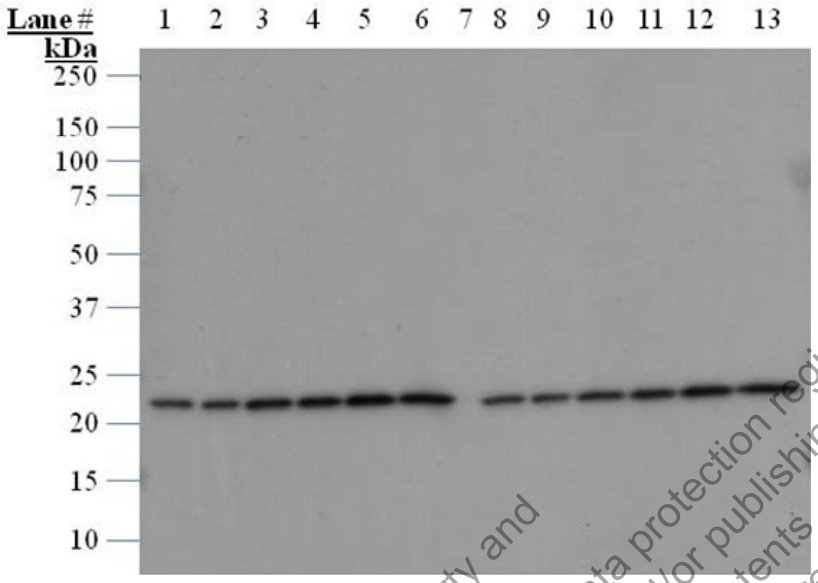


Figure VI-9. Western Blot Analysis of the MON 88701- and *E. coli*-produced PAT (*bar*) Proteins

Aliquots of the MON 88701-produced PAT (*bar*) protein and the *E. coli*-produced PAT (*bar*) protein were subjected to SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with anti-PAT (*bar*) antibodies and immunoreactive bands were visualized using an ECL system (GE Healthcare, Piscataway, NJ). Approximate molecular weights (kDa) are shown on the left. Lanes loaded with molecular weight markers were cropped, and lanes were renumbered relative to the original gel loading. The 1 min exposure is shown. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	*E. coli*-produced PAT (*bar*) protein	2
2	*E. coli*-produced PAT (*bar*) protein	2
3	*E. coli*-produced PAT (*bar*) protein	4
4	*E. coli*-produced PAT (*bar*) protein	4
5	*E. coli*-produced PAT (*bar*) protein	6
6	*E. coli*-produced PAT (*bar*) protein	6
7	Empty	-
8	MON 88701-produced PAT (*bar*) protein	2
9	MON 88701-produced PAT (*bar*) protein	2
10	MON 88701-produced PAT (*bar*) protein	4
11	MON 88701-produced PAT (*bar*) protein	4
12	MON 88701-produced PAT (*bar*) protein	6
13	MON 88701-produced PAT (*bar*) protein	6

Table VI-9. Comparison of Immunoreactive Signals between MON 88701- and *E. coli*-produced PAT (*bar*) Proteins

Mean Signal Intensity from MON 88701-produced PAT (*bar*) (OD x mm^2)	Mean Signal Intensity from *E. coli*-produced PAT (*bar*) (OD x mm^2)	Preset Acceptance Limits for MON 88701-produced PAT (*bar*)[1] (OD x mm^2)
4.167	4.669	3.035 – 6.303

[1]The acceptance limits for the MON 88701-produced PAT (*bar*) are based on the interval between +35% (4.669 × 1.35) and -35% (4.669 × 0.65) of the overall mean of the *E. coli*-produced PAT (*bar*) signal intensity across all six loads (Appendix B.5.3.).

VI.C.3.4. Results of the MON 88701-produced PAT (*bar*) Protein Apparent Molecular Weight and Purity Analysis

The molecular weight and purity of the PAT (*bar*) protein was determined to be 24.1 kDa and 99%, respectively. To assess apparent molecular weight (MW) and purity, the MON 88701-produced PAT (*bar*) protein was subjected to SDS-PAGE. The gel was stained with Brilliant Blue G Colloidal stain and analyzed by densitometry (Figure VI-10). *E. coli*-produced PAT (*bar*) protein was loaded in a single lane for reference (Figure VI-10, Lane 2). The MON 88701-produced PAT (*bar*) protein (Figure VI-10, Lanes 3-8) had an apparent MW of 24.1 kDa (Table VI-10). The apparent molecular weight of the *E. coli*-produced PAT (*bar*) protein as reported on its Certificate of Analysis was 25.0 kDa (Table VI-10). Because the apparent MW of MON 88701-produced PAT (*bar*) protein was within the preset acceptance limits (Appendix B.5.7; Table VI-10), the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins were determined to have equivalent apparent MWs.

The purity of the MON 88701-produced PAT (*bar*) protein was calculated based on the six loads on the gel (Appendix B.5.4.2; Figure VI-10, Lanes 3-8). The average purity was determined to be more than 99%.

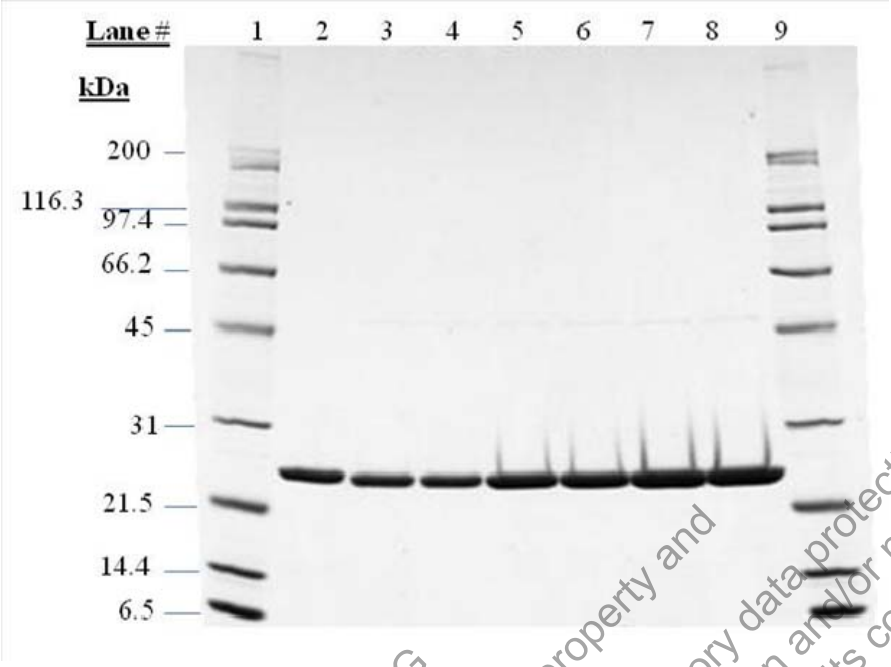


Figure VI-10. Molecular Weight and Purity Analysis of the MON 88701-produced PAT (*bar*) Protein

Aliquots of the MON 88701-produced and the *E. coli*-produced PAT (*bar*) proteins were subjected to SDS-PAGE and then stained with Brilliant Blue G Colloidal stain. Approximate molecular weights are shown on the left and correspond to the markers loaded in Lanes 1 and 9. Empty lane was partially cropped. Lane designations are as follows:

Lane	Sample	Amount (µg)
1	Broad Range Molecular Weight Markers	4.5
2	*E. coli*-produced PAT (*bar*) protein	1
3	MON 88701-produced PAT (*bar*) protein	1
4	MON 88701-produced PAT (*bar*) protein	1
5	MON 88701-produced PAT (*bar*) protein	2
6	MON 88701-produced PAT (*bar*) protein	2
7	MON 88701-produced PAT (*bar*) protein	3
8	MON 88701-produced PAT (*bar*) protein	3
9	Broad Range Molecular Weight markers	4.5

Table VI-10. Molecular Weight Comparison Between the MON 88701- and *E. coli*-produced PAT (*bar*) Proteins Based on SDS-PAGE

Apparent Molecular Weight of MON 88701-produced PAT (*bar*)[1] (kDa)	Apparent Molecular Weight of *E. coli*-produced PAT (*bar*)[2] (kDa)	Preset Acceptance Limits for MON 88701-produced PAT (*bar*)[3] (kDa)
24.1	25.0	23.9-25.4

[1]The reported value is the mean molecular weight across all six loads.
[2]The molecular weight of the *E. coli*-produced PAT (*bar*) protein as reported on its Certificate of Analysis.
[3]See Appendix B.5.7.

VI.C.3.5. PAT (*bar*) Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). To test whether PAT (*bar*) protein was glycosylated when expressed in the cottonseed of MON 88701, the MON 88701-produced PAT (*bar*) protein was analyzed using an ECL Glycoprotein Detection Module (GE, Healthcare, Piscataway, NJ). Transferrin, a glycosylated protein, was used as a positive control in the assay. To assess equivalence of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins, the *E. coli*-produced PAT (*bar*) protein, was also analyzed. The positive control was clearly detected at the expected molecular weight (~80 kDa) and the band intensity increased with increasing concentration (Figure VI-11, Panel A, Lanes 1-2). In contrast, signals were not observed in the lanes containing the MON 88701- or *E. coli*-produced protein at the expected molecular weight for the PAT (*bar*) protein (Figure VI-11 Panel A, Lanes 7-8 and Lanes 4-5, respectively). To assess whether the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were loaded appropriately for glycosylation analysis, a second membrane (with identical loadings and transfer times) was stained with Coomassie Blue R250 for protein detection (Figure VI-11 Panel B). Both the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were clearly detected (Figure VI-11 Panel B, Lanes 7-8 and Lanes 4-5, respectively). These data indicate that the glycosylation status of MON 88701-produced PAT (*bar*) protein and *E. coli*-produced PAT (*bar*) protein are equivalent and that neither is glycosylated.

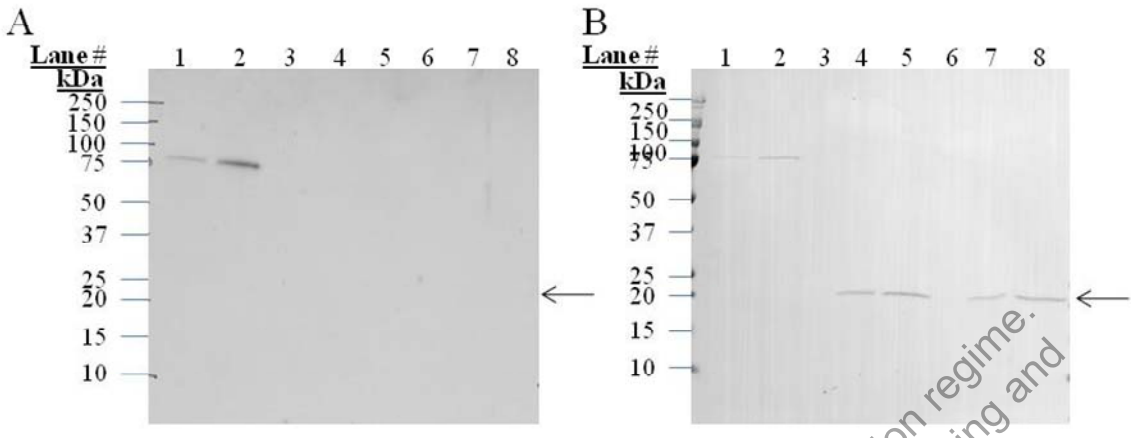


Figure VI-11. Glycosylation Analysis of the MON 88701-produced PAT (*bar*) Protein

Aliquots of the transferrin (positive control), *E. coli*-produced PAT (*bar*) protein and MON 88701-produced PAT (*bar*) protein were subjected to SDS-PAGE and electrotransferred to PVDF membranes. Panel A corresponds to detection of labeled crabohydrate moieties, where present, using the ECL-based system with exposure to Hyperfilm. A 7 min exposure is shown. Panel B corresponds to Coomassie Blue R250 staining of an equivalent blot to confirm the presence of proteins. The signal was captured using a Bio-Rad GS-800 with Quantity One software (version 4.4.0). Approximate molecular weights (kDa) correspond to the Precision Plus, dual color markers (used to verify transfer and MW). Lanes loaded with molecular weight markers were cropped, and lanes were renumbered relative to the original gel loading. Arrows indicate the expected migration of PAT (*bar*) protein. Lane designations are as follows:

Lane	Sample	Amount (ng)
1	Transferrin (positive control)	50
2	Transferrin (positive control)	100
3	Empty	-
4	*E. coli*-produced PAT (*bar*) (negative control)	50
5	*E. coli*-produced PAT (*bar*) (negative control)	100
6	Empty	-
7	MON 88701-produced PAT (*bar*)	50
8	MON 88701-produced PAT (*bar*)	100

VI.C.3.6. PAT (*bar*) Functional Activity

The functional activities of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were assessed using a colorimetric assay that measures PAT (*bar*) catalyzed release of coenzyme A (CoA) from acetyl-CoA upon transfer of an acetyl-group to phosphinothricin. In this assay, protein-specific activity is expressed as $\mu mol \times minute^{-1} \times mg^{-1}$ of PAT enzyme.

The experimentally-determined specific activities for the MON 88701- and *E. coli*-produced PAT (*bar*) proteins are presented in Table VI-11. The specific activities of MON 88701- and *E. coli*-produced PAT (*bar*) proteins were 36.4 and 46.2 $\mu mol \times minute^{-1} \times mg^{-1}$ of PAT (*bar*), respectively. Because the specific activities of the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins fall within the preset acceptance criterion (Appendix B.5.7; Table VI-11), the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were determined to have equivalent functional activity.

Table VI-11. PAT (*bar*) Functional Activity

MON 88701-produced PAT (*bar*)[1] ($\mu mol \times minute^{-1} \times mg^{-1}$)	*E. coli*-produced PAT (*bar*)[1] ($\mu mol \times minute^{-1} \times mg^{-1}$)	Preset Acceptance Limits for MON 88701-produced PAT (*bar*)[2] ($\mu mol \times minute^{-1} \times mg^{-1}$)
36.4 ± 1.3	46.2 ± 2.1	30.17 - 51.70

[1]Value refers to mean and standard deviation calculated based on n = 5.
[2]See Appendix B.5.7.

VI.C.4. MON 88701-produced PAT (*bar*) Protein Identity and Equivalence Conclusion

The MON 88701-produced PAT (*bar*) protein purified from cottonseed of MON 88701 was characterized and the equivalence of the immunoreactive and physicochemical characteristics and functional activity between the MON 88701- and the *E. coli*-produced PAT (*bar*) proteins was established using a panel of analytical tests: 1) N-terminal sequence analysis of the MON 88701-produced PAT (*bar*) protein established identity; 2) MALDI-TOF MS analysis yielded peptide masses consistent with the expected peptide masses from the theoretical trypsin digest of the MON 88701-produced PAT (*bar*) sequence; 3) MON 88701-produced PAT (*bar*) protein was detected on a western blot probed with antibodies specific for PAT (*bar*) protein and the immunoreactive properties of the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent; 4) the electrophoretic mobility and apparent molecular weight of the MON 88701-produced and *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent; 5) glycosylation status of MON 88701- and *E. coli*-produced MON 88701 PAT (*bar*) proteins were determined to be equivalent; and 6) functional activity of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were demonstrated to be equivalent.

Taken together, these data provide a detailed characterization of the MON 88701-produced PAT (*bar*) protein and establish the equivalence of the MON 88701-produced and the *E. coli*-produced PAT (*bar*) proteins. This equivalence justifies the use of protein safety studies conducted in which the *E. coli*-produced PAT (*bar*) protein was used as a test substance.

VI.D. Expression Levels of MON 88701 DMO and PAT (*bar*) Proteins in MON 88701

MON 88701 DMO and PAT (*bar*) protein levels in various tissues of MON 88701 relevant to the risk assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA). Tissues of MON 88701 were collected from four replicate plots planted in a randomized complete block field design during the 2010 growing season from the following eight field sites in the U.S.: Arkansas (ARTI), Georgia (GACH), Kansas (KSLA), Louisiana (LACH), North Carolina (NCBD), New Mexico (NMLC), South Carolina (SCEK) and Texas (TXPL). MON 88701 plots were treated at the 3-5 leaf stage with glufosinate herbicide at the label rate (0.5 lb acid equivalence a.e./acre) and at the 6-10 leaf stage with dicamba herbicide at the label rate (0.5 lb a.e./acre). The field sites were representative of cotton producing regions suitable for commercial production. Seed, pollen, root, and over-season leaf (OSL-1 through OSL-4) tissue samples were collected from each replicated plot at all field sites, except OSL1 at TXPl and OSL4 at LACH.

VI.D.1. MON 88701 DMO Expression Levels

MON 88701 DMO protein levels were determined in all seven tissue types. The results obtained from ELISA analyses are summarized in Table VI-12 and the details of the materials and methods are described in Appendix C. Due to a limited amount of tissue, moisture content was not measured for pollen; therefore, pollen is reported on a fresh weight (fw) basis only. MON 88701 DMO protein levels in MON 88701 across tissue types ranged from <LOD to 410 µg/g dw. The mean MON 88701 DMO protein levels were determined across eight sites, with the exception of OSL-1 (7 sites) and OSL-4 (7 sites). Samples <LOD were not included in mean determinations. The mean MON 88701 DMO protein levels were highest in leaf (ranging from OSL-2 and OSL-3 at 240 µg/g dw, OSL-4 at 230 µg/g dw to OSL-1 at 180 µg/g dw), followed by root at 43 µg/g dw, seed at 21 µg/g dw, and pollen at 14 µg/g fw.

Table VI-12. Summary of MON 88701 DMO Protein Levels in Tissues from MON 88701 Grown in 2010 U.S. Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)	MON 88701 DMO Mean (SD) Range (µg/g fw)[3]	MON 88701 DMO Mean (SD) Range (µg/g dw)[4]	LOQ/LOD[5] (µg/g fw)
OSL-1	2-4 leaf	14-25	27 (7.6) 13 – 42	180 (52) 110 – 280	0.168/0.313
OSL-2	4-7 leaf	25-37	41 (12) 19 – 65	240 (69) 110 – 380	0.168/0.313
OSL-3	9 leaf - Full flower	35-99	52 (17) 24 – 97	240 (75) 91 – 410	0.168/0.313
OSL-4	Cutout – Full flower	70-121	57 (18) 0.70 – 91	230 (59) 2.8 – 310	0.168/0.313
Root	50% open flower – Full flower	62-99	14 (3.7) 8.2 – 21	43 (12) 26 – 72	0.136/0.313
Pollen	50% open flower – Full Flower	68-99	14 (28) 0.31 – 110	NA (NA) NA	0.043/0.125
Seed	Maturity	148-183	20 (4.6) 8.2 – 29	21 (5.0) 8.9 – 33	0.059/0.313

[1]OSL = over-season leaf. Seed = black seed (ginned and delinted).

[2]The crop development stage each tissue was collected ([redacted] et al., 2007).

[3]Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=32, except OSL-3 n=31 due to one sample <LOD, OSL-1 and OSL-4 n=28 due to missed sample collections, and pollen n=29 due to two samples expressing <LOD and one being inconclusive).

[4]Protein levels are expressed as µg/g on a dry weight (dw) basis. The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factors obtained from moisture analysis data. NA= Not Applicable.

[5]LOQ=limit of quantitation; LOD=limit of detection.

VI.D.2. MON 88701-produced PAT (*bar*) Expression Levels

PAT (*bar*) protein levels were determined in all seven tissue types. The results obtained from ELISA are summarized in Table VI-13 and the details of the materials and methods are described in Appendix C. Due to a limited amount of tissue, moisture content was not measured for pollen; therefore, pollen is reported on a fresh weight (fw) basis only. PAT (*bar*) protein levels in MON 88701 across tissue types ranged from <LOQ to 10 µg/g dw. The mean PAT (*bar*) protein levels were determined across eight sites, with the exception of OSL-1 (7 sites) and OSL-4 (7 sites). Samples <LOD were not included in mean determinations. The mean PAT (*bar*) protein levels were highest in seed at 6.6 µg/g dw, followed by leaf (ranging from OSL-2 at 6.4 µg/g dw, OSL-1 at 5.5 µg/g dw, OSL-3 at 4.8 µg/g dw to OSL-4 at 3.2 µg/g dw), root at 1.8 µg/g dw, and pollen at 0.56 µg/g fw.

Table VI-13. Summary of PAT (*bar*) Protein Levels in Tissues from MON 88701 Grown in 2010 U.S. Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)	PAT (*bar*) Mean (SD) Range (µg/g fw)[3]	PAT (*bar*) Mean (SD) Range (µg/g dw)[4]	LOD/LOQ[5] (µg/g fw)
OSL-1	2-4 leaf	14-25	0.84 (0.21) 0.46 – 1.4	5.5 (1.5) 3.7 – 9.1	0.162/0.188
OSL-2	4-7 leaf	25-37	1.1 (0.26) 0.68 – 1.6	6.4 (1.4) 3.8 – 9.4	0.162/0.188
OSL-3	9 leaf – Full flower	35-99	1.0 (0.34) 0.34 – 1.7	4.8 (2.0) 1.3 – 10	0.162/0.188
OSL-4	Cutout – Full flower	70-121	0.78 (0.29) 0.42 – 1.7	3.2 (1.2) 2.0 – 6.7	0.162/0.188
Root	50% open flower	62-99	0.56 (0.18) 0.27 – 0.89	1.8 (0.75) 0.93 – 3.3	0.096/0.188
Pollen	50% open flower – Full flower	68-99	0.56 (0.24) 0.27 – 0.90	NA (NA) NA	0.021/0.188
Seed	Maturity	148-183	6.1 (0.95) 4.8 – 8.8	6.6 (1.1) 5.2 – 9.6	0.032/0.188

[1]OSL= over-season leaf. Seed = black seed (ginned and delinted).
[2]The crop development stage each tissue was collected ([redacted] et al., 2007).
[3]Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=32, except OSL-1 n=28 due to missed sample collections, OSL-4 n=27 due to missed sample collections and one sample expressing <LOD, OSL-3 n=31 due to one sample expressing <LOD, and pollen n=6 due to 26 samples expressing <LOQ).
[4]Protein levels are expressed as µg/g on a dry weight (dw) basis. The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factors obtained from moisture analysis data. NA= Not Applicable.
[5]LOQ=limit of quantitation; LOD=limit of detection.

VI.E. Generational Stability of MON 88701 DMO and PAT (*bar*) Protein Expression in MON 88701

In order to assess the presence of the MON 88701 DMO and PAT (*bar*) proteins in MON 88701 across multiple generations, western blot analysis of MON 88701 was conducted on leaf tissue collected from generations R_2, R_3, R_4, R_5, and R_6 (Figure V-8) of MON 88701, and on leaf tissue of the conventional control. Materials and methods are detailed in Appendix D.

VI.E.1. MON 88701 DMO Protein Generational Stability

The presence of the MON 88701 DMO protein in harvested leaf tissue of the R_2, R_3, R_4, R_5, and R_6 generations of MON 88701 (Figure V-8) was demonstrated (Figure VI-12). An *E coli*-produced MON 88701 DMO standard (1 ng) was used as a reference for the identification of the MON 88701 DMO protein. The presence of MON 88701 DMO protein in MON 88701 leaf tissue samples was determined by visual comparison of the bands produced in multiple breeding generations (Figure VI-12, Lanes 3–7) to the MON 88701 DMO reference standard (Figure VI-12, Lane 1). As shown in Figure VI-12, MON 88701 DMO protein was present in multiple generations of MON 88701 tissue samples and migrated with a mobility indistinguishable from that of the *E. coli*-produced protein standard analyzed on the same western blot. As expected, the MON 88701 DMO protein was not detected in the conventional control extract (Figure VI-12, Lane 8).

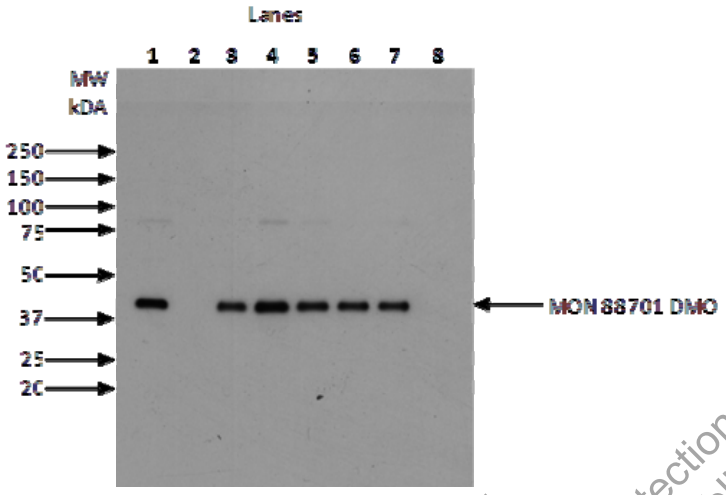


Figure VI-12. Presence of MON 88701 DMO Protein in Multiple Generations of MON 88701

Extracts from five generations of MON 88701 leaf tissues, conventional control leaf tissues, *E. coli*-produced MON 88701 DMO protein standard, and molecular weight markers were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was incubated with goat anti-DMO antibody and immunoreactive bands visualized through the use of ECL reagents. The image represents a 20 sec exposure. Arrows denote the size of the protein, in kiloDaltons (kDa), obtained from the Precision Plus Protein Dual Color Standards (Bio-Rad) transferred to the western membrane. Lane designations are as follows:

Lane	Sample	Amount (µl)
1	*E. coli*-produced MON 88701 DMO protein	20 (1 ng)
2	Empty	-
3	R_2 Generation	20
4	R_3 Generation	20
5	R_4 Generation	20
6	R_5 Generation	20
7	R_6 Generation	20
8	Conventional Control – Coker 130	20

VI.E.2. MON 88701-produced PAT (*bar*) Protein Generational Stability

The presence of the PAT (*bar*) protein in harvested leaf tissue of the R_2, R_3, R_4, R_5, and R_6 generations of MON 88701 was demonstrated (Figure VI-13). An *E. coli*-produced PAT (*bar*) standard (0.5 ng) was used as a reference for the identification of the PAT (*bar*) protein. The presence of PAT (*bar*) protein in MON 88701 leaf tissue samples was determined by visual comparison of the bands produced in the multiple breeding generations (Figure VI-13, Lanes 3–7) to the PAT (*bar*) reference standard (Figure VI-13, Lane 1). As shown in Figure VI-13, PAT (*bar*) protein was present in multiple generations of MON 88701 tissue samples and migrated with a mobility indistinguishable from that of the *E. coli*-produced protein standard analyzed on the same western blot. As expected, the PAT (*bar*) protein was not detected in the conventional control extract (Figure VI-13, Lane 8).

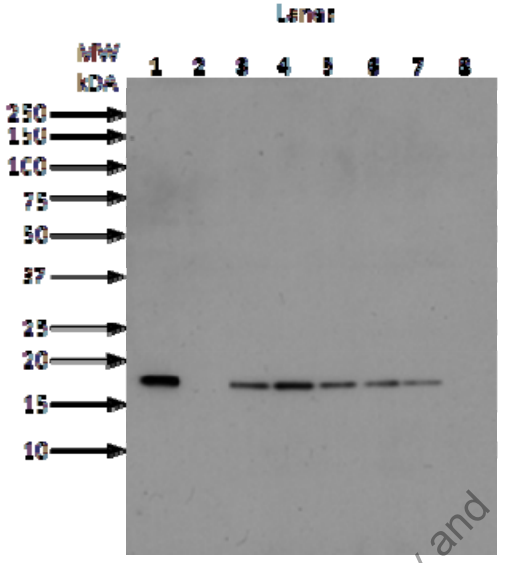


Figure VI-13. Presence of PAT (*bar*) Protein in Multiple Generations of MON 88701

Aliquots of extracts from five generations of MON 88701 leaf tissues, conventional control leaf tissues, *E. coli*-produced PAT (*bar*) protein standard, and molecular weight markers were subjected to SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membrane was incubated with goat anti-PAT (*bar*) antibody and immunoreactive bands visualized through the use of ECL reagents. The image represents a 20 sec exposure. Arrows denote the size of the protein, in kiloDaltons (kDA), obtained from the Precision Plus Protein Dual Color Standards (Bio-Rad) transferred to the Western membrane. Lane designations are as follows:

Lane	Sample	Amount (µl)
1	*E. coli*-produced PAT (*bar*) protein	20 (0.5 ng)
2	Empty	-
3	R_2 Generation	20
4	R_3 Generation	20
5	R_4 Generation	20
6	R_5 Generation	20
7	R_6 Generation	20
8	Conventional Control – Coker 130	20

VI.F. Assessment of the Potential Allergenicity, Toxicity and Dietary Safety of the MON 88701 DMO and PAT (*bar*) Proteins

History of safe use of the introduced protein is a key consideration in the potential for allergenicity and toxicity and for assessment of dietary safety. The history of safe use of MON 88701 DMO and PAT (*bar*) proteins have been previously addressed in Section VI.B.

According to the Codex Alimentarius Commission "if the protein(s) resulting from the inserted gene is present in the food, it should be assessed for potential allergenicity in all cases" (Codex Alimentarius, 2009). The only human food currently produced from cottonseed is refined, bleached, and deodorized (RBD) oil, and to a smaller extent, linters. RBD oil contains undetectable amounts of protein (Reeves and Weihrauch, 1979) and linters are a highly processed product composed of nearly pure (*i.e.*, >99.9%) cellulose (NCPA, 2002a; Nida et al., 1996). Because RBD oil and linters are processed fractions that contain negligible amounts of total protein and the MON 88701 DMO and PAT (*bar*) proteins represents a very small portion of the total protein in the cottonseed of MON 88701 (Sections VI.F.1.2. and VI.F.2.2.), an allergenicity, toxicity, and dietary safety assessment is primarily considered a theoretical assessment due to the extremely low exposure anticipated for food uses of MON 88701.

Nevertheless, following the guidelines adopted by the Codex Alimentarius Commission, an assessment of potential allergenicity of the introduced proteins has been conducted by comparing the characteristics of the introduced protein to characteristics of known allergens (Codex Alimentarius, 2009). 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; 4) the protein is rapidly digested in mammalian gastrointestinal systems; and 5) the protein is not stable to heat treatment. The MON 88701 DMO and PAT (*bar*) proteins in MON 88701 have been assessed for their potential allergenicity according to these safety assessment guidelines.

The assessment of the potential toxicity of an introduced protein is based on comparing the biochemical characteristics of the introduced protein to characteristics of known toxins. The MON 88701 DMO and PAT (*bar*) proteins in MON 88701 have been assessed for their potential toxicity based on these criteria and are discussed in Section VI.F.3.

VI.F.1. Assessment of Potential Allergenicity of MON 88701 DMO Protein

VI.F.1.1. Safety of the Donor Organism

As described in Section III, the *dmo* gene is derived from the bacterium *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993). *S. maltophilia* is

ubiquitous in the environment and is found associated with the rhizosphere of plants. *S. maltophilia* can be found in a variety of foods and feeds, and is widespread in the home environment (Section III). Exposure to *S. maltophilia* is incidental to its presence in food. It has been isolated from "ready to eat" salads, vegetables, frozen fish, milk, and poultry (Qureshi et al., 2005; Ryan et al., 2009). *S. maltophilia* can be found in healthy individuals without causing any harm to human health (Denton et al., 1998) and infections caused by *S. maltophilia* are extremely uncommon (Cunha, 2010). Strains have been found in the transient flora of hospitalized patients as a commensal organism (Echemendia, 2010) and, similar to the indigenous bacteria of the gastrointestinal tract, *S. maltophilia* can be an opportunistic pathogen (Berg, 1996). As such, *S. maltophilia* is of low virulence in immuno-compromised patients where a series of risk factors (severe debilitation, the presence of indwelling devices such as ventilator tubes or catheters, for prolonged periods of time and prolonged courses of antibiotics) must occur for colonization by *S. maltophilia* in humans (Ryan et al., 2009). Therefore, infections by *S. maltophilia* almost exclusively occur in hospital settings, in which case they are only present in a minimal percentage of infections (Ryan et al., 2009). Finally, *S. maltophilia* has not been reported to be source of allergens.

The ubiquitous presence of *S. maltophilia* in the environment, the presence in healthy individuals without causing infections, the incidental presence in foods without any adverse safety reports, and the lack of reported allergenicity establishes the safety of the donor organism.

VI.F.1.2. The MON 88701 DMO Protein as a Proportion of Total Protein

The MON 88701 DMO protein was detected in all plant tissues assayed, at a number of time points during the growing season (Table VI-12). Concerns for assessing potential allergenicity are less relevant to MON 88701 since the only human food currently produced from cottonseed is refined, bleached, and deodorized (RBD) oil, and to a smaller extent, linters. RBD oil contains undetectable amounts of protein (Reeves and Weihrauch, 1979) and linters are a highly processed product composed of nearly pure (*i.e.*, >99%) cellulose (NCPA, 2002a; Nida et al., 1996). Because RBD oil and linters are processed fractions that contain negligible amounts of total protein an allergenicity assessment is primarily considered a theoretical assessment. However, since cottonseed is the source of cottonseed oil and linters, cottonseed is the most appropriate tissue to assess the potential food allergenicity of MON 88701. The mean level of MON 88701 DMO protein in cottonseed of MON 88701 is 21 µg/g dw. The mean percent dry weight of total protein in seed of MON 88701 is 27.91% (or 279,100 µg/g; Table VII-2). The percentage of MON 88701 DMO protein in MON 88701 seed is calculated as follows:

(21 µg/g ÷ 279,100 µg/g) × 100% ≈ 0.008% or 80 ppm of total cottonseed protein

Therefore, the MON 88701 DMO protein represents a very small portion of the total protein in the cottonseed of MON 88701 and due to the harsh conditions used in

cottonseed processing is most likely absent in the oil and linters that are used for food production.

VI.F.1.3. Structural Similarity of MON 88701 DMO to Known Allergens

The Codex guidelines for the evaluation of the allergenicity potential of introduced proteins (Codex Alimentarius, 2009) are based on the comparison of amino acid sequences between introduced proteins and allergens, where 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 recommends that a sliding window search with a scientifically justified peptide size could be used to identify immunologically relevant peptides in otherwise unrelated proteins. Therefore, the extent of sequence similarities between the MON 88701 DMO protein sequence and known allergens, gliadins, and glutenins was assessed using the FASTA sequence alignment tool and an eight-amino acid sliding window search (Codex Alimentarius, 2009; Thomas et al., 2005). The methods used are summarized below and detailed in Appendix E. The data generated from these analyses confirm that the MON 88701 DMO protein does not share amino acid sequence similarities with known allergens, gliadins, or glutenins.

The FASTA program directly compares amino acid sequences (*i.e.*, primary, linear protein structure). This 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. By definition, homologous proteins have common secondary structures, and three-dimensional configuration, and, consequently, may share similar functions. The allergen, gliadin, and glutenin sequence database (AD_2011) was obtained from Food Allergy Research and Resource Program Database (FARRP, 2011) and was used for the evaluation of sequence similarities shared between the MON 88701 DMO protein and all proteins. The AD_2011 database contains 1,491 sequences. When used to align the sequence of the introduced protein to each protein in the database, the FASTA algorithm produces an *E*-score (expectation score) for each alignment. 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 low degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences which have an *E*-score of less than or equal to 1×10^{-5} are considered to have meaningful homology. Results indicate that the MON 88701 DMO protein sequence does not share meaningful similarity with sequences in the allergen database. No alignment met nor exceeded the threshold of 35% identity over 80 amino acids recommended by Codex Alimentarius (2009) or had an *E*-score of less than or equal to 1×10^{-5}.

A second bioinformatic tool, an eight-amino acid sliding window search, was used to specifically identify short linear polypeptide matches to known allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may contain

smaller immunologically meaningful epitopes. An amino acid sequence may have allergenic potential if it has an exact sequence identity of at least eight linearly contiguous amino acids with a potential allergen epitope (Hileman et al., 2002; Metcalfe et al., 1996). Using a sliding window of less than eight amino acids can produce matches containing considerable uncertainty depending on the length of the query sequence (Silvanovich et al., 2006) and are not useful to the allergy assessment process (Thomas et al., 2005). No eight contiguous amino acid identities were detected when the MON 88701 DMO protein sequence was compared to the proteins in the AD_2011 sequence database.

The bioinformatic results demonstrated there were no biologically relevant sequence similarities to allergens when the MON 88701 DMO protein sequence was used as a query for a FASTA search of the AD_2011 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the MON 88701 DMO protein sequence and proteins in the allergen database. These data show that the MON 88701 DMO protein sequence lacks both structurally and immunologically relevant similarities to known allergens, gliadins, and glutenins.

VI.F.1.4. Digestibility of MON 88701 DMO Protein

Proteins introduced into commercial crops using biotechnology are evaluated for their safety for human and animal consumption. Proteins are an essential dietary component for humans and animals, and most are rapidly degraded to the component amino acids for nutritional purposes (Hammond and Jez, 2011). Therefore, evaluating a protein's intrinsic sensitivity to proteolytic digestion with enzymes of the gastrointestinal tract is a key aspect to understanding the safety of any introduced proteins in GM crops. One characteristic of protein toxins and many allergens is their ability to withstand proteolytic digestion by enzymes present in the gastrointestinal tract (Astwood et al., 1996; Moreno et al., 2005; Vassilopoulou et al., 2006; Vieths et al., 1999). Allergenic proteins or their fragments, when presented to the intestinal immune system, can lead to a variety of gastrointestinal and systemic manifestations of immune-mediated allergy. The complete enzymatic degradation of an ingested protein by exposure to gastric pepsin and intestinal pancreatic proteases makes it highly unlikely that either the intact protein or protein fragment(s) will reach the absorptive epithelial cells of the small intestine where antigen processing cells reside (Moreno et al., 2005). To reach these cells, protein or protein fragment(s) must first pass through the stomach where they are exposed to pepsin and then the duodenum where they are exposed to pancreatic fluid containing a mixture of enzymes called pancreatin. Therefore, the digestive fate of MON 88701 DMO was assessed using assays with both simulated gastric fluid (SGF) containing pepsin and simulated intestinal fluid (SIF) containing pancreatin.

A correlation between protein digestibility in simulated gastric fluid (SGF) and the likelihood of the protein being an allergen has been previously reported (Astwood et al., 1996), but this correlation is not complete (Fu et al., 2002). The SGF assay protocol has been standardized based on results obtained from an international, multi-laboratory ring

study (Thomas et al., 2004). The study showed that the results of *in vitro* pepsin digestion assays were reproducible when a standard protocol was followed. The susceptibility of the MON 88701 DMO protein to pepsin digestion was assessed using this standardized *in vitro* pepsin digestion protocol that utilized a physiologically relevant acidic pH to simulate conditions in a stomach.

VI.F.1.4.1. Digestibility of MON 88701 DMO in SGF

The digestibility of *E. coli*-produced MON 88701 DMO in SGF was assessed using two methods: visual analysis of a Brilliant Blue G Colloidal stained SDS-PAGE gel and visual analysis of a western blot probed with an anti-DMO polyclonal antibody. Methods related to SGF analysis are described in Appendix F.

Digestibility of *E. coli*-produced MON 88701 DMO in SGF was evaluated over time by analyzing digestion mixtures incubated for targeted time intervals. For SDS-PAGE analysis, approximately 1 µg of total protein was analyzed for each time point (Figure VI-14). The controls, SGF N0 and SGF N7 (Figure VI-14, Lanes 2 and 13), which evaluate the stability of the pepsin in the test system (SGF) lacking the MON 88701 DMO protein, demonstrated that the pepsin was observed as a stained protein band at ~38 kDa throughout the experimental phase.

Visual examination of SDS-PAGE data showed that the intact *E. coli*-produced MON 88701 DMO protein was completely digested within 0.5 min of incubation in SGF (Figure VI-14, Lane 5). The pepsin (~38 kDa) and the MON 88701 DMO (~39 kDa) protein migrated to similar positions in this gel system. However, the intensity of the stained protein band at SGF time zero (T0) (Figure VI-14, Lane 4 pepsin plus DMO) appears to be the combination of the intensity of both proteins when they are each run separately (Figure VI-14, Lane 2, pepsin alone and Lane 3, DMO alone). After 0.5 min digestion (SGF T1), the intensity of the ~38 kDa band was reduced to approximately the same level as observed for pepsin alone (SGF N0) (Figure VI-14, compare Lanes 2 and 5) suggesting that the intact MON 88701 DMO protein was digested. In addition, no fragments of the MON 88701 DMO protein were observed at 0.5 min of digestion or thereafter.

No change in the *E. coli*-produced MON 88701 DMO protein band intensity was observed in the absence of pepsin in the controls SGF P0 and SGF P7 (Figure VI-14, Lanes 3 and 12) indicating that the digestion of the MON 88701 DMO protein 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.

For the SDS-PAGE analysis, the LOD of the MON 88701 DMO protein was not determined because intact MON 88701 DMO protein and pepsin were not separated in this gel system. Therefore, the percent digestion of intact MON 88701 DMO protein was not estimated on Brilliant Blue G Colloidal stained gel. Digestion of MON88701 DMO protein was confirmed and the LOD was calculated using western blot analysis Figure VI-15). In summary the results from visual analysis of a Brilliant Blue G Colloidal

stained SDS-PAGE gel show that *E coli*-produced MON 88701 DMO protein is rapidly digested in SGF.

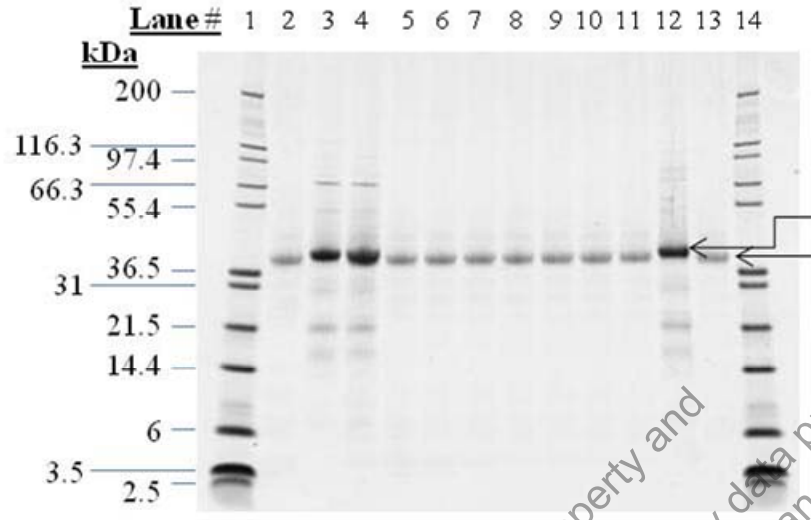


Figure VI-14. Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-produced MON 88701 DMO Protein in Simulated Gastric Fluid

Brilliant Blue G Colloidal stained SDS-PAGE gels were used to assess the digestibility of MON 88701 DMO in SGF. Proteins were subjected to SDS-PAGE and detected by staining with Brilliant Blue G Colloidal stain. *E. coli*-produced MON 88701 DMO protein was loaded at 1 µg per lane based on pre-digestion concentrations. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. Empty lanes on the gel were cropped from the image. T = time. Lane designations are as follows:

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0 (No DMO control)	0
3	SGF P0 (No pepsin control)	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 (No pepsin control)	60
13	SGF N7 (No DMO control)	60
14	Mark 12 MWM	-

For the western blot analysis of MON 88701 DMO digestibility in SGF, the blot used to assess the stability of the MON 88701 DMO protein to pepsin digestion (Figure VI-15 Panel A) was run concurrently with a blot to estimate the LOD of the intact MON 88701 DMO protein (Figure VI-15 Panel B). Twenty ng of *E. coli*-produced MON 88701 DMO protein was analyzed by western blot for each time point. No immunoreactive bands were observed in controls SGF N0 and SGF N7 (Figure VI-15 Panel A, Lanes 2 and 13). This result indicates that non-specific interactions between the test system components and the DMO-specific antibody did not occur under these experimental conditions.

Western blot analysis demonstrated that the MON 88701 DMO protein was digested below the LOD within 0.5 min of incubation in SGF (Figure VI-15 Panel A, Lane 5). The LOD of the MON 88701 DMO protein was visually estimated to be 0.2 ng (Figure VI-15 Panel B, Lane 7). The LOD estimated for the MON 88701 DMO protein was used to calculate the maximum amount of intact MON 88701 DMO protein that could remain visually undetected after digestion. This corresponded to approximately 1.0% of the total MON 88701 DMO protein loaded. Based on the western blot LOD for the MON 88701 DMO protein, it can be concluded that within 0.5 min more than 99% (100% - 1.0% = 99%) of the intact MON 88701 DMO protein was digested and no other fragments were observed.

No change in the MON 88701 DMO protein band intensity was observed in the absence of pepsin in the controls SGF P0 and P7 (Figure VI-15 Panel A, Lanes 3 and 12). This result reaffirms that the MON 88701 DMO protein was stable in the test system without pepsin.

As indicated on the LOD blot, 2 ng of intact MON 88701 DMO was readily detected by the antibody and blotting methods used for this analysis (Figure VI-15, Panel B, Lane 4). Thus, the 20 ng per lane loaded to assess digestibility in SGF represented a heavy loading of the MON 88701 DMO protein for western blot analysis; this amount of MON 88701 DMO protein was applied to increase the probability that any intact protein or protein fragments of MON 88701 DMO would be visible. Under those loading conditions, minor aggregation and breakdown products of the MON 88701 DMO protein were observed in the absence of digestion (Figure VI-15, Panel A, Lanes 3, 4, and 12).

In summary, the western analysis demonstrate that greater than 99% of the *E coli*-produced MON 88701 DMO protein was digested in SGF within 0.5 min. and other immunoreactive bands were not detected.

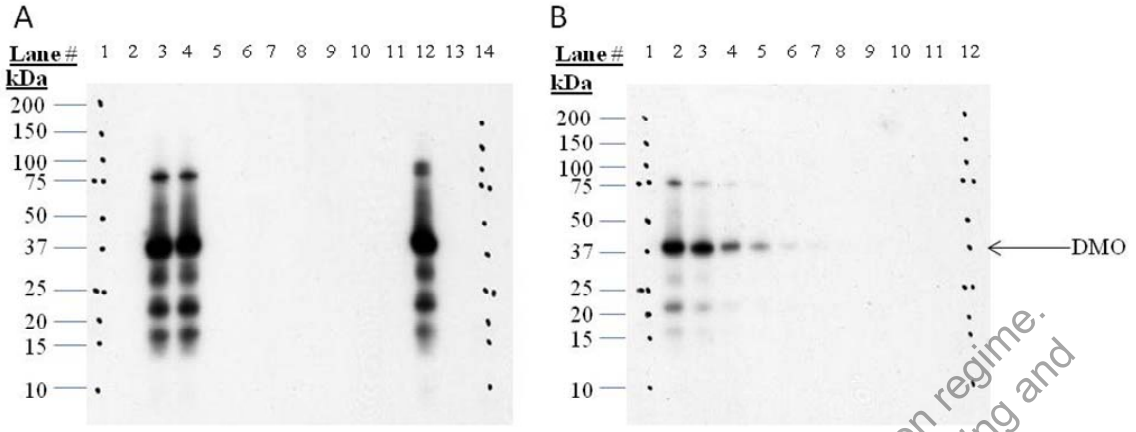


Figure VI-15. Western Blot Analysis of Purified *E. coli*-produced MON 88701 DMO Protein in Simulated Gastric Fluid

Western blots probed with an anti-DMO antibody were used to assess the digestibility of MON 88701 DMO in SGF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. Empty lanes on the gels were cropped from the images. A 0.5 min exposure is shown. T = time. Panel A corresponds to *E. coli*-produced MON 88701 DMO protein digestion in SGF. Based on pre-digestion protein concentrations, 20 ng of total protein was loaded in each lane containing DMO protein (SGF T0-SGF T7). Panel B corresponds to the analysis to determine LOD of *E. coli*-produced MON 88701 DMO. Indicated amounts of the DMO protein from the SGF T0 sample were loaded to estimate the LOD of the protein. Lane designations are as follows:

Panel A		
Lane	**Sample**	**Incubation Time (min)**
1	Precision Plus MWM	-
2	SGF N0 (No DMO control)	0
3	SGF P0 (No Pepsin Control)	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	Precision Plus MWM	-

Panel B		
Lane	**Sample**	**Amount (ng)**
1	Precision Plus MWM	-
2	T0, protein+SGF	10.0
3	T0, protein+SGF	4.0
4	T0, protein+SGF	2.0
5	T0, protein+SGF	1.0
6	T0, protein+SGF	0.4
7	T0, protein+SGF	0.2
8	T0, protein+SGF	0.1
9	T0, protein+SGF	0.05
10	T0, protein+SGF	0.02
11	T0, protein+SGF	0.01
12	Precision Plus MWM	-

VI.F.1.4.2. Digestibility of MON 88701 DMO in SIF

The digestibility of the *E. coli*-produced MON 88701 DMO protein in SIF was assessed by western blot (Figure VI-16). The western blot used to assess the *in vitro* digestibility of the MON 88701 DMO protein in SIF (Figure VI-16, Panel A) was run concurrently with a western blot used to estimate the LOD (Figure VI-16 Panel B) of the intact MON 88701 DMO protein in this assay. Methods for SIF analysis are described in Appendix F. The gel used to assess the digestibility of the MON 88701 DMO protein in SIF by western blot was loaded with 20 ng total protein (based on pre-digestion protein concentrations) for each of the incubation time points. No immunoreactive bands were observed in controls SIF N0 and SIF N8, which represent the SIF test system without *E. coli*-produced MON 88701 DMO protein (Figure V1-16, Panel A, Lanes 2 and 14). This result demonstrates the absence of non-specific antibody interactions with the SIF test system.

Western blot analysis demonstrated *E. coli*-produced MON 88701 DMO protein was digested to a level below the LOD within 5 min of incubation in SIF (Figure V1-16 Panel A, Lane 5), the first time point assessed. The LOD of the MON 88701 DMO protein was visually estimated to be 0.2 ng (Figure VI-16, Panel B, Lane 7). This LOD was used to calculate the maximum amount of MON 88701 DMO protein that could remain visually undetected after digestion, which corresponded to approximately 1% of the total protein loaded. Therefore, based on the LOD, more than 99% (100% - 1% = 99%) of the MON 88701 DMO protein was digested in SIF within 5 min. A faint immunodetectable band of less than ~12 kDa was observed at the 5 min time point in SIF, but was gone by 15 min. No other immunoreactive bands were detected in any other digestion specimens.

Comparison of the signal for *E. coli*-produced MON 88701 DMO protein in the controls SIF P0 and SIF P8 (Figure VI-16 Panel A, Lanes 3 and 13), which represent the test system without pancreatin, suggests that MON 88701 DMO showed some tendency to aggregate when incubated in the test system buffer at 37 °C for 24 h. However, the MON 88701 DMO protein is still readily observed in the SIF P8 sample, indicating that the lack of MON 88701 DMO in the SIF T1 sample is due to pancreatin activity rather than protein aggregation.

In summary, the results from this analysis demonstrate that greater than 99% of the *E coli*-produced MON 88701 DMO protein was digested in SIF within 5 min.

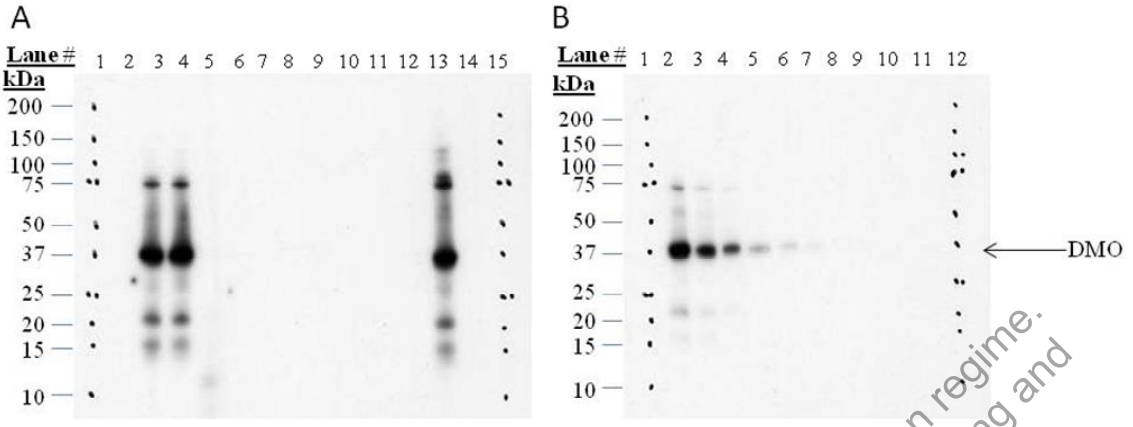


Figure VI-16. Western Blot Analysis of Purified *E. coli*-produced MON 88701 DMO Protein in Simulated Intestinal Fluid

Western blots probed with an anti-DMO antibody were used to assess the digestibility of MON 88701 DMO in SIF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. A 0.5 min exposure is shown. T = time. Panel A corresponds to *E. coli*-produced MON 88701 DMO protein digestion in SIF. Based on pre-digestion protein concentrations, 20 ng of the total protein was loaded in each lane containing DMO protein. Panel B corresponds to the analysis to determine LOD of *E. coli*-produced MON 88701 DMO. Indicated amounts of the DMO protein from the SIF T0 sample were loaded to estimate the LOD of the protein. Empty lanes on the blot were cropped from the image. Lane designations are as follows:

Panel A

Lane	Sample	Incubation Time
1	Precision Plus MWM	-
2	SIF N0 (No DMO control)	0
3	SIF P0 (No pancreatin control)	0
4	SIF T0	0
5	SIF T1	5 min
6	SIF T2	15 min
7	SIF T3	30 min
8	SIF T4	1 hr
9	SIF T5	2 hr
10	SIF T6	4 hr
11	SIF T7	8 hr
12	SIF T8	24 hr
13	SIF P8	24 hr
14	SIF N8	24 hr
15	Precision Plus MWM	-

Panel B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	T0, protein+SIF	10
3	T0, protein+SIF	4.0
4	T0, protein+SIF	2.0
5	T0, protein+SIF	1.0
6	T0, protein+SIF	0.4
7	T0, protein+SIF	0.2
8	T0, protein+SIF	0.1
9	T0, protein+SIF	0.05
10	T0, protein+SIF	0.02
11	T0, protein+SIF	0.01
12	Precision Plus MWM	-

VI.F.1.4.3. Digestibility of MON 88701 DMO - Conclusions

Digestibility of the MON 88701 DMO protein was evaluated in SGF and SIF. The results of the study demonstrate that greater than 99% of the *E coli*-produced MON 88701 DMO protein was digested in SGF within 0.5 min, when analyzed by Brilliant Blue G Colloidal stained SDS-PAGE and by western blot using a DMO-specific antibody. Additionally, at least 99% of the MON 88701 DMO protein was digested within 5 min during incubation in SIF.

Results from the digestibility experiments show that *E coli*-produced MON 88701 DMO protein is rapidly digested in the *in vitro* model gastrointestinal digestive system. Rapid digestion of the *E coli*-produced MON 88701 protein in SGF and SIF support the conclusion that the MON 88701 DMO protein is highly unlikely to pose a safety concern to human and animal health.

VI.F.1.5. Heat Stability of the Purified MON 88701 DMO Protein

Temperature can have a profound effect on the structure and function of proteins. Cottonseed processing involves treatment of cottonseed for hours with temperatures from 88 ºC to greater than 130 ºC for meal processing and up to 230 ºC for deodorization of the oil (Harris, 1981; NCPA, 1993). In addition the processing of linters involves processing at temperatures greater than 100 ºC (AOCS, 1991). Therefore it is reasonable to assume that the conditions encountered during the processing of cottonseed and linters from MON 88701 will have an effect on the functional activity and structure of MON 88701 DMO protein when consumed in different feed products derived from MON 88701 and in human food products in the unlikely event protein is present.

The effect of heat treatment on the activity of *E coli*-produced MON 88701 DMO protein was evaluated using purified protein. The method for evaluating heat stability is described in Appendix G. Heat-treated samples and an unheated control sample of *E coli*-produced MON 88701 DMO protein were analyzed: 1) using a functional assay to assess the impact of temperature on the enzymatic activity of MON 88701 DMO protein; and 2) using SDS-PAGE to assess the impact of temperature on protein integrity.

Aliquots of *E coli*-produced MON 88701 DMO were heated to 25, 37, 55, 75, and 95 °C for 15 and 30 min, while a separate aliquot of *E coli*-produced MON 88701 DMO was maintained on ice for the duration of the heat treatments to serve as a temperature control. The effect of heat treatment on the activity of MON 88701 DMO was evaluated using a functional activity assay (Appendix B, Section B.5.6.1., and Appendix G). The effect of heat treatment on the integrity of the MON 88701 DMO protein was evaluated using SDS-PAGE analysis of the heated and temperature control MON 88701 DMO samples.

The effects of heating on the functional activity of *E coli*-produced MON 88701 DMO are presented in Tables VI-14 and VI-15. The functional activity of MON 88701 DMO was unaffected at 25 ºC and 37 ºC for 15 and 30 min. The functional activity of MON 88701 DMO was below the LOQ of the assay following incubation at 55 ºC or

higher for 15 min or more, indicating that the majority of the functional activity of MON 88701 DMO had been lost during heating. These results suggest that temperature has a considerable effect on the activity of MON 88701 DMO.

Analysis by SDS-PAGE stained with Brilliant Blue G Colloidal demonstrated that the MON 88701 DMO control treatment and reference standard contain a major band at ~38 kDa, corresponding to the MON 88701 DMO protein (Figures VI-17 and VI-18, Lanes 2 and 8). No apparent decrease in the intensity of this band was observed in heat-treated MON 88701 DMO at any temperatures at 15 or 30 min (Figure VI-17, Lanes 3–7 and Figure VI-18, Lanes 3–7).

These data demonstrate that *E. coli*-produced MON 88701 DMO remains intact, but is deactivated at temperatures 55 ºC and above. Therefore, in the unlikely event that processed cottonseed oil or linters contain protein (NCPA, 2002a; Nida et al., 1996; Reeves and Weihrauch, 1979), it is reasonable to conclude that MON 88701 DMO protein would not be consumed as an active protein in food or feed products due to standard processing practices that include heat treatment.

Table VI-14. Activity of *E. coli*-produced MON 88701 DMO Protein after 15 Minutes at Elevated Temperatures

Temperature	Functional Activity (nmol DCSA × minute^{-1} × mg^{-1})[1]	Relative Activity[2] (% of unheated control)
Unheated Control (0 ºC)	6.74	100%
25 ºC	6.29	93%
37 ºC	7.48	111%
55 ºC	Below LOQ[3]	<22%
75 ºC	Below LOQ[3]	<22%
95 ºC	Below LOQ[3]	<22%

[1] Mean specific activity determined from n=3.
[2] Relative activity = [activity of sample/ activity of unheated control] × 100; DMO protein activity of unheated control was assigned 100%.
[3] The LOQ is 1.5 nmol DCSA×min^{-1}×mg^{-1} of *E. coli*-produced MON 88701 DMO protein.

Table VI-15. Activity of *E. coli*-produced MON 88701 DMO Protein after 30 Minutes at Elevated Temperatures

Temperature	Functional Activity (nmol DCSA × minute^{-1} × mg^{-1})[1]	Relative Activity (% of unheated control)
Unheated Control (0 ºC)	6.74	100%
25 ºC	7.36	109%
37 ºC	7.28	108%
55 ºC	Below LOQ[3]	<22%
75 ºC	Below LOQ[3]	<22%
95 ºC	Below LOQ[3]	<22%

[1] Mean specific activity determined from n=3.
[2] Relative activity = [activity of sample/ activity of unheated control] × 100; DMO protein activity of unheated control was assigned 100%.
[3] The LOQ is 1.5 nmol DCSA×min^{-1}×mg^{-1} of *E. coli*-produced MON 88701 DMO protein.

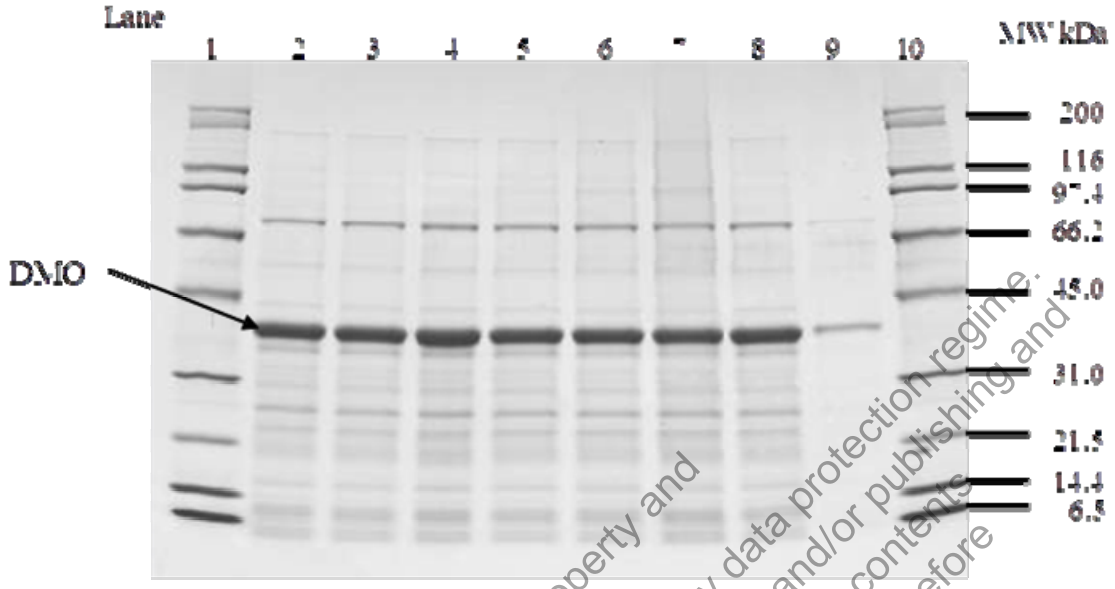


Figure VI-17. SDS-PAGE of *E. coli*-produced MON 88701 DMO Protein Following Heat Treatment for 15 Minutes

Heat-treated samples of *E. coli*-produced MON 88701 DMO (3.3 µg total protein) subjected to SDS-PAGE and stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10. Lane designations are as follows:

Lane	Description	Amount (µg)
1	Broad Range MWM	5.0
2	*E. coli*-produced MON 88701 DMO Unheated Control (0 ºC)	3.3
3	*E. coli*-produced MON 88701 DMO 25 ºC	3.3
4	*E. coli*-produced MON 88701 DMO 37 ºC	3.3
5	*E. coli*-produced MON 88701 DMO 55 ºC	3.3
6	*E. coli*-produced MON 88701 DMO 75 ºC	3.3
7	*E. coli*-produced MON 88701 DMO 95 ºC	3.3
8	*E. coli*-produced MON 88701 DMO Reference	3.3
9	*E. coli*-produced MON 88701 DMO Reference	0.33
10	Broad Range MWM	5.0

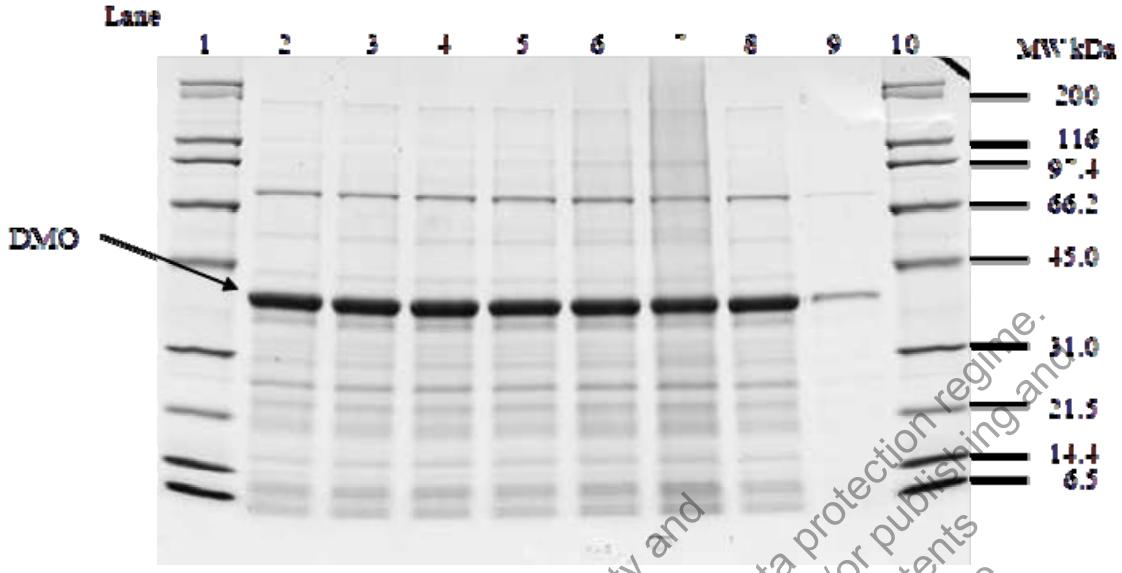


Figure VI-18. SDS-PAGE of *E. coli*-produced MON 88701 DMO Protein Following Heat Treatment for 30 Minutes

Heat-treated samples of *E. coli*-produced MON 88701 DMO (3.3 µg total protein) subjected to SDS-PAGE and stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10. Lane designations are as follows:

Lane	Description	Amount (µg)
1	Broad Range MWM	5.0
2	*E. coli*-produced MON 88701 DMO Unheated Control (0 ºC)	3.3
3	*E. coli*-produced MON 88701 DMO 25 ºC	3.3
4	*E. coli*-produced MON 88701 DMO 37 ºC	3.3
5	*E. coli*-produced MON 88701 DMO 55 ºC	3.3
6	*E. coli*-produced MON 88701 DMO 75 ºC	3.3
7	*E. coli*-produced MON 88701 DMO 95 ºC	3.3
8	*E. coli*-produced MON 88701 DMO Reference	3.3
9	*E. coli*-produced MON 88701 DMO Reference	0.33
10	Broad Range MWM	5.0

VI.F.2. Assessment of Potential Allergenicity of PAT (*bar*) Protein

The safety of PAT protein present in biotechnology-derived crops has been extensively assessed (ILSI-CERA, 2011) and in 1997 a tolerance exemption was issued for PAT proteins by U.S. EPA (1997). Numerous glufosinate-tolerant products including those in cotton, corn, soy, canola, sugarbeet, and rice have been reviewed by the FDA (U.S. FDA, 1995a; 1995c; 1995b; 1997; 1998b; 1998a; 1999; 2002) with no concerns identified. Further, a comprehensive study on the safety of PAT proteins present in biotechnology-derived crops (Hérouet et al., 2005) demonstrated the safety of the donor organism, lack of sequence homology to know allergens, rapid degradation in gastric and intestinal fluids and loss of functional activity following heat treatment. Hérouet et al. concluded that there is a reasonable certainty of no harm resulting from the inclusion of PAT proteins in human food or animal feed. The data below were generated to confirm the previously documented safety assessments.

VI.F.2.1 Safety of the Donor Organism

As described in Section III, the *bar* gene is derived from the bacterium *Streptomyces hygroscopicus* (Thompson et al., 1987). The ubiquitous presence of *S. hygroscopicus* in the environment, the widespread human exposure without any adverse safety or allergenicity reports, and the successive reviews of several glufosinate-tolerant events with no safety or allergenicity issues identified establishes the safety of the donor organism.

VI.F.2.2. The MON 88701-produced PAT (*bar*) Protein as a Proportion of Total Protein

The MON 88701-produced PAT (*bar*) protein was detected in all plant tissues assayed, at a number of time points during the growing season (Table VI-13). Concerns for assessing potential allergenicity are less relevant to MON 88701 since the only human food currently produced from cottonseed is refined, bleached, and deodorized (RBD) oil, and to a smaller extent, linters. RBD oil contains undetectable amounts of protein (Reeves and Weihrauch, 1979) and linters are a highly processed product composed of nearly pure (*i.e.*, >99%) cellulose (NCPA, 2002a; Nida et al., 1996). Because RBD oil and linters are processed fractions that contain negligible amounts of total protein an allergenicity assessment is primarily considered a theoretical assessment. However, since cottonseed is the source of cottonseed oil and linters, cottonseed is the most appropriate tissue to assess the potential food allergenicity of MON 88701. The mean level of MON 88701-produced PAT (*bar*) protein in seed of MON 88701 is 6.6 µg/g dw. The mean percent dry weight of total protein in seed of MON 88701 is 27.91% (or 279,100 µg/g; Table VII-2). The percentage of MON 88701-produced PAT (*bar*) protein in MON 88701 seed is calculated as follows:

(6.6 µg/g ÷ 279,100 µg/g) × 100% ≈ 0.002% or 20 ppm of total cottonseed protein

Therefore, the MON 88701-produced PAT (*bar*) protein represents a very small portion of the total protein in the cottonseed of MON 88701 and due to the harsh conditions used in cottonseed processing is most likely absent in the oil and linters that are used for food production.

VI.F.2.3. Structural Similarity of PAT (*bar*) to Known Allergens

The scientific justification and methodology for these analyses are provided in Section VI.F.1.3. The methods used are detailed in Appendix E. The results are provided below.

The FASTA analysis (as described in Section VI.F.1.3.) indicate that the PAT (*bar*) protein sequence does not share significant similarity with sequences in the allergen database. No alignment met or exceeded the threshold of 35% identity over 80 amino acids recommended by Codex Alimentarius (2009) or had an *E*-score of less than or equal to 1×10^{-5}.

The bioinformatic analysis of the eight-amino acid sliding window search (as described in Section VI.F.1.3) demonstrated there were no biologically-relevant sequence similarities to allergens when the PAT (*bar*) protein sequence was used as a query for a FASTA search of the AD_2011 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the PAT (*bar*) protein sequence and proteins in the allergen database. These data show that the PAT (*bar*) protein sequence lacks both structurally and immunologically relevant sequence similarities to known allergens, gliadins, and glutenins. This is comparable with previously published safety assessments of PAT (*bar*) protein (Hérouet et al., 2005).

VI.F.2.4. Digestibility of the PAT (*bar*) Protein

The scientific justification and basic methodology are described in Section VI.F.1.4. Materials and methods for the SGF and SIF assays are detailed in Appendix F. The results are described below.

VI.F.2.4.1. Digestibility of PAT (*bar*) in SGF

The digestibility of *E. coli*-produced PAT (*bar*) in SGF was assessed using two methods: visual analysis of a Brilliant Blue G Colloidal stained SDS-PAGE gel and visual analysis of a western blot probed with an anti-PAT (*bar*) polyclonal antibody. For this assessment, a separate SDS-PAGE gel containing dilutions of the pre-digestion test sample was run concurrently to estimate the limit of detection (LOD) of the undigested *E. coli*-produced PAT (*bar*) protein. Methods related to SGF analysis are described in Appendix F.

Digestibility of *E. coli*-produced PAT (*bar*) in SGF was evaluated over time by analyzing digestion mixtures incubated for targeted time intervals. For SDS-PAGE analysis, approximately 1 µg of total protein was analyzed for each timepoint (Figure VI-19 Panel A). The controls, SGF N0 and SGF N7 (Figure VI-19 Panel A, Lanes 2 and 13), which

evaluate the stability of the pepsin in the test system (SGF) lacking the PAT (*bar*) protein, demonstrated that the pepsin was observed as a stained protein band at ~38 kDa throughout the experimental phase.

No change in the PAT (*bar*) protein band intensity was observed over time in the absence of pepsin (compare SGF P0 to SGF P7; Figure VI-19 Panel A, Lanes 3 and 12) indicating that the digestion of the PAT (*bar*) protein 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.

Visual examination of SDS-PAGE data showed that the intact PAT (*bar*) protein was completely digested within 0.5 min of incubation in SGF (Figure VI-19 Panel A, Lane 5). For the SDS-PAGE analysis, the LOD of the PAT (*bar*) protein was visually estimated to be 13 ng, or 0.013 µg (Figure VI-19 Panel B, Lane 6). This LOD used to calculate the maximum amount of intact PAT (*bar*) protein that could remain visually undetected after digestion, which corresponded to approximately 1.3% of the total protein loaded. Based on that LOD, more than 98.7% (100% - 1.3% = 98.7%) of the intact PAT (*bar*) protein was digested within 0.5 min of incubation in SGF. This is comparable with previously published safety assessments of PAT (*bar*) protein (Hérouet et al., 2005).

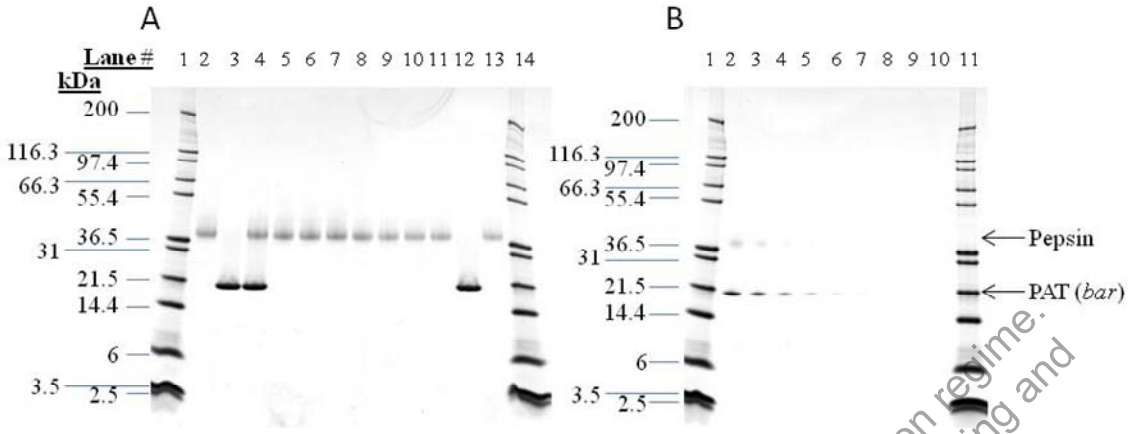


Figure VI-19. Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-produced PAT (*bar*) Protein in Simulated Gastric Fluid

Brilliant Blue G Colloidal stained SDS-PAGE gels were used to assess the digestibility of PAT (*bar*) in SGF. Proteins were subjected to SDS-PAGE and detected by staining with Brilliant Blue G Colloidal stain. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. Empty lanes on the gels were cropped from the images. T = time. Panel A corresponds to PAT (*bar*) protein digestion in SGF. *E. coli*-produced PAT (*bar*) protein was loaded at 1 µg per lane based on pre-digestion concentrations. Panel B corresponds to the analysis to determine Limit of Detection (LOD) of PAT (*bar*). Sample amount indicates the amount of *E. coli*-produced PAT (*bar*) protein in the SGF T0 sample loaded to estimate the LOD of the PAT (*bar*) protein. Lane designations are as follows:

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0 (No PAT (*bar*) control)	0
3	SGF P0 (No pepsin control)	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	Mark 12 MWM	-

Lane	Sample	Amount (ng)
1	Mark 12 MWM	-
2	T0, protein+SGF	200
3	T0, protein+SGF	100
4	T0, protein+SGF	50
5	T0, protein+SGF	25
6	T0, protein+SGF	13
7	T0, protein+SGF	6.3
8	T0, protein+SGF	3.1
9	T0, protein+SGF	1.6
10	T0, protein+SGF	0.8
11	Mark 12 MWM	-

For the western blot analysis of PAT (*bar*) digestibility in SGF, the blot used to assess the stability of the PAT (*bar*) protein to pepsin digestion (Figure VI-20 Panel A) was run concurrently with a blot to estimate the LOD of the PAT (*bar*) protein (Figure VI-20 Panel B). Ten ng of total protein was analyzed by western blot for each time point. No immunoreactive bands were observed in controls SGF N0 and SGF N7 (Figure VI-20 Panel A, Lanes 2 and 13). This result indicates that non-specific interactions between the test system components and the PAT (*bar*) -specific antibody did not occur under these experimental conditions.

No change in the intact PAT (*bar*) protein band intensity was observed in the absence of pepsin (compare SGF P0 to P7) (Figure VI-20 Panel A, Lanes 3 and 12). This result reaffirms that the PAT (*bar*) protein was stable in the test system without pepsin.

Western blot analysis demonstrated that the *E. coli*-produced PAT (*bar*) protein was digested below the LOD within 0.5 min of incubation in SGF (Figure VI-20 Panel A, Lane 5). The LOD of the PAT (*bar*) protein was visually estimated to be 0.16 ng (Figure VI-20 Panel B, Lane 7). The LOD estimated for the PAT (*bar*) protein was used to calculate the maximum amount of PAT (*bar*) protein that could remain visually undetected after digestion, which corresponded to approximately 1.6% of the total PAT (*bar*) protein loaded. Based on the western blot LOD for the PAT (*bar*) protein, the conclusion was that more than 98.4% (100% - 1.6% = 98.4%) of the intact PAT (*bar*) protein was digested within 0.5 min. This is comparable with previously published safety assessments of PAT (*bar*) protein (Hérouet et al., 2005).

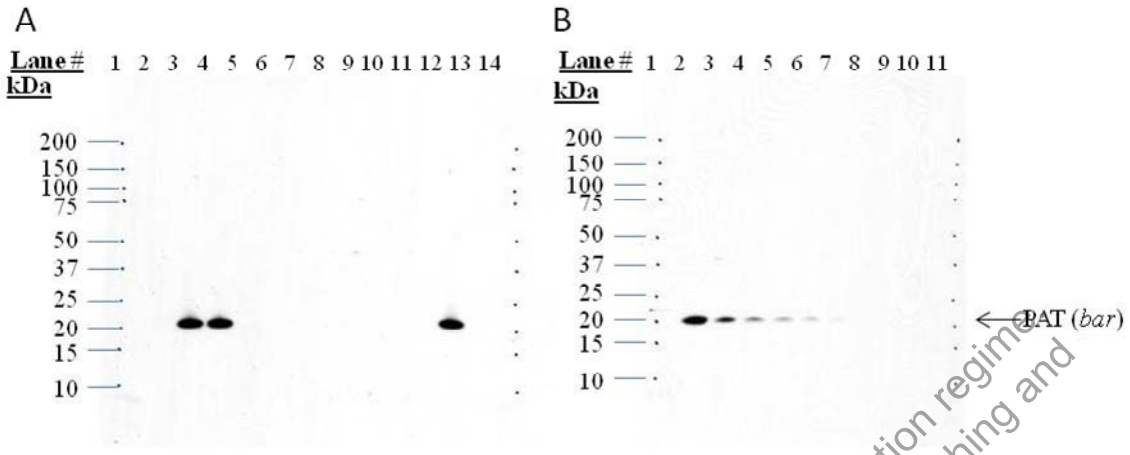


Figure VI-20. Western Blot Analysis of Purified *E. coli*-produced PAT (*bar*) Protein in Simulated Gastric Fluid

Western blots probed with an anti-PAT (*bar*) antibody were used to assess the digestibility of PAT (*bar*) in SGF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. Empty lanes on the gels were cropped from the images. A 0.5 min exposure is shown. T = time. Panel A corresponds to *E. coli*-produced PAT (*bar*) protein digestion in SGF. Based on pre-digestion protein concentrations, 10 ng of total protein was loaded in each lane containing PAT (*bar*) protein (SGF T0-SGF T7). Panel B corresponds to the analysis to determine LOD of *E. coli*-produced PAT (*bar*) protein. Indicated amounts of the PAT (*bar*) protein from the SGF T0 sample were loaded to estimate the LOD of the protein. Lane designations are as follows:

Panel A		
Lane	**Sample**	**Incubation Time (min)**
1	Precision Plus MWM	-
2	SGF N0 (No PAT (*bar*) control)	0
3	SGF P0 (No pepsin control)	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	Precision Plus MWM	-

Panel B		
Lane	**Sample**	**Amount (ng)**
1	Precision Plus MWM	-
2	T0, protein+SGF	5
3	T0, protein+SGF	2.5
4	T0, protein+SGF	1.3
5	T0, protein+SGF	0.63
6	T0, protein+SGF	0.31
7	T0, protein+SGF	0.16
8	T0, protein+SGF	0.08
9	T0, protein+SGF	0.04
10	T0, protein+SGF	0.02
11	Precision Plus MWM	-

VI.F.2.4.2. Digestibility of PAT (*bar*) in SIF

The digestibility of the PAT (*bar*) protein in SIF was assessed by western blot (Figure VI-21). The western blot used to assess the *in vitro* digestibility of the PAT (*bar*) protein in SIF (Figure VI-21 Panel A) was run concurrently with a western blot used to estimate the LOD (Figure VI-21 Panel B) of the intact PAT (*bar*) protein in this assay. Methods for SIF analysis are described in Appendix F. The gel used to assess the digestibility of the PAT (*bar*) protein in SIF by western blot was loaded with 10 ng total protein (based on pre-digestion protein concentrations) for each of the incubation time points. No immunoreactive bands were observed in controls SIF N0 and SIF N8, which represent the SIF test system without *E. coli*- -produced PAT (*bar*) protein (Figure V1-21 Panel A, Lanes 2 and 14). This result demonstrates the absence of non-specific antibody interactions with the SIF test system.

No change in PAT (*bar*) protein band intensity was observed in the controls SIF P0 and SIF P8 (Figure VI-21 Panel A, Lanes 3 and 13), which represent the test system without pancreatin. This result reaffirms that PAT (*bar*) was stable in the test system without pancreatin.

Western blot analysis demonstrated that a band corresponding to the PAT (*bar*) protein was digested to a level below the LOD within 5 min of incubation in SIF (Figure V1-21 Panel A, Lane 5), the first time point assessed. The LOD was visually estimated to be 0.16 ng (Figure VI-16 Panel B, Lane 7). This LOD was used to calculate the maximum amount of PAT (*bar*) protein that could remain visually undetected after digestion, which corresponded to approximately 1.6% of the total protein loaded. Therefore, based on the LOD, more than 98.4% (100% - 1.6% = 98.4%) of the PAT (*bar*) protein was digested in SIF within 5 min. A faint immunodetectable band of less than 10 kDa was observed at the 5 and 15 min time point in SIF, but was gone by 30 min. No other immunoreactive bands were detected in any other digestion specimens. This is comparable with previously published safety assessments of PAT (*bar*) protein (Hérouet et al., 2005).

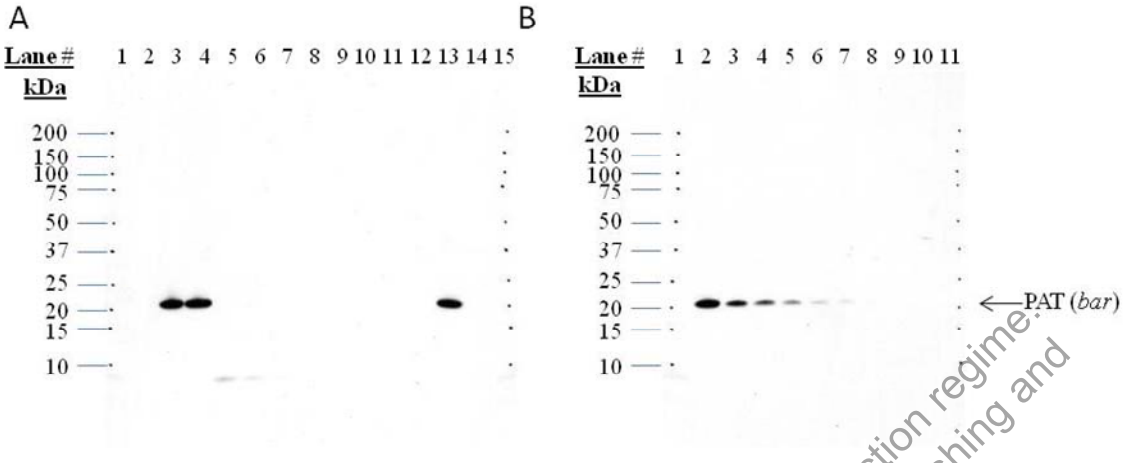


Figure VI-21. Western Blot Analysis of Purified *E. coli*-produced PAT (*bar*) Protein in Simulated Intestinal Fluid

Western blots probed with an anti- PAT (*bar*) antibody were used to assess the digestibility of PAT (*bar*) in SIF. Approximate molecular weights (kDa) are shown on the left of each gel, and correspond to the markers loaded. A 1 min exposure is shown. T = time. Panel A corresponds to *E. coli*-produced PAT (*bar*) protein digestion in SIF. Ten ng of total protein was loaded per lane based on pre-digestion concentrations. Panel B corresponds to the analysis to determine Limit of Detection (LOD) of *E. coli*-produced PAT (*bar*) protein. Indicated amounts of the PAT (*bar*) protein from the SIF T0 sample were loaded to estimate the LOD of the protein. Empty lanes on the blot were cropped from the image. Lane designations are as follows:

Panel A

Lane	Sample	Incubation Time
1	Precision Plus MWM	-
2	SIF N0 (No PAT (*bar*) control)	0
3	SIF P0 (No pepsin control)	0
4	SIF T0	0
5	SIF T1	5 min
6	SIF T2	15 min
7	SIF T3	30 min
8	SIF T4	1 hr
9	SIF T5	2 hr
10	SIF T6	4 hr
11	SIF T7	8 hr
12	SIF T8	24 hr
13	SIF P8	24 hr
14	SIF N8	24 hr
15	Precision Plus MWM	-

Panel B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	-
2	T0, protein+SIF	5
3	T0, protein+SIF	2.5
4	T0, protein+SIF	1.3
5	T0, protein+SIF	0.63
6	T0, protein+SIF	0.31
7	T0, protein+SIF	0.16
8	T0, protein+SIF	0.08
9	T0, protein+SIF	0.04
10	T0, protein+SIF	0.02
11	Precision Plus MWM	-

VI.F.2.4.3. Digestibility of PAT (*bar*) - Conclusions

Digestibility of the PAT (*bar*) protein was evaluated in SGF and SIF. Comparable to previously published safety assessment data on PAT (bar) protein (Hérouet et al., 2005), the results of the present studies demonstrate that greater than 98.4% of the *E. coli*-produced PAT (*bar*) protein was digested in SGF within 0.5 min, when analyzed by Brilliant Blue G Colloidal stained SDS-PAGE and by western blot using a PAT (*bar*)-specific antibody. Additionally, at least 98.4% of the PAT (*bar*) protein was digested within 5 min during incubation in SIF.

Results from the digestibility experiments show that the PAT (*bar*) protein is rapidly digested in the *in vitro* model gastrointestinal digestive system. Rapid digestion of the *E. coli*-produced PAT (*bar*) protein in SGF and SIF supports the conclusion that the PAT (*bar*) protein is highly unlikely to pose a safety concern to human and animal health.

VI.F.2.5. Heat Stability of the Purified PAT (*bar*) Protein

Temperature can have a profound effect on the structure and function of proteins. Cottonseed processing involves treatment of cottonseed for hours with temperatures from 88 ºC to greater than 130 ºC for meal processing and up to 230 ºC for deodorization of the oil (Harris, 1981; NCPA, 1993). In addition, the processing of linters involves processing at temperatures greater than 100 ºC (AOCS, 1991). Therefore is reasonable to assume that the conditions encountered during the processing of cottonseed and linters from MON 88701 will have an effect on the functional activity and structure of MON 88701 PAT (*bar*) protein when consumed in different feed products derived from MON 88701 and in human food products in the unlikely event protein is present.

The effect of heat treatment on the activity of *E. coli*-produced PAT (*bar*) protein was evaluated using purified protein. The method for evaluating heat stability is described in Appendix G. Heat-treated samples and an unheated control sample of *E. coli*-produced PAT (*bar*) protein were analyzed: 1) using a functional assay to assess the impact of temperature on the enzymatic activity of PAT (*bar*) protein; and 2) using SDS-PAGE to assess the impact of temperature on protein integrity.

Aliquots of *E. coli*-produced PAT (*bar*) were heated to 25, 37, 55, 75, and 95 °C for 15 and 30 min, while a separate aliquot of *E. coli*-produced PAT (*bar*) was maintained on ice for the duration of the heat treatments to serve as a temperature control. The effect of heat treatment on the activity of PAT (*bar*) was evaluated using a functional activity assay (Appendix B, Section B.5.6.2, and Appendix G). The effect of heat treatment on the integrity of the PAT (*bar*) protein was evaluated using SDS-PAGE analysis of the heated and temperature control PAT (*bar*) samples.

The effects of heating on the functional activity of PAT (*bar*) are presented in Tables VI-16 and VI-17. The functional activity of PAT (*bar*) was unaffected at 25 ºC and 37 ºC for 15 and 30 min. The functional activity of PAT (*bar*) heated to 55 ºC demonstrated a substantial reduction in *E. coli*-produced PAT (*bar*) activity with 40% activity remaining

at the 15 min incubation time and 24% activity remaining after the 30 min incubation. The functional activity of PAT (*bar*) heated to 75 ºC or higher for 15 min or more demonstrated a >90% loss of functional activity relative to the temperature control PAT (*bar*) sample. These results suggest that temperature has a considerable effect on the activity of functional activity of PAT (*bar*).

Analysis by SDS-PAGE stained with Brilliant Blue G Colloidal demonstrated that the PAT (*bar*) control treatment and reference standard contain a major band at ~25 kDa, corresponding to the PAT (*bar*) protein (Figures VI-22 and VI-23, Lanes 2 and 8). No apparent decrease in the intensity of this band was observed in heat-treated PAT (*bar*) at all temperatures at 15 or 30 min (Figure VI-22, Lanes 3–7 and Figure VI-23, Lanes 3–7). There was a slight visible appearance of higher molecular weight species at heat treatments of 75 ºC and 95 ºC, presumably due to protein aggregation.

These data demonstrate that PAT (*bar*) remains intact, but is deactivated at temperatures of 75 ºC and above. This is comparable with what has been previously published on the safety assessment of PAT (*bar*) protein (Hérouet et al., 2005; Wehrmann et al., 1996). Therefore, in the unlikely event that cottonseed oil contains protein (Reeves and Weihrauch, 1979), it is reasonable to conclude that PAT (*bar*) protein would not be consumed as an active protein in food or feed products due to standard processing practices that include heat treatment.

Table VI-16. Activity of *E. coli*-produced PAT (*bar*) Protein after 15 Minutes at Elevated Temperatures

Temperature	Functional Activity ($\mu mol \times minute^{-1} \times mg^{-1}$)[1]	Relative Activity (% of unheated control)[2]
Unheated Control (0 ºC)	27.2	100%
25 ºC	22.1	81%
37 ºC	22.4	82%
55 ºC	10.9	40%
75 ºC	2.3	8%
95 ºC	2.4	9%

[1] Mean specific activity determined from n = 3.
[2] Relative activity = [activity of sample/ activity of unheated control] × 100; PAT (*bar*) protein activity of unheated control was assigned 100%.

Table VI-17. Activity of *E. coli*-produced PAT (*bar*) Protein after 30 Minutes at Elevated Temperatures

Temperature	Functional Activity ($\mu mol \times minute^{-1} \times mg^{-1}$)[1]	Relative Activity (% of unheated control)[2]
Unheated Control (0 ºC)	27.2	100%
25 ºC	21.5	79%
37 ºC	24.1	89%
55 ºC	6.6	24%
75 ºC	2.3	8%
95 ºC	2.4	9%

[1] Mean specific activity determined from n = 3.
[2] Relative activity = [activity of sample/ activity of unheated control] × 100; PAT (*bar*) protein activity of unheated control was assigned 100%.

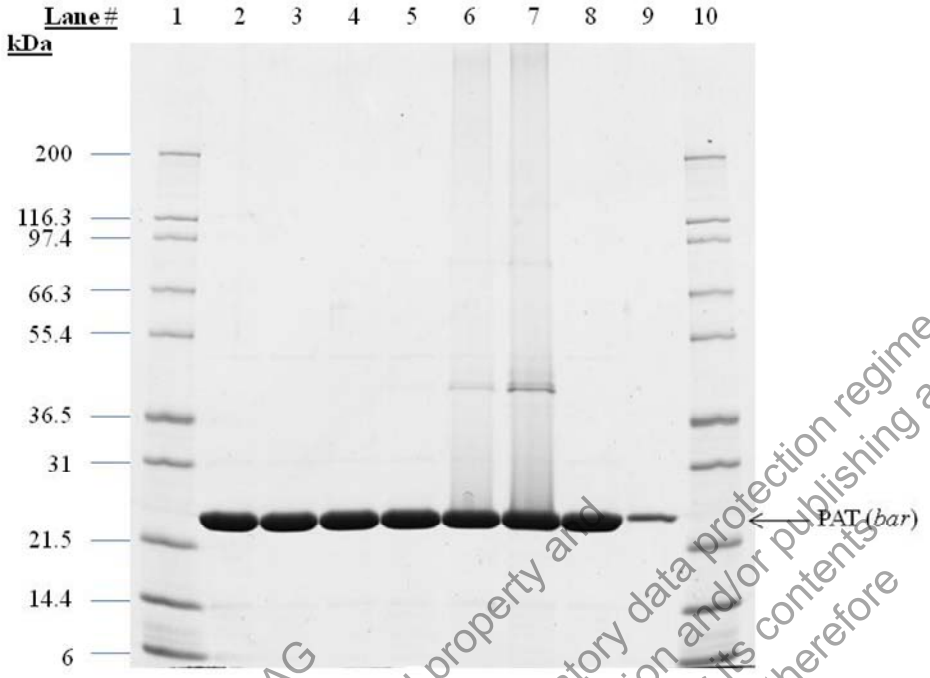


Figure VI-22. SDS-PAGE of *E. coli*-produced PAT (*bar*) Protein Following Heat Treatment for 15 Minutes

Heat-treated samples of PAT (*bar*) (3.0 µg total protein) subjected to SDS-PAGE and stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10. Lane designations are as follows:

Lane	Description	Amount (µg)
1	Mark 12 MWM	-
2	*E. coli*-produced PAT (*bar*) Unheated Control (0 ºC)	3.0
3	*E. coli*-produced PAT (*bar*) 25 ºC	3.0
4	*E. coli*-produced PAT (*bar*) 37 ºC	3.0
5	*E. coli*-produced PAT (*bar*) 55 ºC	3.0
6	*E. coli*-produced PAT (*bar*) 75 ºC	3.0
7	*E. coli*-produced PAT (*bar*) 95 ºC	3.0
8	*E. coli*-produced PAT (*bar*) Reference	3.0
9	*E. coli*-produced PAT (*bar*) Reference	0.3
10	Mark 12 MWM	-

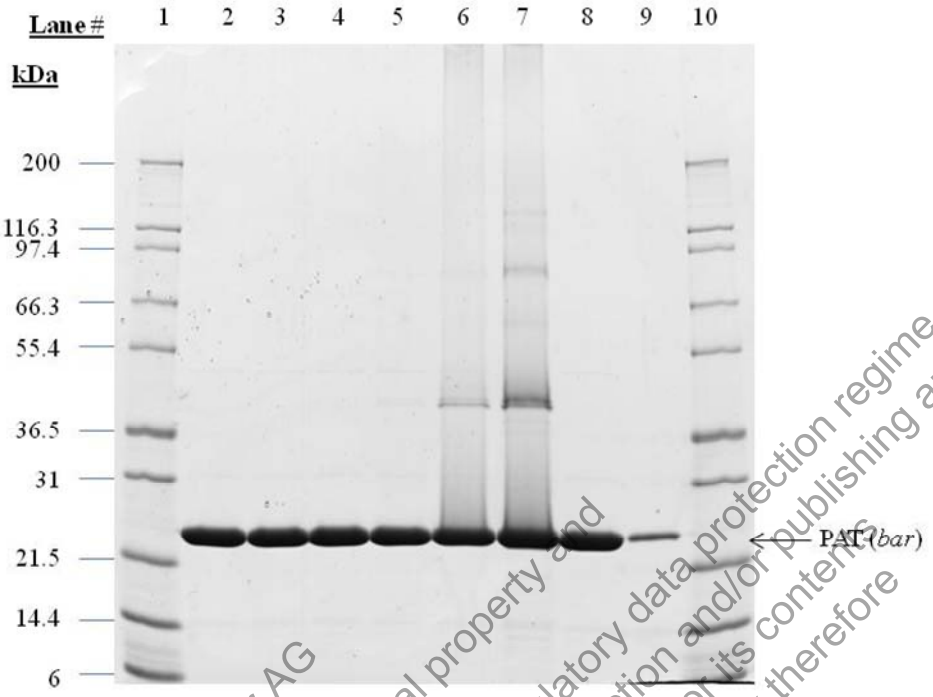


Figure VI-23. SDS-PAGE of *E. coli*-produced PAT (*bar*) Protein Following Heat Treatment for 30 Minutes

Heat-treated samples of PAT (*bar*) protein (3.0 µg total protein) subjected to SDS-PAGE and stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10. Lane designations are as follows:

Lane	Description	Amount (µg)
1	Mark 12 MWM	-
2	*E. coli*-produced PAT (*bar*) Unheated Control (0 ºC)	3.0
3	*E. coli*-produced PAT (*bar*) 25 ºC	3.0
4	*E. coli*-produced PAT (*bar*) 37 ºC	3.0
5	*E. coli*-produced PAT (*bar*) 55 ºC	3.0
6	*E. coli*-produced PAT (*bar*) 75 ºC	3.0
7	*E. coli*-produced PAT (*bar*) 95 ºC	3.0
8	*E. coli*-produced PAT (*bar*) Reference	3.0
9	*E. coli*-produced PAT (*bar*) Reference	0.3
10	Mark 12 MWM	-

VI.F.3. Assessment for the Potential for Toxicity of the MON 88701 DMO and PAT (*bar*) Proteins

The assessment of the potential toxicity of an introduced protein is based on comparing the biochemical characteristics of the introduced protein to characteristics of known toxins. A protein is not likely to be associated with toxicity if: 1) the protein lacks any structural similarity to known toxins or other biologically-active proteins that could cause adverse effects in humans or animals; 2) the protein is rapidly digested in mammalian gastrointestinal systems; and 3) the protein is unstable to heat treatment. The lack of any effects in an acute oral mammalian toxicity study performed at dose levels substantially greater than anticipated human exposure levels can provide further confirmation that an introduced protein is unlikely to pose a significant risk to human or animal health. The MON 88701 DMO and PAT (*bar*) proteins in MON 88701 have been assessed for their potential toxicity based on these criteria.

VI.F.3.1. Structural Similarity of MON 88701 DMO and PAT (*bar*) Proteins to Known Toxins

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 MON 88701 DMO and PAT (*bar*) proteins with 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 often have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions (Caetano-Anollés et al., 2009; Illergård et al., 2009).

FASTA bioinformatic alignment searches using the MON 88701 DMO amino acid sequence and the PAT (*bar*) amino acid sequence were performed with the toxin database to identify possible homology with proteins that may be harmful to human and animal health. The toxin database, TOX_2011, is a subset of sequences derived from the PRT_2011 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. The TOX_2011 database contains 10,570 sequences.

An E-score acceptance criteria of $1x10^{-5}$ or less for any alignment was used to identify proteins from the TOX_2011 database with potential for significant shared structural similarity and function with MON 88701-produced DMO and PAT (*bar*) proteins. As described above (Section VI.F.1.3.), 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 less to be considered to have sufficient sequence similarity to infer homology. The results of the search comparisons showed that no relevant alignments were observed against proteins in the TOX_2011 database.

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the MON 88701 DMO or PAT (bar) proteins and any sequence in the TOX_2011 database, as no alignments displaying an E-score < $1e^{-5}$ were observed. This is comparable with previously published safety assessments of PAT (*bar)* protein (Hérouet et al., 2005).

VI.F.3.2. Digestibility and Heat Stability of the MON 88701 DMO and PAT (*bar*) Proteins

The stability of a protein to heat or its degradation in simulated mammalian gastrointestinal fluids is a factor in the assessment of its potential toxicity. The digestibility of MON 88701 DMO and PAT (*bar*) proteins were evaluated by incubation with simulated gastric fluid and simulated intestinal fluid, and the results show that both MON 88701 DMO and PAT (*bar*) proteins were readily digested (Section VI.F.1.4. and VI.F.2.4., respectively). Exposure to heat during food processing or cooking, and to digestive fluids is likely to have a profound effect on the structure and function of proteins. The effect of heat treatment on the activity of MON 88701 DMO and PAT (*bar*) proteins was evaluated using functional assays to assess the impact of temperature on enzymatic activity, and using SDS-PAGE to assess the impact of temperature on protein integrity. The results show that MON 88701 DMO protein was completely deactivated by heating at temperatures 55 ºC or higher (Section VI.F.1.5.) and PAT (*bar)* protein was substantially deactivated by heating at temperatures 75 ºC or above (Section VI.F.2.5.). In addition, RBD oil and linters are processed fractions that contain undetectable or negligible amounts of protein, respectively and minimal, if any, dietary exposure to MON 88701 DMO and PAT (*bar*) proteins is expected from consumption of foods derived from MON 88701. Therefore, it is anticipated that exposure to functionally active MON 88701 DMO or PAT (*bar*) protein from the consumption of MON 88701 or foods derived from MON 88701 is unlikely.

VI.F.3.3. Acute Oral Toxicity Study with the MON 88701 DMO and PAT (*bar*) Proteins

Most known protein toxins act through acute mechanisms to exert toxicity (Hammond and Fuchs, 1998; Pariza and Johnson, 2001; Sjoblad et al., 1992). The primary exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, which manifest toxicity in a short term (few weeks) feeding study (Liener, 1994). The amino acid sequence of both MON 88701 DMO and PAT (*bar*) proteins produced in MON 88701 are not similar to any of these anti-nutritional proteins or to any other known protein toxins (see section VI.F.3.1.). Further, MON 88701 DMO

and PAT proteins have a history of safe use (see section VI.B), are functionally inactivated at temperatures below those used in processing (see sections V1.F.1.5 and V1.F.2.5), and are digested in gastric and intestinal model systems (see sections V1.F.1.4.3 and V1.F.2.4.3). In additional the safety of PAT proteins has been previously demonstrated and there is a long history of safe use (Hérouet et al., 2005; ILSI-CERA, 2011; Wehrmann et al., 1996). These assessments satisfy the criteria described in the Codex guidelines specific to the safety assessment of biotechnology-derived plants; thus, it was not necessary to conduct an acute toxicity assay with MON 88701 DMO and PAT (*bar*) proteins. Further, there is no anticipated human exposure to these proteins because only highly processed cotton products (oil and linters) containing non-detectable or negligible amounts of protein are consumed by humans (see section V1.F.4.1). Nevertheless, an acute gavage assay was conducted with MON 88701 DMO and PAT (*bar*) proteins to provide additional support for the primary toxicity assessment studies.

The *E. coli*-produced MON 88701 DMO protein and *E. coli*-produced PAT (*bar*), in independent studies, were administered as a single dose by oral gavage to 10 male and 10 female CD-1 mice. The MON 88701 DMO protein was administered at a dose level of 283 mg/kg body weight (bw) and PAT (*bar*) was administered at a dose level of 1086 mg/kg bw. The dose levels were selected based on the risk assessment principles of hazard identification and margin of exposure, as well as consideration of the physicochemical properties of the E. coli-produced MON 88701 DMO protein (i.e. solubility in a suitable dosing formulation). The selected doses are sufficiently high for potential hazard identification in light of the lack of anticipated human exposure and the other evaluations of safety described above. Each study contained an additional group of 10 male and 10 female mice to serve as a concurrent control. These control groups in each study were administered an amount of bovine serum albumin (BSA) comparable to the amount of test substance (i.e., MON 88701 DMO or PAT (*bar*)) administered to the test group in each study on a mg/kg bw basis. The BSA was suspended in the appropriate buffer at a volume comparable to that received by the test substance group. Following dosing, all mice were observed twice daily throughout the study for general health, mortality and moribundity. A detailed clinical observation was performed on each animal once prior to and twice following treatment on the day of dosing (i.e., three times on the day of dosing) and daily thereafter. Food consumption was measured weekly. Body weights were measured prior to dosing (day 0) and on study days 3, 7, 10 and 14. All animals were euthanized on day 14 and subjected to a gross necropsy. There were no treatment-related effects on survival, clinical observations, body weight gain, food consumption or gross pathology. Therefore, the No Observable Effect Levels, adverse or otherwise, (NOELs and NOAELs) for MON 88701 DMO and PAT (*bar*) were considered to be 283 mg/kg bw and 1086 mg/kg bw, respectively.

VI.F.4. Dietary Risk Assessment of the MON 88701 DMO and PAT (*bar*) Proteins

VI.F.4.1. Estimated Human Exposure to the MON 88701 and PAT (*bar*) Proteins from MON 88701

Cottonseed is not consumed by humans because the majority of commercial cotton varieties contain the anti-nutrients gossypol and cyclopropenoid fatty acids. The primary human food currently produced from cottonseed is refined, bleached, and deodorized (RBD) oil, and to a smaller extent, linters. RBD oil contains undetectable amounts of protein (Reeves and Weihrauch, 1979); therefore, oil produced from MON 88701 will contain negligible levels of MON 88701 DMO and PAT (*bar*) proteins. Linters are an industrial byproduct of ginning, and can be consumed as a highly processed product composed of nearly pure (*i.e.*, >99%) cellulose (NCPA, 2002a; Nida et al., 1996). Because RBD oil and linters are processed fractions that contain undetectable or negligible amounts of protein there is minimal, if any, dietary exposure to MON 88701 DMO and PAT (*bar*) proteins from consumption of foods derived from MON 88701. Therefore, making an estimate of human exposure to the proteins is unnecessary.

VI.F.4.1.1 Dietary Exposure Assessment: Margin of Exposure for MON 88701 DMO and PAT (*bar*) Proteins from MON 88701

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 and female mice were administered doses of 283 mg/kg bw of MON 88701 DMO protein and 1086 mg/kg bw of PAT (*bar*) protein. Therefore, based on an apparent absence of hazard and lack of dietary exposure as discussed in section VI.F.4.1, a dietary risk assessment for these proteins is unnecessary.

VI.F.4.2. Estimated Animal Exposure to MON 88701 DMO and PAT (*bar*) Proteins from MON 88701

Cotton is planted primarily for production of cotton fibers, but an important by-product is cottonseed. In fact, yield of cottonseed is greater than cotton fibers. In 2009, U.S. growers harvested 777 lb/acre cotton fiber and 1102 lb/acre of cottonseed (USDA-ERS, 2010; 2011). Cottonseed oil and feeds derived from cotton include whole cottonseed, cottonseed hulls, and cottonseed meal. Of these, whole cottonseed and cottonseed meal have the greatest amounts of protein.

Cottonseed is an important feed for dairy cows because it supplies significant amounts of energy, protein and fiber to the dairy ration (Hutjens, 2003), which is a unique combination of nutrients. Cottonseed meal is a high-protein feed produced by extraction of the oil and cottonseed meal is a more economical source of protein that replaces soybean meal in dairy cattle diets (Hutjens, 2007). In contrast, feeding whole cottonseed and cottonseed meal to non-ruminants such as poultry and swine is limited due to the

presence of the anti-nutrients such as gossypol and cyclopropenoid fatty acids (NCPA, 2002b; NRC, 1994; OECD, 2009b; Smith, 1970; U.S. EPA, 1996).

Dairy cows would be exposed to MON 88701 DMO and PAT (*bar*) proteins in MON 88701 through dietary intake of high-protein feeds from MON 88701 seed, such as whole cottonseed or cottonseed meal. Since livestock diets typically contain a much higher level of the protein from meal than from whole seed, dietary intake estimates of cottonseed will assume the worst case, consumption as cottonseed meal.

For this assessment, a worst-case scenario assessment was conducted that assumed cottonseed meal from MON 88701 as the only cottonseed feedstuff in the diet. The maximum amount of cottonseed recommended in dairy diets is 15% of the ration dry weight (dw) (Shaver, 2008; U.S. EPA, 1996). Assuming that a dairy cow consumed 24 kg of total feed (dw) and weighed 600 kg (OECD, 2009a), it would consume 6 g of cottonseed meal/kg body weight (Table VI-18).

Table VI-18. Dairy Cow Dietary Intake of Cottonseed Meal

Amount in Diet[1]	Total Diet Intake on Dry Weight (dw) Basis[2]	Body Weight (bw)[2]	Daily Dietary Intake (DDI) of Cottonseed Meal[3]
(%)	(kg/d)	(kg)	(g dw/kg bw/d)
15	24	600	6

[1] (U.S. EPA, 1996).
[2] (OECD, 2009a).
[3] DDI of cottonseed meal = (Total Diet Intake × Amount in Diet) / kg body weight.

VI.F.4.3. Animal Dietary Intake of MON 88701 DMO and PAT (*bar*) Proteins from MON 88701

The quantity of MON 88701-derived cottonseed meal consumed on a daily basis as well as the levels of MON 88701 DMO and PAT (*bar*) proteins in MON 88701 are necessary to derive an estimate of daily dietary intake (DDI) of each protein. DDI is calculated as follows:

DDI (g MON 88701 DMO/kg bw/d) = cottonseed meal intake (g/kg bw/d) × MON 88701 DMO protein in cottonseed meal (ng/g) × 10^{-9} (g/ng)

DDI (g PAT (*bar*)/kg bw/d) = cottonseed meal intake (g/kg bw/d) × PAT (*bar*) protein in cottonseed meal (ng/g) × 10^{-9} (g/ng)

Expression of MON 88701 DMO and PAT (*bar*) were measured from MON 88701 planted in eight sites in the U.S. during 2010 (Section VI.D.). To calculate the greatest possible animal exposure to these expressed proteins, the following assumptions were made: 1) cottonseed meal from MON 88701 would be the only source of cottonseed in

the diet and would not be commingled with non-MON 88701 sources, even though the typical commercial situation would involve commingling of MON 88701 with other varieties of cotton; 2) no loss in protein from heat or other processing effects; and 3) the yield of cottonseed meal from cottonseed is approximately 46% (USDA, 2011). This processing yield was based on statistics from USDA for Aug. 2009 to Sep. 2010, indicating that during the crushing process 883 thousand short tons of cottonseed meal were produced from 1901 thousand short tons of cottonseed (USDA, 2011). Mean and maximum MON 88701 DMO protein expression levels in cottonseed were 21 µg/g dw and 33 µg/g dw, respectively and mean and maximum PAT (*bar*) protein expression levels in cottonseed were 6.6 µg/g dw and 9.6 µg/g dw. Therefore, using a crushing yield of 46% and no loss of MON 88701 DMO or PAT (*bar*) proteins during crushing, the mean and maximum amounts of MON 88701 DMO protein were calculated in cottonseed meal to be 45 µg/g dw and 71 µg/g dw, respectively. The mean and maximum amounts of PAT (*bar*) protein were calculated to be 14.2 µg/g dw and 20.7 µg/g dw.

The estimated maximum dietary exposures of dairy cows to MON 88701 DMO and PAT (*bar*) proteins from MON 88701 are shown in Table VI-19. Assuming that the lactating dairy cow would typically consume diets containing 15.6% crude protein (NRC, 2001), they would consume 6.2 g dietary protein/kg bw. The maximum percentages of MON 88701 DMO and PAT (*bar*) proteins (g/kg bw) consumed as a percent of total dietary protein consumed were 0.007% and 0.0020% (g/g), respectively.

Table VI-19. Mean and Maximum Daily Intake of the MON 88701 DMO and PAT (*bar*) Protein by Dairy Cows (g/kg body weight/day)[1]

Dietary Intake	MON 88701 DMO	PAT (*bar*)
Mean Intake		
g/kg of body weight/day[2]	0.00027	0.000085
% of dietary protein	0.004%	0.0014%
Maximum Intake		
g/kg of body weight/day[2]	0.00043	0.000124
% of dietary protein	0.007%	0.0020%

[1] dw = dry weight

[2] Cottonseed meal consumed × concentration of MON 88701 DMO or PAT (*bar*) protein in the meal.

VI.F.5. Potential Allergenicity or Toxicity of MON 88701 DMO and PAT (*bar*) Proteins Produced in MON 88701: Summary and Conclusion

MON 88701 DMO and PAT (*bar*) proteins both possess a strong safety profile. The donor organisms for the MON 88701 DMO and PAT (*bar*) protein coding sequences, *Stenotrophomonas maltophilia* and *Streptomyces hygroscopicus*, respectively, are ubiquitous in the environment and are not commonly known for human or animal pathogenicity, or allergenicity. Furthermore, *S. maltophilia* has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding dicamba-tolerant soybean, MON 87708, and *S. hygroscopicus* has been previously reviewed as a part of the safety assessment of the donor organism during consultations with the regulatory agencies in 11 different countries, including the FDA for more than 38 transgenic events in eight different species (ILSI-CERA, 2011). MON 88701 DMO and PAT (*bar*) proteins are present at a very low level in the cottonseed of MON 88701. In addition, only oil and linters extracted from cottonseed are used in food applications, and these fractions contain negligible amounts of total protein (NCPA, 2002a; Nida et al., 1996; Reeves and Weihrauch, 1979). Therefore, MON88701 DMO and PAT (*bar*) proteins are essentially non-existent in food derived from MON 88701. Bioinformatic analyses of MON 88701 DMO and PAT (*bar*) proteins demonstrated a lack of structural similarity to allergens or toxins or other proteins known to have adverse effects on mammals. The MON 88701 DMO and PAT (*bar*) proteins were rapidly digested in SGF and SIF, lost activity upon heating at temperatures well below standard cotton processing temperatures, and demonstrated no oral toxicity in mice at the levels tested. In addition, no consumption of the MON 88701 DMO or PAT (*bar*) proteins derived from MON 88701 is expected for the U.S. general population at the present time given the nature of cottonseed fractions consumed by humans. Finally, the overall animal exposure as a percent of total protein is demonstrated to be small for both proteins.

Based on the above information, the consumption of the MON 88701 DMO and PAT (*bar*) proteins from foods derived from processed fractions of cottonseed of MON 88701 or products derived from MON 88701 is considered safe for humans and animals, though human exposure is unlikely.

VI.G. Bioinformatic Assessment of Putative Open Reading Frames (ORFs) of MON 88701 Insert and Flanking Sequences

The 2009 Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2009) includes an assessment element on the identification and evaluation of "open reading frames within the inserted DNA or created by the insertion with contiguous plant genomic DNA." These assessments examine the potential homology of any putative polypeptides or proteins that could be produced from open reading frames (ORFs) in the insert or at the plant-insert junction to known toxins or allergens. These analyses are conducted even if there is no evidence that such ORFs at the plant-insert junction or alternative reading frames in the

insert are capable of being transcribed or translated into a protein. Results from these bioinformatics analyses demonstrate that any putative polypeptides in MON 88701 are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

In addition to the bioinformatic analyses conducted on MON 88701 DMO and PAT (*bar*) protein sequences (Sections VI.F.1.3., VI.F.2.3., and VI.F.3.1.), bioinformatic analyses were also performed on the MON 88701 insert and flanking genomic DNA sequences to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by all six reading frames present in the MON 88701 insert DNA, as well as, ORFs present in the 5' and 3' flanking sequence junctions (Table V-2). These various bioinformatic evaluations are depicted in Figure VI-24. ORFs spanning the 5' and 3' flanking sequence DNA-inserted DNA junctions were translated from stop codon to stop codon in all six reading frames (three forward reading frames and three reading frames in reverse orientation)[3]. Polypeptides of eight amino acids or greater from each reading frame were then compared to toxin, allergen and all proteins databases using bioinformatic tools. Similarly, the entire MON 88701 insert DNA sequence was translated in all six reading frames and the resulting deduced amino acid sequence was subjected to bioinformatic analyses. There are no analytical data that indicate any putative polypeptides subjected to bioinformatic evaluation other than the MON 88701 DMO and PAT (*bar*) proteins, which are part of the insert DNA sequence analysis are produced. Moreover, the data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than MON 88701 DMO and PAT (*bar*) proteins were derived from frames 1 to 6 of the insert DNA or the ORFs spanning the insert junctions, they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Therefore, there is no evidence for concern regarding the relatedness of the putative polypeptides for MON 88701 to known toxins, allergens, or biologically active putative peptides.

VI.G.1. Bioinformatics Assessment of Insert DNA Reading Frames

Bioinformatic analyses were performed to assess the potential of toxicity, allergenicity or biological activity of any putative peptides encoded by translation of reading frames 1 through 6 of the inserted DNA in MON 88701 (Figure VI-24).

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2011, TOX_2011, and PRT_2011 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness

[3] An evaluation of sequence translated from stop codon to stop codon represents the most conservative approach possible for flank junction analysis as it does not assume that a start codon is necessary for the production of a protein sequence.

was evaluated by detailed visual inspection of the alignment, the calculated percent identity and alignment length to ascertain if alignments exceeded Codex (Codex Alimentarius, 2009) thresholds for FASTA searches of the AD_2011 database, and the *E*-score. Alignments having an *E*-score less than 1×10^{-5} are deemed significant because they may reflect shared structure and function among sequences (Ladics et al., 2007). In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope (Silvanovich et al., 2006) and evaluated against the AD_2011 database.

The results of the search comparisons showed that no relevant structural similarity to known allergens or toxins were observed for any of the putative polypeptides when compared to proteins in the allergen (AD_2011) or toxin (TOX_2011) databases. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database.

When used to query the PRT_2011 database, translations of frame 2, 3, 5 and 6 yielded alignments with *E*-scores less than or equal to $1e^{-5}$. Inspection of the two alignments with frame 2 revealed that they contained a stop codon within the aligning region and required numerous gaps to optimize the alignment. As a result, it is unlikely these alignments reflect conserved structure. Translation of frame 3 positively identified the DMO protein (GI# 314865630) disclosed in a patent filed by Monsanto. The alignment obtained showed 100% identity over 340 amino acids with an *E*-score of $4.4e^{-149}$. In addition, alignment with frame 3 also positively identified PAT (*bar*) protein (GI# 32265028) with an alignment that displayed 100% identity in 183 amino acids and an *E*-score of $9.8e^{-77}$. Translation of frame 5 yielded one alignment with an *E*-score less than or equal to $1e^{-5}$ when used to search the PRT_2011 database. The alignment obtained showed 97.9% identity over 49 amino acids with an *E*-score of $5.9e^{-10}$ with GI-3327940. Inspection of the alignment revealed that there was one stop codon in the query sequence and it is unlikely the alignment reflects conserved structure. Translation of frame 6 yielded five alignments with *E*-scores less than or equal to $1e^{-5}$ when used to search the PRT_2011 database. Inspection of the top five alignments revealed that they were all with an unnamed protein product derived from the translation of the reverse complement strand of the *bar* coding sequence in a synthetic protein construct. These alignments are not unexpected because the identified *bar* gene is contained in MON 88701. Taken together, these data demonstrate the lack of relevant similarities between known allergens or toxins for putative peptides derived from all six reading frames from the inserted DNA sequence of MON 88701. As a result, in the unlikely event that a translation product other than the DMO or PAT (*bar*) protein sequences were derived from reading frames 1 to 6, these putative polypeptides are not expected to be cross-reactive allergens, toxins, or display adverse biological activity.

VI.G.2. Insert Junction Open Reading Frame Bioinformatics Analysis

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' genomic junctions of the MON 88701 inserted DNA were performed using a bioinformatic comparison strategy (Figure VI-24). The purpose of the assessment is to evaluate the potential for novel open reading frames (ORFs) that may have homology to known allergens, toxins, or proteins that display adverse biological activity. Sequences spanning the 5' and 3' flanking sequence DNA-inserted DNA junctions (Figure VI-24) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. Putative polypeptides from each reading frame of eight amino acids or greater in length, were compared to AD_2011, TOX_2011, and PRT_2011 databases using FASTA and to the AD_2011 database using an eight amino acid sliding window search.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and protein sequences in the AD_2011, TOX_2011, and PRT_2011 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity and the alignment length to ascertain if alignments exceeded Codex (Codex Alimentarius, 2009) thresholds for FASTA searches of the AD_2011 database, and the *E*-score. In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope, and evaluated against the AD_2011 database.

No biologically relevant structural similarity to known allergens or toxins, or proteins that display adverse biological activity was observed for any of the putative polypeptides. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. As a result, in the unlikely event that a translation product was derived from DNA spanning the 5' or 3' genomic DNA insert junctions of MON 88701, these putative polypeptides are not expected to be allergens, toxins, or display adverse biological activity.

VI.G.3. Bioinformatic Assessment of Allergenicity, Toxicity, and Adverse Biological Activity Potential of MON 88701 Polypeptides Putatively Encoded by the Insert and Flanking Sequences: Summary and Conclusions

A conservative bioinformatic assessment of allergenicity, toxicity and adverse biological activity for putative polypeptides that are encoded on all reading frames and spanning the 5' and 3' junctions of MON 88701 was conducted. The data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than MON 88701 DMO or PAT (*bar*) protein sequences were derived from frames 1 to 6 for the insert DNA, or the insert junctions, they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic,

toxic, or have other safety implications. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. Therefore, there is no evidence for concern regarding health implications of putative polypeptides for MON 88701.

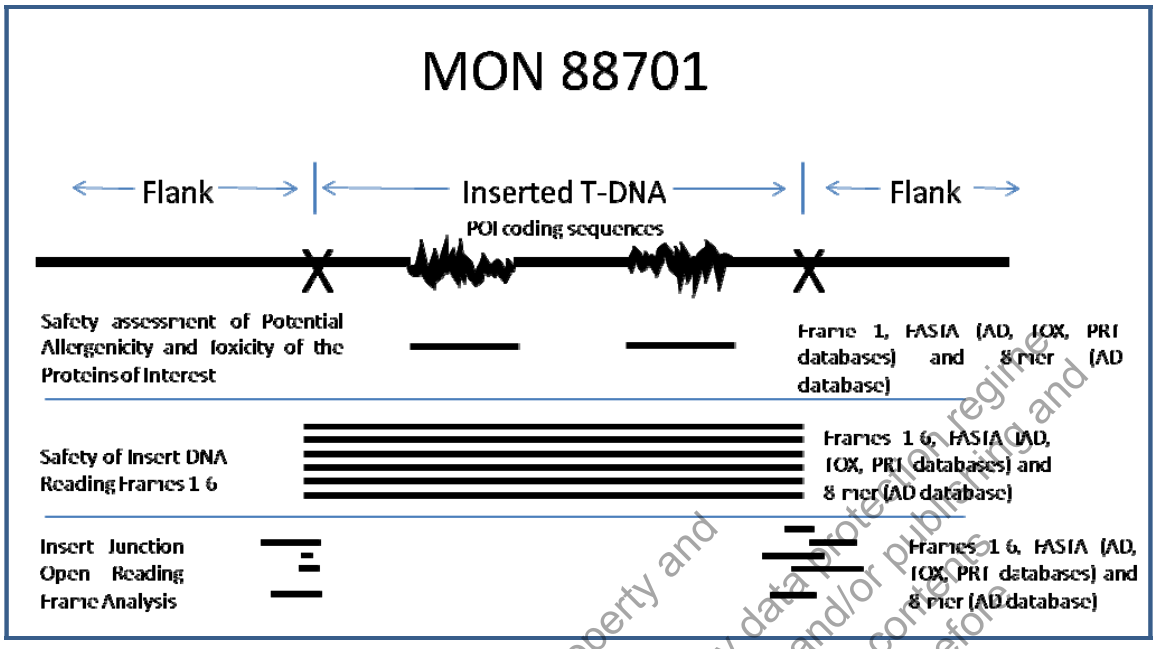


Figure VI-24. Schematic Summary of MON 88701 Bioinformatic Analyses

VI.H. Safety Assessment of Expressed Products: Summary and Conclusion

The data and information provided in this section address the questions important to the food and feed safety assessment of the MON 88701 DMO and PAT (*bar*) proteins in MON 88701 including its potential allergenicity and toxicity. To summarize, the MON 88701 DMO and PAT (*bar*) proteins are present at very low levels in the harvested cottonseed of MON 88701. Furthermore, only oil and linters obtained from cottonseed are used for food applications, both of which contain undetectable or negligible levels of protein. Therefore, MON 88701 DMO and PAT (*bar*) proteins are essentially a non-existent portion of the total protein present in food derived from MON 88701. Nevertheless, further safety assessments were completed in accordance with Codex guidelines. The physicochemical and functional characteristics of the MON 88701 DMO and MON 88701-produced PAT (*bar*) proteins were determined. MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were shown to be equivalent. Similarly, MON 88701- and *E. coli*-produced PAT (*bar*) proteins were shown to be equivalent. This equivalence justifies the use of the *E. coli*-produced proteins as test subtances in the protein safety studies. The expression levels of the MON 88701 DMO and PAT (*bar*) proteins in selected tissues of MON 88701 were determined. The donor organisms for the MON 88701 DMO and PAT (*bar*) protein coding sequences, *Stenotrophomonas maltophilia* and *Streptomyces hygroscopicus*, respectively, are ubiquitous in the environment and are not commonly known for human or animal pathogenicity, or allergenicity. A bioinformatic analysis confirmed that the MON 88701 DMO and PAT (*bar*) proteins lack structural similarity to known allergens and toxins, or other proteins known to have adverse effects on mammals. The MON 88701 DMO and PAT (*bar*) proteins were rapidly digested in simulated gastrointestinal fluids. The MON 88701 DMO and PAT (*bar*) proteins demonstrated substantial loss of activity upon heating at temperatures well below standard cottonseed processing temperatures and therefore, it is reasonable to conclude that they would not be consumed as an active protein. The MON 88701 DMO and PAT (*bar*) proteins demonstrated no oral toxicity in mice at the levels tested and the overall animal exposure as a percent of total protein is demonstrated to be small for both proteins. Based on the above information, the consumption of the MON 88701 DMO and PAT (*bar*) proteins from MON 88701 or its progeny and the consumption of food and feed products derived from MON 88701 or its progeny are considered safe for humans and animals. Though no consumption of the MON 88701 DMO or PAT (*bar*) proteins derived from MON 88701 is expected for the U.S. general population at the present time given the nature of processed cottonseed fractions consumed by humans. Finally, bioinformatics analyses demonstrate the lack of relevant similarities between known allergens and toxins and all putative peptides derived from all six reading frames from the entire inserted DNA sequence of MON 88701 or its flanking sequences.

Taken together, this safety assessment reaffirms the earlier conclusion that the MON 88701 DMO and PAT (*bar*) proteins expressed in MON 88701, do not pose a significant health risk to humans or animals. Finally, in the unlikely event that translation

products other than the MON 88701 DMO and PAT (*bar*) proteins were to be produced, they would pose no allergenic or toxic risk.

VII. COMPOSITIONAL ASSESSMENT OF MON 88701

Safety assessments of biotechnology-derived crops follow the comparative safety assessment process (Codex Alimentarius, 2009) in which the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop is compared to the appropriate parental conventional control that has a history of safe use. Compositional assessments are performed using the principles and analytes outlined in the OECD consensus document for cotton composition (OECD, 2009b).

A recent review of compositional assessments conducted according to OECD guidelines that encompassed a total of seven biotechnology-derived crop varieties, nine countries and eleven growing seasons concluded that incorporation of biotechnology-derived agronomic traits has had little impact on natural variation in crop composition. Most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan et al., 2010). Compositional quality, therefore, implies a very broad range of endogenous levels of individual constituents. Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients and anti-nutrients that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Reynolds et al., 2005). This observation extends to publications specific to cotton (Berberich et al., 1996; Hamilton et al., 2004; Nida et al., 1996).

Compositional equivalence between biotechnology-derived and conventional crops supports an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2002b). The OECD consensus document on considerations for new varieties of cotton emphasize quantitative measurements of key nutrients and known anti-nutrients (OECD, 2009b). This is based on the premise that such comprehensive and detailed analyses will most effectively discern any compositional changes that imply potential nutritional or safety (*e.g.*, anti-nutritional) concerns. Levels of the components in seed of the biotechnology-derived crop are compared to: 1) corresponding levels in a conventional comparator, the genetically similar conventional line, grown concurrently, under field conditions; and 2) natural ranges generated from an evaluation of commercial reference varieties grown concurrently and from data published in the scientific literature. The comparison to data published in the literature places any potential differences between the assessed crop and its comparator in the context of the well-documented variation in the concentrations of crop nutrients, and anti-nutrients.

This section provides analyses of concentrations of key nutrients and anti-nutrients of MON 88701 compared with equivalent analyses of a conventional counterpart grown and harvested under the same conditions, as appropriate. In addition, commercial cotton reference varieties were included in the composition analyses to establish a range of

natural variability for each analyte, defined by a 99% tolerance interval. The production of materials for the compositional analyses used field designs to allow accurate assessments of compositional characteristics over a range of environmental conditions under which MON 88701 is expected to be grown. The field trial design parameters included a sufficient number of trial sites to allow adequate exposure to the variety of conditions cotton plants typically encounter in nature. Field sites were replicated with an adequate number of plants sampled, and the methods of analysis were sufficiently sensitive and specific to detect variations in the components measured to allow statistically rigorous analyses. The information provided in this section also addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45 for compositional analyses (Codex Alimentarius, 2009).

VII.A. Compositional Equivalence of MON 88701 Cottonseed to Conventional Cotton

Compositional analyses comparing MON 88701 treated with dicamba and glufosinate herbicides to the conventional control variety (Coker 130) and commercial reference varieties demonstrated that MON 88701 is compositionally equivalent to conventional cotton. Samples of acid-delinted cottonseed were collected from MON 88701 and the conventional control grown in a 2010 U.S. field production. Nine unique conventional cotton varieties, known as reference substances, were included across all sites of the field production with four varieties per site to provide data on natural variability of each compositional component analyzed. The field production was conducted at eight sites: Arkansas (ARTI), Georgia (GACH), Kansas (KSLA), Louisiana (LACH), North Carolina (NCBD), New Mexico (NMLC), South Carolina (SCEK) and, Texas (TXPL). The sites were planted in a randomized complete block design with four blocks per site. All cotton plants including MON 88701, the conventional control, and the reference varieties were grown under normal agronomic field conditions for their respective geographic regions, including maintenance pesticides as needed. In addition, MON 88701 plots were treated at the 3-5 leaf stage with glufosinate herbicide at the label rate (0.5 lb a.e. /acre) and at the 6-10 leaf stage with dicamba herbicide at the label rate (0.5 lb a.e. /acre).

Compositional analyses were conducted to assess whether levels of key nutrients and anti-nutrients in MON 88701 were equivalent to levels in the conventional control and also comparable to the composition of conventional reference varieties. A description of nutrients and anti-nutrients present in cotton is provided in the OECD consensus document on compositional considerations for cottonseed (OECD, 2009b). Nutrients assessed in this analysis included proximates (ash, calories and carbohydrates by calculation, fat, moisture, and protein), acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber (CF), total dietary fiber (TDF), amino acids (AA, 18 components), fatty acids (FA, C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc) and vitamin E. The anti-nutrients assessed in this analysis included gossypol and cyclopropenoid fatty acids (dihydrosterculic, malvalic and sterculic). Methods used in the assessments of nutrients and anti-nutrients are found in Appendix H. In all, 65 different analytical components were measured. Due to

statistical constraints, in order to proceed with the statistical analysis of any component in this study, at least 50% of the observed values for that analyte needed to be greater than the assay limit of quantitation (LOQ). Of the 65 components measured, 13 had more than 50% of the observations below the assay LOQ and were excluded from statistical analysis. Therefore, 52 components were statistically assessed using a mixed-model analysis of variance method. Values for all components were expressed on a dry weight basis with the exception of moisture, expressed as percent fresh weight, and fatty acids, expressed as percent of total FA.

For MON 88701, nine sets of statistical comparisons to the conventional control were conducted. One comparison was based on compositional data combined across all eight field sites (the combined-site analysis) and eight separate comparisons to the control were conducted on data from each of the eight individual field sites. Statistically significant differences were identified at a 5% level of significance ($p < 0.05$). Compositional data from the reference substances, grown concurrently in the same trial as MON 88701 and the conventional control Coker 130, were combined across all sites and used to calculate a 99% tolerance interval for each component to define the natural variability in cotton varieties that have a history of safe consumption.

For the combined-site analysis, statistically significant differences ($p < 0.05$) in nutrient and anti-nutrient components were evaluated further using considerations relevant to the safety and nutritional quality of MON 88701 when compared to the conventional control. The evaluation included: 1) the relative magnitude of the significant difference in the mean values of nutrient and anti-nutrient components of MON 88701 compared to the conventional control; 2) whether the MON 88701 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of conventional reference varieties grown concurrently in the same trial; 3) analyses of the reproducibility of the significant combined-site component differences at individual sites; and 4) assessing the combined-site statistically significant differences and reproducible individual site significant differences within the context of natural variability of commercial cottonseed composition published in the scientific literature and/or in the International Life Sciences Institute Crop Composition Database (ILSI, 2011). Statistical summaries of nutrients and anti-nutrients for individual sites are found in Appendix H.

This analysis provides a comprehensive comparative assessment of the levels of key nutrients and anti-nutrients in cottonseed of MON 88701 and the conventional control discussed in the context of natural variability in composition of commercial cotton. Results of the comparison indicate that the composition of the cottonseed of MON 88701 is equivalent to that of conventional cotton.

VII.A.1. Nutrient Levels in Cottonseed

In the combined-site analysis of nutrient levels in cottonseed, the following components had no statistically significant differences ($p < 0.05$) in mean values between MON 88701 and the control: one proximate (protein), one type of fiber (crude fiber), 15 amino acids (alanine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, serine, threonine, tryptophan, tyrosine and valine), seven fatty acids (16:0 palmitic acid, 16:1 palmitoleic acid, 18:0 stearic acid, 18:1 oleic acid, 18:3 linolenic acid, 20:0 arachidic acid and 22:0 behenic acid), and four minerals (copper, iron, phosphorus and sodium) (Table VII-2).

The components that had significant differences in mean values between MON 88701 and the conventional control in the combined-site analysis were: five proximates (ash, calories, carbohydrates, moisture and total fat), three types of fiber (ADF, NDF and TDF), three amino acids (arginine, methionine and proline), two fatty acids (14:0 myristic acid and 18:2 linoleic acid), five minerals (calcium, magnesium, manganese, potassium and zinc) and vitamin E (Table VII-1).

The statistical significant differences in nutrients were further evaluated using the four previously described considerations relevant to the safety and nutritional quality of MON 88701 when compared to the conventional control:

1) All nutrient component differences observed in the combined-site statistical analysis, whether reflecting increased or decreased MON 88701 mean values with respect to the conventional control, were 14.09% or less. The relative magnitudes of the differences were : 0.66 to 5.00% for proximates, 4.08 to 5.72% for fibers, 2.61 to 4.82% for amino acids, 0.69 to 2.69% for fatty acids, 4.94 to 14.09% for minerals and 6.70% for vitamin E.

2) With the exception of methionine, mean values for all significantly different nutrient components from the combined-site analysis of MON 88701 were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial.

3) Assessment of the reproducibility of the combined-site differences at the eight individual sites showed significant differences for: NDF, methionine, proline and 18:2 linoleic acid at one site; carbohydrates, total fat, ADF, manganese and zinc at two sites; TDF, arginine, 14:0 myristic acid, potassium, and vitamin E at three sites; magnesium at four sites, ash at six sites and calcium at seven sites. Moisture and calories were not affected at any site. With the exception of methionine, arginine and zinc, all individual site mean values of MON 88701 for all nutrient components with significant differences were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial.

4) All combined-site mean values and individual mean values of MON 88701 for all nutrient components, including those that were significantly different, were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

Five of the 19 cottonseed nutrient statistically significant differences between MON 88701 and the conventional control that were observed in the combined-site data analysis were attributable to small differences in proximates (ash, calories, carbohydrates, total fat expressed as % dw, and moisture expressed as % fw). For ash, calories and total fat the relative magnitude of the differences between the mean value for MON 88701 and the conventional control were all small increases (5.00% for ash, 0.66% for calories, and 3.71% for total fat). The differences for carbohydrates and moisture between the mean value for MON 88701 and the conventional control were both small decreases (2.60% for carbohydrates and 4.51% for moisture). All of the nutrient mean values for MON 88701 observed in the combined-site analysis for proximates were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for most proximate mean values between MON 88701 and the conventional control were not consistently observed among individual sites. Total fat was increased at two sites ranging from 6.47 to 8.46% and carbohydrates were decreased at two sites, with decreases ranging from 4.33 to 5.08%. There were no significant differences at any of the individual sites for calories or moisture. Although ash was increased in MON 88701 when compared to the conventional control at six sites, increases ranged from 4.95 to 11.50%, which was less than the variability for the control samples (range 3.46 to 4.29, a relative difference of 24.0%, Table VII-1). Overall, observed differences in proximate values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because the magnitudes of combined-site differences were 5.00% or less, most were not consistently reproduced across the individual sites, and the mean MON 88701 combined-site values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial, and were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

Three of the 19 cottonseed nutrient statistically significant differences between MON 88701 and the conventional control observed in the combined-site data analysis were attributable to small differences in fiber (ADF, NDF and TDF all expressed as % dw). All relative magnitudes of the differences for fiber between the mean values for MON 88701 and the conventional control were small decreases (4.94% for ADF, 5.72% for NDF and 4.08% for TDF). All of the nutrient mean values for MON 88701 observed in the combined-site analysis for fiber were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for fiber mean values between MON 88701 and the conventional control were not consistently observed among individual sites. TDF and ADF were

decreased at three and two sites, respectively, with decreases ranging from 4.55 to 8.15% for TDF and 9.27 to 9.86% for ADF. NDF was significantly different at one site with a small decrease of 7.40%. Overall, observed differences in fiber values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because they were small in magnitude, not consistently reproduced across the individual sites, and the mean MON 88701 combined-site values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial, and were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

Three other combined-site nutrient statistically significant differences between MON 88701 and the conventional control observed in the combined-site analysis were attributed to small differences in amino acids (arginine, methionine and proline expressed as % dw). For both arginine and proline, the relative magnitude of the differences between the mean values for MON 88701 and the conventional control were small decreases (3.80% for arginine and 2.61% for proline). Methionine was increased 4.82% when MON 88701 was compared to the conventional control. With the exception of methionine, the nutrient mean values for MON 88701 observed in the combined-site analysis for amino acids were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. The combined-site difference value for methionine was within the context of natural variation of methionine found in commercial cotton as published in the scientific literature or as found in the ILSI Crop Composition Database (ILSI, 2011). Significant differences for amino acid mean values between MON 88701 and the conventional control were not consistently observed at all eight individual sites. Arginine and proline were decreased at three sites and one site, respectively, with decreases ranging from 6.10 to 8.35% for arginine and 6.16% for proline. Methionine was increased 12.03% at only one site. Overall, observed differences in amino acid values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because they were small in magnitude, not consistently reproduced across the individual sites, and with the exception of methionine, the mean MON 88701 combined-site values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. In addition, all MON 88701 amino acid values were within the context of the natural variability of commercial cotton composition as published in the scientific literature or available in the ILSI Crop Composition Database (ILSI, 2011).

Two of the combined-site nutrient statistically significant differences between MON 88701 and the conventional control were attributed to the fatty acids 14:0 myristic acid and 18:2 linoleic acid (expressed as % total FA). The relative magnitudes of the differences between the mean fatty acid values for MON 88701 and the conventional control in the combined-site analysis were small decreases (2.69% for 14:0 myristic acid and 0.69% for 18:2 linoleic acid). The nutrient mean values for MON 88701 observed in the combined-site analysis for both 14:0 myristic acid and 18:2 linoleic acid were within

the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for fatty acid mean values between MON 88701 and the conventional control were not consistently observed among individual sites. 14:0 myristic acid was decreased at three sites, while 18:2 linoleic acid was decreased at one site with differences ranging from 4.43 to 8.36% for 14:0 myristic acid and 1.93% for 18:2 linoleic acid. Overall, observed differences in fatty acid values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety and nutritional perspective because they were small, not consistently reproduced across the individual sites, and the mean MON 88701 values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial and were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

Five of the 19 cottonseed nutrient statistically significant differences between MON 88701 and the conventional control observed in the combined-site analysis were attributed to small differences in minerals (calcium, magnesium, and potassium expressed as % dw and manganese and zinc expressed as mg/kg dw). For calcium, magnesium, potassium and manganese the relative magnitudes of the differences between the mean values for MON 88701 and the conventional control were increases of (14.09% for calcium, 5.63% for magnesium, 9.20% for manganese, and 4.94% for potassium). The relative magnitude of the difference for zinc between the mean value for MON 88701 and the conventional control was a decrease of 6.39%. All of the nutrient mean values for MON 88701 observed in the combined-site analysis for minerals were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for mineral mean values between MON 88701 and the conventional control were not consistently observed among individual sites. Although calcium was significantly different at seven sites, with increases ranging from 6.92 to 22.70%, this was less than the variability observed for the control samples (range 0.091 to 0.18, a relative difference of 97.8%, Table VII-1).

Magnesium, potassium, and manganese were significantly different at four, three and two sites, respectively with increases ranging from 5.54 to 9.36% for magnesium, 8.01 to 16.37% for potassium and from 16.52 to 20.59% for manganese. Zinc was significantly different at two sites with decreases ranging from 7.68 to 17.66%. Overall, observed differences in mineral values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because they were small in magnitude, not consistently reproduced across the individual sites (with the exception of calcium), and the mean MON 88701 combined-site values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial and within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

The last nutrient statistically significant difference observed in the combined-site analysis between MON 88701 and the conventional control was attributed to vitamin E (expressed as mg/kg dw). The relative magnitude of the difference between the mean vitamin E value for MON 88701 and the conventional control in the combined-site analysis was a small increase of 6.70%. The nutrient mean value for MON 88701 observed in the combined-site analysis for vitamin E was within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for vitamin E mean values between MON 88701 and the conventional control were not consistently observed among individual sites, with significant increases ranging from 7.78 to 13.28% observed at three sites. Overall, the observed differences in the vitamin E values between MON 88701 and the conventional control in the combined-site analysis were not considered to be meaningful from a food and feed safety and nutritional perspective because they were 13.28% or less, not consistently reproduced across the individual sites, and the mean MON 88701 values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial and were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

In summary, statistical analyses found no consistent differences between the levels of nutrient components in cottonseed from MON 88701 and the conventional control. Differences were observed for calcium and ash in combined analyses and most individual sites, but the magnitudes of differences for these nutrients were less than the variability for the control samples, and values were within the range of natural variability for cottonseed. These findings support the conclusion of compositional equivalence of MON 88701 to conventional cotton.

VII.A.2. Anti-Nutrient Levels in Cottonseed

Cottonseed was analyzed for five anti-nutrients and in the combined-site analysis of anti-nutrient levels in cottonseed no statistically significant differences ($p < 0.05$) in mean values between MON 88701 and the conventional control: for two cyclopropenoid fatty acids (malvalic and sterculic) (Table VII-3).

The components that showed statistically significant differences in mean values between MON 88701 and the conventional control in the combined-site analysis were the cyclopropenoid fatty acid dihydrosterculic, free gossypol, and total gossypol (Table VII-1).

The statistically significant differences in anti-nutrients were further evaluated using the four previously described considerations relevant to the safety and nutritional quality of MON 88701 when compared to the conventional control:

1) All anti-nutrient component differences observed in the combined-site statistical analysis, which reflected an increase in MON 88701 mean values with respect to

the conventional control, were small in magnitude. The relative magnitude of the differences for dihydrosterculic acid, free gossypol and total gossypol were 9.59%, 6.23% and 6.75%, respectively.

2) Mean values for all significantly different anti-nutrient components from the combined-site analysis of MON 88701 were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial. ,

3) Assessment of the reproducibility of the combined-site differences at the eight individual sites showed significant differences for: dihydrosterculic at one site; free gossypol at two sites; and total gossypol at three sites. All individual site mean values of MON 88701 for all anti-nutrient components with significant differences were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial.

4) All combined-site mean values of MON 88701 for all anti-nutrient components including those that were significantly different were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

The three cottonseed anti-nutrient statistically significant differences between MON 88701 and the conventional control observed in the combined-site data analysis were attributed to small differences in one cyclopropenoid fatty acid (dihydrosterculic expressed as % total fatty acid), free gossypol and total gossypol (expressed as % dw). For dihydrosterculic acid, free gossypol and total gossypol, the relative magnitude of the differences between the mean values for MON 88701 and the conventional control were increases of 9.59% for dihydrosterculic acid, 6.23% for free gossypol and 6.75% for total gossypol. These anti-nutrient differences between MON 88701 and the conventional control observed in the combined-site analysis were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial. Significant differences for the three anti-nutrient mean values between MON 88701 and the conventional control were not consistently observed across all eight individual sites. Dihydrosterculic acid, free gossypol, and total gossypol were significantly different at one, two and three sites respectively, with an increases of 28.35% for dihydrosterculic acid, and ranging from 12.69 to 22.32% for free gossypol and 9.54 to 15.53% for total gossypol. Overall, observed differences in anti-nutrient values between MON 88701 and the conventional control were not considered to be meaningful from a food and feed safety or nutritional perspective because they were generally small, not consistently reproduced across the individual sites, and the mean MON 88701 values were within the 99% tolerance interval established by conventional commercial reference varieties grown concurrently in the same trial and within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

In summary, statistical analyses found no consistent statistically significant differences between the levels of anti-nutrient components in cottonseed from MON 88701 and the conventional control and mean values for anti-nutrients were within the natural variability found for cottonseed. These findings supported the conclusion of compositional equivalence of MON 88701 to conventional cotton.

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in Combined-Site Analysis						
Cottonseed Proximate (% dw)						
Ash	4.31	4.11	5.00	0.001	3.77 - 4.74	3.42, 4.65
Calories	498.50	495.24	0.66	0.013	482.46 - 517.46	457.61, 527.56
Carbohydrates	44.64	45.83	-2.60	<0.001	41.40 - 48.89	40.26, 56.45
Moisture (% fw)	7.15	7.48	-4.51	0.005	5.93 - 9.67	4.79, 9.92
Total Fat	23.14	22.31	3.71	0.001	19.79 - 26.78	15.01, 28.51
Cottonseed Fiber (% dw)						
Acid Detergent Fiber	25.27	26.58	-4.94	0.002	23.26 - 27.74	22.24, 31.96
Neutral Detergent Fiber	30.73	32.59	-5.72	<0.001	25.13 - 34.42	27.03, 42.49

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in Combined-Site Analysis						
Cottonseed Fiber (% dw)						
Total Dietary Fiber	39.44	41.12	-4.08	<0.001	36.91 - 42.13	34.52, 52.58
Cottonseed Amino Acid (% dw)						
Arginine	3.03	3.15	-3.80	0.002	2.33 - 3.60	2.38, 3.47
Methionine	0.40	0.38	4.82	0.026	0.35 - 0.46	0.32, 0.38
Proline	1.00	1.03	-2.61	0.037	0.82 - 1.21	0.83, 1.08
Cottonseed Fatty Acid (% Total FA)						
14:0 Myristic	0.77	0.79	-2.69	0.009	0.66 - 0.95	0.16, 1.37
18:2 Linoleic	55.77	56.15	-0.69	0.026	54.24 - 58.22	47.49, 63.18

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in Combined-Site Analysis						
Cottonseed Mineral						
Calcium (% dw)	0.15	0.13	14.09	<0.001	0.10 - 0.22	0.058, 0.21
Magnesium (% dw)	0.40	0.38	5.63	<0.001	0.35 - 0.44	0.28, 0.47
Manganese (mg/kg dw)	12.81	11.73	9.20	0.001	10.18 - 14.81	9.07, 17.33
Potassium (% dw)	1.12	1.07	4.94	0.021	0.98 - 1.24	0.92, 1.21
Zinc (mg/kg dw)	37.58	40.14	-6.39	0.005	27.31 - 46.74	27.27, 44.95
Cottonseed Vitamin (mg/kg dw)						
Vitamin E	140.14	131.33	6.70	<0.001	86.23 - 179.34	41.91, 205.89
Cottonseed Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15	0.14	9.59	0.003	0.11 - 0.19	0.078, 0.25

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in Combined-Site Analysis						
Cottonseed Gossypol (% dw)						
Free Gossypol	0.94	0.89	6.23	0.016	0.80 - 1.18	0.099, 1.57
Total Gossypol	1.04	0.97	6.75	<0.001	0.84 - 1.24	0.064, 1.76
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 7 Sites						
Calcium (% dw) Site ARTI	0.15	0.12	22.70	0.010	0.14 - 0.16	0.058, 0.21
Calcium (% dw) Site GACH	0.13	0.11	17.57	<0.001	0.13 - 0.13	0.058, 0.21
Calcium (% dw) Site KSLA	0.20	0.18	14.74	0.007	0.19 - 0.22	0.058, 0.21
Calcium (% dw) Site NCBD	0.15	0.14	6.92	0.007	0.14 - 0.15	0.058, 0.21
Calcium (% dw) Site NMLC	0.15	0.13	16.83	0.003	0.14 - 0.15	0.058, 0.21

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 7 Sites						
Calcium (% dw) Site SCEK	0.11	0.091	17.98	0.027	0.10 - 0.11	0.058, 0.21
Calcium (% dw) Site TXPL	0.16	0.14	15.31	<0.001	0.16 - 0.16	0.058, 0.21
Cottonseed Proximate (% dw) - 6 Sites						
Ash Site GACH	4.53	4.21	7.56	<0.001	4.45 - 4.57	3.42, 4.65
Ash Site KSLA	4.53	4.29	5.64	0.027	4.25 - 4.66	3.42, 4.65
Ash Site LACH	4.35	4.12	5.56	0.013	4.23 - 4.47	3.42, 4.65
Ash Site NCBD	4.34	4.14	4.95	0.033	4.29 - 4.40	3.42, 4.65
Ash Site SCEK	4.11	3.74	9.95	0.010	3.99 - 4.28	3.42, 4.65

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Proximate (% dw) - 6 Sites						
Ash Site TXPL	3.85	3.46	11.50	0.001	3.77 - 3.92	3.42, 4.65
Cottonseed Fatty Acid (% Total FA) - 5 Sites						
18:0 Stearic Site ARTI	2.68	2.51	6.70	0.019	2.65 - 2.72	1.98, 2.95
18:0 Stearic Site LACH	2.68	2.52	6.04	0.001	2.64 - 2.73	1.98, 2.95
18:0 Stearic Site NCBD	2.50	2.34	6.85	0.036	2.39 - 2.64	1.98, 2.95
18:0 Stearic Site NMLC	2.51	2.64	-5.13	<0.001	2.47 - 2.56	1.98, 2.95
18:0 Stearic Site TXPL	2.35	2.46	-4.67	0.006	2.30 - 2.43	1.98, 2.95
Cottonseed Mineral - 4 Sites						
Magnesium (% dw) Site GACH	0.41	0.38	6.92	<0.001	0.40 - 0.41	0.28, 0.47

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 4 Sites						
Magnesium (% dw) Site KSLA	0.43	0.40	6.85	0.002	0.41 - 0.43	0.28, 0.47
Magnesium (% dw) Site SCEK	0.39	0.36	9.36	0.005	0.37 - 0.41	0.28, 0.47
Magnesium (% dw) Site TXPL	0.35	0.34	5.54	0.003	0.35 - 0.37	0.28, 0.47
Cottonseed Fiber (% dw) - 3 Sites						
Total Dietary Fiber Site KSLA	38.32	40.14	-4.55	0.034	37.62 - 38.75	34.52, 52.58
Total Dietary Fiber Site LACH	39.82	43.35	-8.15	0.002	39.02 - 40.86	34.52, 52.58
Total Dietary Fiber Site NMLC	39.16	41.10	-4.73	0.016	37.46 - 40.44	34.52, 52.58
Cottonseed Amino Acid (% dw) - 3 Sites						
Arginine Site GACH	2.95	3.21	-8.35	0.008	2.87 - 3.02	2.38, 3.47

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Amino Acid (% dw) - 3 Sites						
Arginine Site KSLA	3.02	3.28	-7.87	0.013	2.95 - 3.10	2.38, 3.47
Arginine Site NMLC	3.48	3.71	-6.10	0.005	3.42 - 3.60	2.38, 3.47
Cottonseed Fatty Acid (% Total FA) - 3 Sites						
14:0 Myristic Site KSLA	0.68	0.72	-5.33	0.007	0.66 - 0.71	0.16, 1.37
14:0 Myristic Site NCBD	0.68	0.75	-8.36	0.002	0.66 - 0.70	0.16, 1.37
14:0 Myristic Site NMLC	0.93	0.98	-4.43	0.001	0.92 - 0.95	0.16, 1.37
Cottonseed Mineral - 3 Sites						
Potassium (% dw) Site GACH	1.21	1.12	8.01	<0.001	1.17 - 1.24	0.92, 1.21
Potassium (% dw) Site SCEK	1.13	1.02	10.88	0.042	1.11 - 1.17	0.92, 1.21

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88701 minus Control) Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 3 Sites						
Potassium (% dw) Site TXPL	1.01	0.87	16.37	0.004	0.98 - 1.06	0.92, 1.21
Cottonseed Vitamin (mg/kg dw) - 3 Sites						
Vitamin E Site GACH	151.03	140.12	7.78	0.025	148.34 - 154.95	41.91, 205.89
Vitamin E Site LACH	169.88	149.96	13.28	0.001	163.34 - 175.33	41.91, 205.89
Vitamin E Site TXPL	114.39	103.66	10.35	0.033	107.81 - 118.39	41.91, 205.89
Cottonseed Gossypol (% dw) - 3 Sites						
Total Gossypol Site KSLA	1.13	1.01	12.00	0.049	1.00 - 1.24	0.064, 1.76
Total Gossypol Site NMLC	0.92	0.80	15.53	0.026	0.84 - 0.97	0.064, 1.76
Total Gossypol Site SCEK	1.17	1.07	9.54	0.017	1.13 - 1.23	0.064, 1.76

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Proximate (% dw) - 2 Sites						
Carbohydrates Site SCEK	46.56	48.67	-4.33	0.031	45.10 - 47.48	40.26, 56.45
Carbohydrates Site TXPL	44.03	46.39	-5.08	0.010	42.73 - 45.99	40.26, 56.45
Total Fat Site NCBD	23.04	21.59	6.74	0.024	21.89 - 23.76	15.01, 28.51
Total Fat Site SCEK	25.65	23.65	8.46	0.019	24.23 - 26.78	15.01, 28.51
Cottonseed Fiber (% dw) - 2 Sites						
Acid Detergent Fiber Site ARTI	24.81	27.53	-9.86	0.007	24.44 - 25.20	22.24, 31.96
Acid Detergent Fiber Site LACH	25.72	28.35	-9.27	0.005	24.16 - 27.08	22.24, 31.96
Cottonseed Amino Acid (% dw) - 2 Sites						
Phenylalanine Site GACH	1.40	1.49	-5.89	0.039	1.37 - 1.43	1.12, 1.58

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Amino Acid (% dw) - 2 Sites						
Phenylalanine Site KSLA	1.44	1.53	-5.88	0.025	1.40 - 1.46	1.12, 1.58
Cottonseed Fatty Acid (% Total FA) - 2 Sites						
16:0 Palmitic Site LACH	24.48	24.04	1.81	0.014	24.37 - 24.55	16.54, 30.55
16:0 Palmitic Site SCEK	24.74	24.39	1.43	0.029	24.59 - 24.94	16.54, 30.55
16:1 Palmitoleic Site NCBD	0.46	0.48	-3.88	0.019	0.44 - 0.47	0.39, 0.70
16:1 Palmitoleic Site NMLC	0.53	0.54	-2.27	0.014	0.52 - 0.53	0.39, 0.70
18:3 Linolenic Site ARTI	0.14	0.13	11.92	0.012	0.14 - 0.15	0.060, 0.24
18:3 Linolenic Site NMLC	0.16	0.14	8.12	0.009	0.15 - 0.16	0.060, 0.24

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Mineral - 2 Sites						
Iron (mg/kg dw) Site NCBD	43.21	48.04	-10.05	0.025	41.96 - 44.44	47.30, 97.12
Iron (mg/kg dw) Site TXPL	60.47	79.02	-23.47	0.039	56.94 - 66.50	47.30, 97.12
Manganese (mg/kg dw) Site GACH	13.41	11.51	16.52	0.003	12.79 - 14.14	9.07, 17.33
Manganese (mg/kg dw) Site TXPL	10.91	9.04	20.59	0.007	10.18 - 11.37	9.07, 17.33
Zinc (mg/kg dw) Site NCBD	40.79	49.54	-17.66	0.006	40.28 - 41.37	27.27, 44.95
Zinc (mg/kg dw) Site NMLC	45.63	49.43	-7.68	0.009	44.12 - 46.74	27.27, 44.95
Cottonseed Gossypol (% dw) - 2 Sites						
Free Gossypol Site KSLA	1.07	0.95	12.69	0.014	1.03 - 1.10	0.099, 1.57

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Cottonseed Gossypol (% dw) - 2 Sites						
Free Gossypol Site NMLC	0.85	0.69	22.32	0.011	0.83 - 0.88	0.099, 1.57
Statistical Differences Observed in One Site						
Cottonseed Proximate (% dw)						
Protein Site TXPL	29.43	28.48	3.33	0.017	29.06 - 30.14	22.30, 29.41
Cottonseed Fiber (% dw)						
Crude Fiber Site KSLA	16.43	17.67	-7.04	0.019	16.06 - 17.24	16.93, 22.68
Neutral Detergent Fiber Site TXPL	29.75	32.12	-7.40	0.006	28.74 - 30.56	27.03, 42.49
Cottonseed Amino Acid (% dw)						
Alanine Site LACH	1.07	1.03	3.73	0.030	1.00 - 1.11	0.86, 1.11
Aspartic Acid Site GACH	2.31	2.45	-6.03	0.019	2.24 - 2.36	1.94, 2.57

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in One Site						
Cottonseed Amino Acid (% dw)						
Glutamic Acid Site GACH	4.57	4.96	-7.95	0.010	4.35 - 4.77	3.74, 5.28
Isoleucine Site GACH	0.90	0.94	-4.21	0.034	0.90 - 0.91	0.75, 0.96
Leucine Site GACH	1.51	1.58	-4.32	0.024	1.49 - 1.54	1.25, 1.62
Lysine Site LACH	1.26	1.18	7.01	0.023	1.17 - 1.31	1.01, 1.30
Methionine Site LACH	0.42	0.38	12.03	0.013	0.37 - 0.44	0.32, 0.38
Proline Site GACH	0.98	1.05	-6.16	0.033	0.97 - 0.99	0.83, 1.08
Threonine Site GACH	0.85	0.90	-5.14	0.049	0.83 - 0.88	0.72, 0.89
Tryptophan Site SCEK	0.35	0.38	-6.70	0.023	0.33 - 0.38	0.34, 0.42

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in One Site						
Cottonseed Amino Acid (% dw)						
Tyrosine Site GACH	0.80	0.84	-4.30	0.037	0.79 - 0.82	0.67, 0.84
Valine Site GACH	1.21	1.26	-4.19	0.017	1.19 - 1.23	1.00, 1.28
Cottonseed Fatty Acid (% Total FA)						
18:1 Oleic Site LACH	14.70	14.29	2.89	0.021	14.48 - 15.01	11.38, 20.64
18:2 Linoleic Site LACH	55.53	56.63	-1.93	0.001	55.15 - 55.99	47.49, 63.18
20:0 Arachidic Site LACH	0.31	0.29	6.78	0.033	0.31 - 0.32	0.17, 0.38
22:0 Behenic Site ARTI	0.14	0.15	-9.92	0.008	0.13 - 0.14	0.070, 0.21
Cottonseed Mineral						
Sodium (% dw) Site KSLA	0.022	0.0080	178.30	0.020	0.019 - 0.025	0, 0.066

Table VII-1. Summary of Differences (p<0.05) for the Comparison of Cottonseed Component Levels for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88701 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 88701 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in One Site						
Cottonseed Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid Site GACH	0.15	0.12	28.35	0.022	0.14 - 0.16	0.078, 0.25

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 was sprayed with dicamba and glufosinate.
[3]Mean = least-square mean.
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table VII-2. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.31 (0.11) (3.77 - 4.74)	4.11 (0.11) (3.34 - 5.00)	0.21 (0.052) (-0.49 - 0.61)	0.094, 0.32	0.001	3.42, 4.65 (3.18 - 4.68)
Calories	498.50 (1.65) (482.46 - 517.46)	495.24 (1.71) (487.70 - 512.65)	3.26 (1.29) (-14.30 - 18.37)	0.70, 5.82	0.013	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	44.64 (0.56) (41.40 - 48.89)	45.83 (0.57) (42.14 - 50.30)	-1.19 (0.32) (-5.19 - 2.45)	-1.82, -0.56	<0.001	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	7.15 (0.26) (5.93 - 9.67)	7.48 (0.27) (6.15 - 9.19)	-0.34 (0.11) (-1.82 - 0.79)	-0.56, -0.11	0.005	4.79, 9.92 (6.05 - 10.50)
Protein	27.91 (0.77) (22.71 - 31.47)	27.79 (0.77) (23.53 - 31.27)	0.13 (0.31) (-1.99 - 3.73)	-0.53, 0.78	0.685	22.30, 29.41 (20.58 - 29.28)
Total Fat	23.14 (0.31) (19.79 - 26.78)	22.31 (0.33) (20.71 - 25.20)	0.83 (0.26) (-2.89 - 3.86)	0.32, 1.34	0.001	15.01, 28.51 (16.58 - 25.25)

Table VII-2. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Fiber (% dw)						
Acid Detergent Fiber	25.27 (0.34) (23.26 - 27.74)	26.58 (0.35) (22.08 - 29.58)	-1.31 (0.35) (-5.42 - 1.77)	-2.06, -0.57	0.002	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	18.17 (0.37) (15.97 - 21.66)	18.54 (0.38) (16.06 - 21.70)	-0.38 (0.32) (-3.36 - 4.75)	-1.02, 0.27	0.246	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	30.73 (0.51) (25.13 - 34.42)	32.59 (0.53) (28.87 - 35.89)	-1.86 (0.41) (-6.95 - 1.16)	-2.68, -1.05	<0.001	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	39.44 (0.39) (36.91 - 42.13)	41.12 (0.41) (39.05 - 44.37)	-1.68 (0.36) (-5.34 - 1.09)	-2.45, -0.91	<0.001	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.06 (0.020) (0.91 - 1.14)	1.05 (0.020) (0.88 - 1.17)	0.0026 (0.0091) (-0.13 - 0.12)	-0.017, 0.022	0.775	0.86, 1.11 (0.83 - 1.22)
Arginine	3.03 (0.10) (2.33 - 3.60)	3.15 (0.10) (2.41 - 3.77)	-0.12 (0.033) (-0.47 - 0.39)	-0.19, -0.049	0.002	2.38, 3.47 (2.30 - 3.55)

Table VII-2. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.39 (0.062) (1.94 - 2.64)	2.41 (0.062) (1.92 - 2.74)	-0.015 (0.027) (-0.29 - 0.29)	-0.072, 0.042	0.575	1.94, 2.57 (1.79 - 2.72)
Cystine	0.41 (0.0091) (0.32 - 0.47)	0.40 (0.0094) (0.31 - 0.46)	0.0096 (0.0070) (-0.063 - 0.082)	-0.0043, 0.023	0.174	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.76 (0.13) (3.80 - 5.38)	4.84 (0.14) (3.66 - 5.70)	-0.079 (0.072) (-0.78 - 0.79)	-0.23, 0.077	0.295	3.74, 5.28 (3.39 - 5.45)
Glycine	1.10 (0.020) (0.93 - 1.19)	1.09 (0.020) (0.91 - 1.20)	0.0014 (0.011) (-0.13 - 0.14)	-0.021, 0.024	0.896	0.90, 1.14 (0.85 - 1.23)
Histidine	0.74 (0.019) (0.58 - 0.85)	0.75 (0.019) (0.61 - 0.84)	-0.0014 (0.0073) (-0.062 - 0.091)	-0.017, 0.014	0.854	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.91 (0.018) (0.75 - 1.01)	0.92 (0.018) (0.77 - 1.03)	-0.0066 (0.0079) (-0.077 - 0.096)	-0.023, 0.010	0.421	0.75, 0.96 (0.72 - 1.03)

Table VII-2. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Amino Acid (% dw)						
Leucine	1.53 (0.032) (1.29 - 1.70)	1.54 (0.032) (1.28 - 1.69)	-0.0018 (0.013) (-0.14 - 0.16)	-0.029, 0.026	0.892	1.25, 1.62 (1.20 - 1.72)
Lysine	1.24 (0.025) (1.05 - 1.38)	1.23 (0.025) (1.06 - 1.39)	0.0069 (0.015) (-0.11 - 0.15)	-0.026, 0.039	0.658	1.01, 1.30 (0.99 - 1.44)
Methionine	0.40 (0.0079) (0.35 - 0.46)	0.38 (0.0084) (0.32 - 0.46)	0.018 (0.0081) (-0.066 - 0.12)	0.0023, 0.035	0.026	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.43 (0.039) (1.14 - 1.66)	1.46 (0.039) (1.15 - 1.66)	-0.022 (0.014) (-0.18 - 0.19)	-0.052, 0.0084	0.144	1.12, 1.58 (1.10 - 1.63)
Proline	1.00 (0.029) (0.82 - 1.21)	1.03 (0.029) (0.81 - 1.25)	-0.027 (0.012) (-0.12 - 0.10)	-0.052, -0.0018	0.037	0.83, 1.08 (0.79 - 1.17)
Serine	1.08 (0.025) (0.90 - 1.23)	1.09 (0.026) (0.86 - 1.24)	-0.0036 (0.015) (-0.18 - 0.16)	-0.035, 0.028	0.807	0.83, 1.21 (0.81 - 1.24)

Table VII-2. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.87 (0.016) (0.74 - 0.94)	0.86 (0.016) (0.73 - 0.95)	0.0057 (0.0083) (-0.10 - 0.10)	-0.012, 0.023	0.504	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.41 (0.0092) (0.33 - 0.52)	0.42 (0.0095) (0.37 - 0.52)	-0.0061 (0.0066) (-0.081 - 0.078)	-0.019, 0.0071	0.361	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.81 (0.017) (0.67 - 0.92)	0.81 (0.018) (0.67 - 0.91)	-0.0011 (0.0083) (-0.074 - 0.12)	-0.019, 0.017	0.898	0.67, 0.84 (0.63 - 0.91)
Valine	1.21 (0.027) (1.00 - 1.40)	1.23 (0.027) (1.00 - 1.40)	-0.012 (0.011) (-0.090 - 0.12)	-0.036, 0.012	0.296	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.77 (0.030) (0.66 - 0.95)	0.79 (0.031) (0.71 - 0.98)	-0.021 (0.0071) (-0.077 - 0.047)	-0.036, -0.0060	0.009	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	23.95 (0.30) (22.34 - 25.28)	23.80 (0.30) (22.69 - 25.05)	0.15 (0.076) (-0.68 - 0.76)	-0.016, 0.31	0.073	16.54, 30.55 (19.11 - 26.73)

Table VII-2. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.50 (0.0094) (0.44 - 0.54)	0.50 (0.0094) (0.45 - 0.54)	0.0022 (0.0038) (-0.025 - 0.039)	-0.0060, 0.010	0.572	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.54 (0.058) (2.29 - 2.85)	2.47 (0.058) (2.15 - 2.76)	0.068 (0.036) (-0.16 - 0.24)	-0.0091, 0.14	0.079	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	15.10 (0.26) (14.15 - 16.45)	14.96 (0.26) (14.06 - 16.44)	0.14 (0.070) (-0.48 - 0.75)	-0.0049, 0.29	0.057	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	55.77 (0.39) (54.24 - 58.22)	56.15 (0.40) (54.04 - 57.93)	-0.39 (0.16) (-1.42 - 0.80)	-0.72, -0.053	0.026	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.18 (0.022) (0.14 - 0.34)	0.17 (0.022) (0.12 - 0.30)	0.011 (0.0068) (-0.0073 - 0.052)	-0.0038, 0.025	0.136	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.29 (0.0086) (0.23 - 0.32)	0.28 (0.0087) (0.23 - 0.32)	0.0044 (0.0047) (-0.027 - 0.046)	-0.0057, 0.015	0.364	0.17, 0.38 (0.20 - 0.36)

Table VII-2. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.15 (0.0051) (0.12 - 0.19)	0.15 (0.0051) (0.13 - 0.21)	-0.0035 (0.0029) (-0.049 - 0.032)	-0.0098, 0.0029	0.260	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.15 (0.0093) (0.10 - 0.22)	0.13 (0.0093) (0.081 - 0.19)	0.018 (0.0022) (-0.012 - 0.038)	0.013, 0.023	<0.001	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	8.90 (0.70) (5.22 - 11.91)	8.93 (0.70) (5.40 - 11.92)	-0.025 (0.16) (-2.59 - 1.29)	-0.34, 0.29	0.875	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	67.21 (4.40) (41.96 - 83.17)	71.33 (4.48) (45.03 - 95.10)	-4.12 (2.74) (-38.15 - 12.79)	-9.96, 1.71	0.153	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.40 (0.0083) (0.35 - 0.44)	0.38 (0.0084) (0.33 - 0.44)	0.021 (0.0032) (-0.036 - 0.054)	0.015, 0.028	<0.001	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	12.81 (0.47) (10.18 - 14.81)	11.73 (0.48) (8.61 - 14.11)	1.08 (0.28) (-1.95 - 2.54)	0.48, 1.68	0.001	9.07, 17.33 (9.07 - 17.14)

Table VII-2. Statistical Summary of Combined-Site Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Mineral						
Phosphorus (% dw)	0.72 (0.031) (0.56 - 0.84)	0.72 (0.031) (0.54 - 0.87)	0.0081 (0.0067) (-0.087 - 0.11)	-0.0053, 0.021	0.230	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.12 (0.028) (0.98 - 1.24)	1.07 (0.028) (0.79 - 1.27)	0.053 (0.020) (-0.12 - 0.27)	0.0089, 0.097	0.021	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.034 (0.0095) (0.018 - 0.12)	0.029 (0.0096) (0.0053 - 0.10)	0.0045 (0.0046) (-0.065 - 0.030)	-0.0053, 0.014	0.346	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	37.58 (2.01) (27.31 - 46.74)	40.14 (2.02) (28.22 - 52.95)	-2.57 (0.77) (-11.57 - 3.27)	-4.22, -0.91	0.005	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	140.14 (9.87) (86.23 - 179.34)	131.33 (9.88) (91.78 - 162.98)	8.80 (2.07) (-6.54 - 26.36)	4.39, 13.22	<0.001	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 was sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table VII-3. Statistical Summary of Combined-Site Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15 (0.0034) (0.11 - 0.19)	0.14 (0.0037) (0.11 - 0.17)	0.013 (0.0044) (-0.026 - 0.068)	0.0044, 0.022	0.003	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.39 (0.015) (0.20 - 0.55)	0.37 (0.016) (0.26 - 0.49)	0.013 (0.015) (-0.16 - 0.16)	-0.016, 0.043	0.371	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.22 (0.0067) (0.13 - 0.29)	0.21 (0.0072) (0.17 - 0.27)	0.0067 (0.0081) (-0.085 - 0.078)	-0.0096, 0.023	0.412	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	0.94 (0.037) (0.80 - 1.18)	0.89 (0.037) (0.68 - 1.20)	0.055 (0.020) (-0.086 - 0.20)	0.012, 0.099	0.016	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	1.04 (0.037) (0.84 - 1.24)	0.97 (0.037) (0.74 - 1.10)	0.066 (0.017) (-0.021 - 0.23)	0.031, 0.10	<0.001	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 was sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table VII-4. Literature and ILSI Ranges for Components in Cottonseed

Cottonseed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Cottonseed Nutrients		
Proximates (% dw)		
Ash	3.87 – 5.29[a]; 3.7 – 4.2[d]	3.761 – 5.342
Carbohydrates by calculation	45.28 – 53.62[a]	39.0 – 53.6
Calories by calculation	471.34 – 506.95[a]	
Moisture (% fw)	2.25 – 7.49[a]	2.3 – 9.9
Protein	24.54 – 30.83[a]; 21.2 – 25.9[b]	21.48 – 32.97
Total Fat	17.37 – 25.16[a]; 14.4 – 16.9[d]	17.201 – 27.292
Fiber (% dw)		
Acid Detergent Fiber	21.10 – 34.8[a]; 37.6 – 40.5[d]	19.74 – 38.95
Neutral Detergent Fiber	32.92 – 45.83[a]; 50.0 – 53.6[d]	25.56 – 51.87
Crude Fiber	13.85 – 17.94[a]	13.86 – 23.10
Total Dietary Fiber	not available	33.69 – 47.55
Amino Acids	**(% total AA)**	**(% dw)**
Alanine	4.16 – 4.41[a]; 3.6 – 4.2[b]	0.80 – 1.22
Arginine	11.28 – 12.51[a]; 10.9 – 12.3[b]	2.06 – 3.72
Aspartic acid	9.73 – 9.99[a]; 8.8 – 9.5[b]	1.82 – 2.94
Cystine/Cysteine	1.60 – 1.92[a]; 2.3 – 3.4[b]	0.35 – 0.56
Glutamic acid	20.76 – 21.61[a]; 20.5 – 22.4[b]	3.91 – 6.72
Glycine	4.44 – 4.58[a]; 3.8 – 4.5[b]	0.83 – 1.32
Histidine	3.00 – 3.12[a]; 2.6 – 2.8[b]	0.57 – 0.91
Isoleucine	3.10 – 3.67[a]; 3.0 – 3.4[b]	0.62 – 1.05
Leucine	6.27 – 6.65[a]; 5.5 – 6.1[b]	1.14 – 1.86
Lysine	4.85 – 5.37[a]; 4.2 – 4.6[b]	0.94 – 1.46
Methionine	1.46 – 1.88[a]; 1.3 – 1.8[b]	0.30 – 0.47
Phenylalanine	5.56 – 5.77[a]; 5.0 – 5.6[b]	1.02 – 1.72
Proline	4.06 – 4.28[a]; 3.1 – 4.0[b]	0.75 – 1.23
Serine	4.45 – 4.86[a]; 3.9 – 4.4[b]	0.91 – 1.35
Threonine	3.26 – 3.59[a]; 2.8 – 3.2[b]	0.55 – 0.92
Tryptophan	0.97 – 1.21[a]; 1.0 – 1.4[b]	0.194 – 0.319
Tyrosine	2.65 – 2.92[a]; 2.8 – 3.3[b]	0.53 – 0.84
Valine	4.76 – 5.14[a]; 4.3 – 4.7[b]	0.87 – 1.49
Fatty Acids (% total FA)		
8:0 Caprylic	not available	not available
10:0 Capric	not available	not available
12:0 Lauric	not available	not available
14:0 Myristic	0.55 – 2.40[a]; 0.6 – 1.5[b]	0.455 – 2.400
14:1 Myristoleic	not available	not available
15:0 Pentadecanoic	0.050 – 0.17[a]	0.103 – 0.481
15:1 Pentadecenoic	not available	not available
16:0 Palmitic	21.23 – 27.9[a]; 17.6 – 24.8[b]	15.11 – 27.90
16:1 Palmitoleic	0.55 – 1.16[a]	0.464 – 1.190
17:0 Heptadecanoic	not available	0.092 – 0.119

Table VII-4. Literature and ILSI Ranges for Components in Cottonseed (continued)

Cottonseed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
17:1 Heptadecenoic	not available	not available
18:0 Stearic	1.99 – 3.11 [a]; 2.0 – 2.5 [b]	0.20 – 3.11
18:1 Oleic	13.90 – 20.10 [a]; 15.0 – 20.7 [b]	12.8 – 25.3
18:2 Linoleic	46.00 – 56.88 [a]	46.0 – 59.4
18:3 Gamma Linolenic	0.050 – 0.25 [a]	0.097 – 0.232
18:3 Linolenic	0.050 – 0.25 [a]	0.11 – 0.35
20:0 Arachidic	0.25 – 0.33 [a]	0.186 – 0.414
20:1 Eicosenoic	not available	0.095 – 0.098
20:2 Eicosadienoic	not available	not available
20:3 Eicosatrienoic	not available	not available
20:4 Arachidonic	not available	not available
22:0 Behenic	0.13 – 0.17 [a]	0.104 – 0.295
Vitamins	**(mg/kg fw)**	**(mg/kg dw)**
Vitamin E	99 – 224[c]	70.825 – 197.243
Minerals (% dw)		
Calcium	0.10 – 0.33[a]	0.10323 – 0.32581
Copper (mg/kg dw)	3.54 – 11.14[a]	3.13 – 24.57
Iron (mg/kg dw)	40.58 – 56.54 [a]	36.71 – 318.38
Magnesium	0.37 – 0.46 [a]	0.34709 – 0.49312
Manganese (mg/kg dw)	11.06 – 18.31 [a]	10.69 – 21.96
Phosphorus	0.60 – 0.84 [a]	0.48254 – 0.99157
Potassium	0.98 – 1.24 [a]	0.98345 – 1.44835
Sodium	0.0054 – 0.74 [a]	0.01118 – 0.73548
Zinc (mg/kg dw)	30.21 – 47.75 [a]	27.0 – 59.5
Cottonseed Anti-Nutrients		
Gossypol, Total (% dw)	0.57 – 1.42[a]; 0.55 – 0.77[d]	0.547 – 1.522
Gossypol, Free (% dw)	0.53 – 1.20[a]	0.454 – 1.399
Cyclopropenoid Fatty Acids (% total FA)		
Dihydrosterculic	0.13 – 0.24 [a]	0.075 – 0.310
Malvalic	0.33 – 0.58 [a]	0.229 – 0.759
Sterculic	0.21 – 0.56 [a]	0.190 – 0.556

[1] fw=fresh weight; dw=dry weight
[2] Literature range references; [a](Hamilton et al., 2004); [b](Lawhon et al., 1977); [c](Smith and Creelman, 2001); [d](Bertrand et al., 2005).
[3] (ILSI, 2011).

VII.B. Compositional Assessment of MON 88701: Summary and Conclusion

Detailed comparisons were conducted on nutrient and anti-nutrient levels in MON 88701 cottonseed and compared to levels in the conventional control. The analytes evaluated are consistent with those identified by the OECD as important to understanding the safety and nutrition of new varieties of biotechnology-derived cotton (OECD, 2009b). These compositional comparisons were made by analyzing the acid-delinted cottonseed harvested from plants grown at each of eight field sites in the U.S. during the 2010 field season. Composition analysis of all samples was conducted in accordance with OECD guidelines and included analysis for nutrients including proximates (ash, calories and carbohydrates by calculation, fat, moisture, and protein), ADF, NDF, CF, TDF, amino acids, fatty acids (C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and vitamin E. The anti-nutrients assessed in this analysis included total and free gossypol and cyclopropenoid fatty acids (dihydrosterculic, malvalic and sterculic). These analyses also included measurements of the same nutrients and anti-nutrients in conventional cotton varieties, known as reference substances, to provide data on natural variability of each compositional component analyzed. All cotton plants including MON 88701, the conventional control, and the commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition, MON 88701 plots were treated at the 3-5 leaf stage with glufosinate herbicide at the label rate (0.5 lb a.e. /acre) and at the 6-10 leaf stage with dicamba herbicide at the label rate (0.5 lb a.e. /acre).

For MON 88701 compared to the conventional control, the combined-site analysis of cottonseed showed no statistically significant differences ($p < 0.05$) between nutrient and anti-nutrient components of MON 88701 and the control for 30 (57.7%) of the 52 mean value comparisons. Combined-site statistical differences for nutrients in cottonseed included mean values for five proximates (ash, calories, carbohydrates, moisture and total fat), three types of fiber (ADF, NDF and TDF), three amino acids (arginine, methionine and proline), two fatty acids (14:0 myristic acid and 18:2 linoleic acid), five minerals (calcium, magnesium, manganese, potassium and zinc), and vitamin E. Combined-site statistical differences for anti-nutrients in cottonseed included mean values for dihydrosterculic acid, free gossypol and total gossypol. All nutrient and anti-nutrient component statistical differences observed in the combined-site statistical analysis, whether reflecting increased or decreased MON 88701 mean values with respect to the conventional control, were 14.09% or less. Mean values for all significantly different nutrient and anti-nutrient components from the combined-site analysis of MON 88701, with the exception of methionine, were within the 99% tolerance interval established from the conventional commercial reference varieties grown concurrently in the same trial. All combined-site mean values including methionine and individual site mean values of MON 88701 for all nutrient and anti-nutrient components were within the context of the natural variability of commercial cotton composition as published in the scientific literature and/or available in the ILSI Crop Composition Database (ILSI, 2011).

For MON 88701 compared to the conventional control, most of the combined-site differences were not reproducible among the individual sites with the exception of ash and calcium; however, all of the combined-site component values were within the range of values reported in the scientific literature and/or in the ILSI Crop Composition Database. Based on the results of this composition analysis it is concluded that cottonseed from MON 88701 is compositionally equivalent to conventional cotton and therefore the food and feed safety and nutritional quality of this product is comparable to that of the conventional cotton.

Conventional cotton processing is described in this document in Section II. The processing of MON 88701 cotton is not expected to be any different from that of conventional cotton. As described in this section, detailed compositional analyses of key components of MON 88701 have been performed and have demonstrated that MON 88701 is compositionally equivalent to conventional cotton. Additionally, the modes-of-action of MON 88701 DMO and PAT (*bar*) proteins, as described in Section VI.A, is well understood, and there is no reason to expect interactions with endogenous toxicants or important nutrients that may be present in cotton. Therefore, when MON 88701 and its progeny are used on a commercial scale as a source of food or feed, these products are not expected to be different from the equivalent foods or feeds originating from conventional cotton.

VIII. USE OF ANTIBIOTIC RESISTANCE MARKER GENES

VIII.A. Presence of Genes that Encode Resistance to Antibiotics

No genes that encode resistance to an antibiotic marker were inserted into the crop genome during development of MON 88701. Molecular characterization data presented in Section V demonstrate the absence of the *aadA* antibiotic resistant marker gene in MON 88701.

IX. SUMMARY OF FOOD AN FEED SAFETY ASSESSMENTS

This section provides a concluding discussion of the safety assessment and addresses the relevant factors in Codex Plant Guidelines, Section 5, paragraph 59 (Codex Alimentarius, 2009).

IX.A. Donor Organisms

As described in more detail in Section III, the *dmo* gene is derived from the bacterium *S. maltophilia*. *S. maltophilia* is ubiquitous in all environments, is found associated with the rhizosphere of plants, and can be found in a variety of foods and feeds. Exposure to *S. maltophilia* is incidental to its presence in food such as "ready to eat" salads, vegetables, frozen fish, milk, and poultry. Infections caused by *S. maltophilia* are extremely uncommon in humans and *S. maltophilia* can be found in healthy individuals without causing any harm to human health. Strains have been found in the transient flora of hospitalized patients as a commensal organism and, similar to the indigenous bacteria of the gastrointestinal tract, *S. maltophilia* can be an opportunistic pathogen. As such, *S. maltophilia* is of low virulence in immuno-compromised patients where a series of factors must occur for colonization by *S. maltophilia* in humans. The ubiquitous presence of *S. maltophilia* in the environment, its presence in healthy individuals, and the incidental presence on foods without any adverse safety reports establishes the safety of the donor organism.

In addition Section III provides details on *S. hygroscopicus*, the donor organism for the *bar* gene. *S. hygroscopicus* is a common soil-borne bacterium with no known safety issues. *Streptomyces* species are widespread in the environment and present no known allergenic or toxicity issues, though human exposure is quite common. The *S. hygroscopicus* history of safe use has been extensively reviewed during the regulatory reviews of numerous glufosinate-tolerant events with no safety or allergenicity issues identified. The ubiquitous presence of *S. hygroscopicus* in the environment, the widespread human exposure without any adverse safety or allergenicity reports, and the successive reviews resulting from the regulatory evaluation of numerous glufosinate-tolerant events with no safety or allergenicity issues identified establishes the safety of the donor organism.

IX.B. Genetic Insert

As described in more detail in Section IV, MON 88701 was produced by *Agrobacterium tumefaciens*-mediated transformation of cotton with PV–GHHT6997, which is a binary vector containing two expression cassettes in a single T-DNA (Figure IV-1). The *dmo* expression cassette contains the *dmo* coding sequence under the regulation of the *PC1SV* promoter, *TEV* leader, the *CTP2* targeting sequence, and the *E6* 3′ non-translated region. The *bar* expression cassette is regulated by the *e35S* promoter, the heat shock protein 70 (*Hsp70)* leader, and the nopaline synthase (*nos)* 3′ untranslated region. During plant

transformation, a single T-DNA was inserted into the cotton genome, with the *bar* expression cassette functioning as a selectable marker, as well as a gene of interest.

Molecular analyses demonstrated that MON 88701 contains a single copy of the inserted T-DNA at a single integration locus. No backbone sequences from the PV–GHHT6997 were detected in the genome of MON 88701. Data confirmed the organization and sequence of the insert and the stability of the insert over several generations.

IX.C. Safety of MON 88701 DMO and PAT (*bar*) Proteins

A history of safe use has been established for both MON 88701 DMO and PAT (*bar*) proteins (Section VI.B.). MON 88701 DMO and PAT (*bar*) lack structural similarity to known allergens (Sections VI.F.1.3. and VI.F.2.3) or toxins (Section VI.F.3.1.) known to have adverse effects on mammals. MON 88701 DMO and PAT (*bar*) are present at very low levels in MON 88701 cottonseed (Section VI.D) and will constitute a small portion of the total protein present in feed derived from MON 88701 (Section VI.F.1.2. and VI.F.2.2). No consumption of the MON 88701 DMO or PAT (*bar*) proteins derived from MON 88701 is expected for the U.S. general population at the present time given that the only foods produced from cottonseed are RBD oil and linter, both of which contain neligible amounts of total protein. MON 88701 DMO and PAT (*bar*) are readily digestible in simulated gastric and simulated intestinal fluids (Sections VI.F.1.4. and VI.F.2.4.), are heat labile (Sections VI.F.1.5. and VI.F.2.5.), and show no oral toxicity in mice (Section VI.F.3.3.). In addition, PAT proteins has been evaluated in several previous safety assessments with no safety concerns identified.

IX.D. Compositional Characteristics of MON 88701

Detailed compositional comparisons were presented (Section VII.A.) to assess whether levels of nutrients and anti-nutrients in cottonseed derived from MON 88701 are comparable to levels in the parental conventional control Coker 130 and several commercially available reference cotton varieties for which there is an established history of safe consumption. The analysis included proximates (ash, fat, moisture, protein, and carbohydrates), calories, fiber, amino acids, fatty acids, minerals, vitamin E, and anti-nutrients (gossypol and cyclopropenoid fatty acids) in cottonseed.

Compositional and nutritional comparisons were conducted using a combined-site analysis to determine statistically significant differences (5% level of significance) between MON 88701 and the conventional control. The results of the combined-site analysis were then evaluated using considerations relevant to food and feed safety and nutritional quality, including relative magnitudes of the difference, whether the mean component value was within the range of natural variability as represented by the 99% tolerance interval of the commercial reference varieties grown concurrently in the same field trial, the reproducibility of differences across individual sites, and whether the mean component value was within the range of natural variability as compared to published values including the ILSI Crop Composition Database.

Assessment of the analytical results confirmed that the differences observed in the combined-site analysis were not meaningful to food and feed safety or the nutritional quality of MON 88701. In addition, all combined-site component values were within the range of values reported in the scientific literature and/or in the ILSI Crop Composition Database. These results support the overall conclusion that MON 88701 cottonseed is compositionally equivalent to conventional cotton in accordance with OECD guidelines.

IX.E. Summary of Food and Feed Safety Assessment of MON 88701

These data, along with the history of safe use of cotton as a limited source of human food and common animal feed, collectively support a conclusion of "no concerns" for every criterion specified in the flowcharts outlined in the FDA's Food Policy document (U.S. FDA, 1992) and shown in Figure IX-1. MON 88701 is not materially different in composition, safety or nutrition from conventional cotton other than the introduction of the dicamba- and glufosinate-tolerance traits. Sales and consumption of cottonseed and processed products derived from MON 88701 and its progeny would be fully consistent with the FDA's Food Policy, the Federal Food, Drug and Cosmetic Act, and current practices for the development and introduction of new cotton varieties and biotechnology-derived traits.

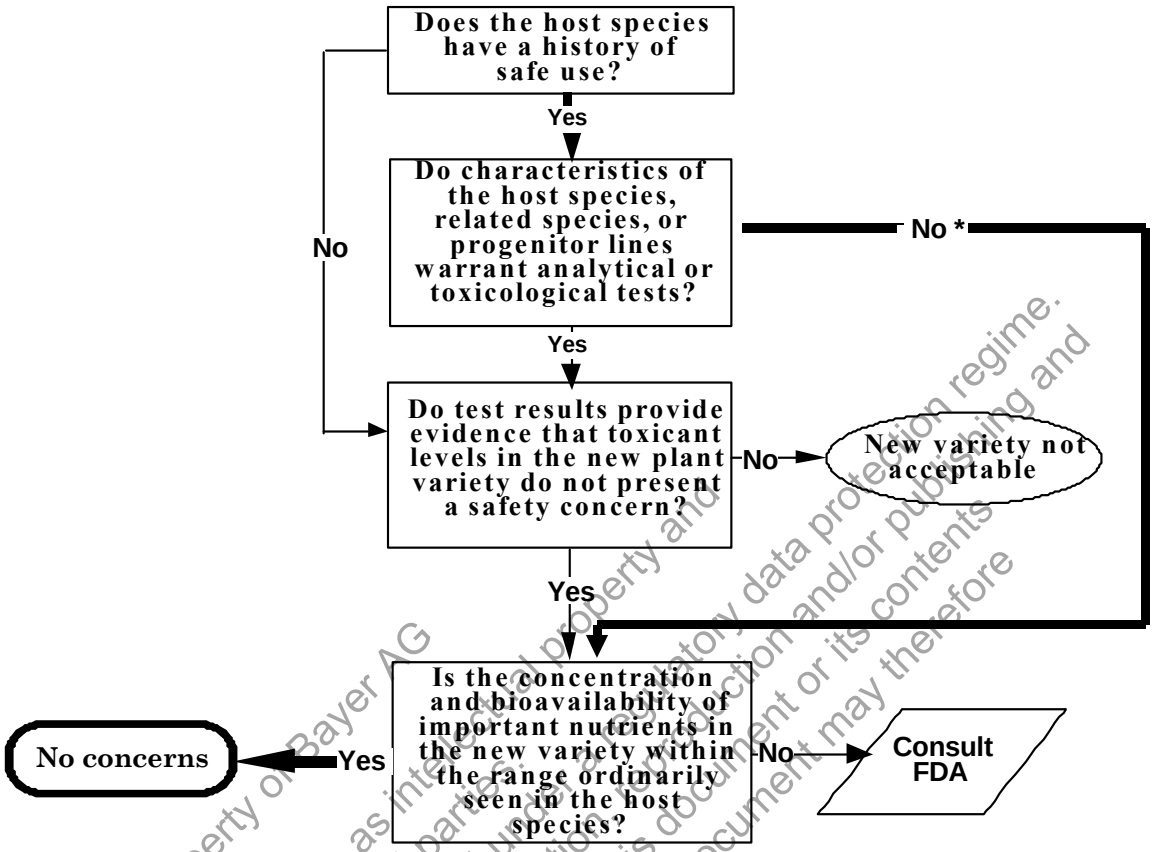


Figure IX-1. Safety Assessment of New Varieties: The Host Plant

REFERENCES

Abou-Donia, M.B. 1976. Physiological effects and metabolism of gossypol. Pages 126-160 in Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment. Volume 61. F.A. Gunther and J.D. Gunther (eds.). Springer-Verlag, New York, New York.

Adrian-Romero, M., G. Blunden, B.G. Carpenter and E. Tyihák. 1999. HPLC quantification of formaldehyde, as formaldemethone, in plants and plant-like organisms. Chromatographia 50:160-166.

AOCS. 1991. Cellulose yield pressure-cook method. Method Bb 3-47, American Oil Chemists' Society, Champaign, Illinois.

Arfin, S.M. and R.A. Bradshaw. 1988. Cotranslational processing and protein turnover in eukaryotic cells. Biochemistry 27:7979-7984.

Astwood, J.D., J.N. Leach and R.L. Fuchs. 1996. Stability of food allergens to digestion in vitro. Nature Biotechnology 14:1269-1273.

Barker, R.F., K.B. Idler, D.V. Thompson and J.D. Kemp. 1983. Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Molecular Biology 2:335-350.

Behrens, M.R., N. Mutlu, S. Chakraborty, R. Dumitru, W.Z. Jiang, B.J. LaVallee, P.L. Herman, T.E. Clemente and D.P. Weeks. 2007. Dicamba resistance: Enlarging and preserving biotechnology-based weed management strategies. Science 316:1185-1188.

Berardi, L.C. and L.A. Goldblat. 1980. Gossypol. Pages 184-238 in Toxic Constituents of Plant Foodstuffs. Academic Press, Inc., New York, New York.

Berberich, S.A., J.E. Ream, T.L. Jackson, R. Wood, R. Stipanovic, P. Harvey, S. Patzer and R.L. Fuchs. 1996. The composition of insect-protected cottonseed is equivalent to that of conventional cottonseed. Journal of Agricultural and Food Chemistry 44:365-371.

Berg, G., P. Marten and G. Ballin. 1996. *Stenotrophomonas maltophilia* in the rhizosphere of oilseed rape - Occurrence, characterization and interaction with phytopathogenic fungi. Microbiological Research 151:19-27.

Berg, G., N. Roskot and K. Smalla. 1999. Genotypic and phenotypic relationships between clinical and environmental isolates of *Stenotrophomonas maltophilia*. Journal of Clinical Microbiology 37:3594-3600.

Berg, G., N. Roskot, A. Steidle, L. Eberl, A. Zock and K. Smalla. 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Applied and Environmental Microbiology 68:3328-3338.

Berg, R.D. 1996. The indigenous gastrointestinal microflora. Trends in Microbiology 4:430-435.

Bertrand, J.A., T.Q. Sudduth, A. Condon, T.C. Jenkins and M.C. Calhoun. 2005. Nutrient content of whole cottonseed. Journal of Dairy Science 88:1470-1477.

Bevan, M., W.M. Barnes and M.-D. Chilton. 1983. Structure and transcription of the nopaline synthase gene region of T-DNA. Nucleic Acids Research 11:369-385.

Biron, D.G., C. Brun, T. Lefevre, C. Lebarbenchon, H.D. Loxdale, F. Chevenet, J.-P. Brizard and F. Thomas. 2006. The pitfalls of proteomics experiments without the correct use of bioinformatics tools. Proteomics 6:5577-5596.

[redacted] **and** [redacted]**. 2006. Pedigrees of upland and pima cotton cultivars released between 1970 and 2005. Mississippi State University, Mississippi State, Mississippi.**

Bradshaw, R.A., W.W. Brickey and K.W. Walker. 1998. N-terminal processing: The methionine aminopeptidase and N^{α}-acetyl transferase families. Trends in Biochemical Sciences 23:263-267.

Breyton, C. 2000. The cytochrome *b_6f* complex: Structural studies and comparison with the *bc_1* complex. Biochimica et Biophysica Acta 1459:467-474.

Brubaker, C.L., E.M. Bourland and J.E. Wendel. 1999. The origin and domestication of cotton. Pages 3-31 in Cotton: Origin, History, Technology and Production W. Smith and J.T. Cothren (eds.). John Wiley & Sons, Inc., New York, New York.

Buchanan, B.B., W. Gruissem and R.L. Jones. 2000. Phenylpropanoid and phenylpropanoid-acetate pathway metabolites. Pages 1286-1289 in Biochemistry and Molecular Biology of Plants. American Society of Plant Biologists, Rockville, Maryland.

Caetano-Anollés, G., M. Wang, D. Caetano-Anollés and J.E. Mittenthal. 2009. The origin, evolution and structure of the protein world. Biochemical Journal 417:621-637.

Cao, J., J.-P. Blond and J. Bézard. 1993. Inhibition of fatty acid Δ^6- and Δ^5-desaturation by cyclopropene fatty acids in rat liver microsomes. Biochimica et Biophysica Acta 1210:27-34.

Chakraborty, S., M. Behrens, P.L. Herman, A.F. Arendsen, W.R. Hagen, D.L. Carlson, X.-Z. Wang and D.P. Weeks. 2005. A three-component dicamba *O*-demethylase from *Pseudomonas maltophilia*, strain DI-6: Purification and characterization. Archives of Biochemistry and Biophysics 437:20-28.

Cherry, J.P. 1983. Cottonseed oil. Journal of the American Oil Chemists' Society 60:360-367.

Clark, S.E. and G.K. Lamppa. 1992. Processing of the precursors for the light-harvesting chlorophyll-binding proteins of photosystem II and photosystem I during import and in an organelle-free assay. Plant Physiology 98:595-601.

Codex Alimentarius. 2009. Foods derived from modern biotechnology. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, Food and Agriculture Organization of the United Nations, Rome, Italy.

Cross, T. 1989. Other genera. Pages 2586-2615 in Bergey's Manual of Systematic Bacteriology Volume 4. S.T. Williams and M.E. Sharpe (eds.). Williams & Wilkins, Balitmore, Maryland.

Cunha, B.A. 2010. *Stenotrophomonas maltophilia*. WebMD, LLC, New York, New York. http://www.emedicine.com/med/topic3457.htm [Accessed January 2, 2010].

D'Ordine, R.L., T.J. Rydel, M.J. Storek, E.J. Sturman, F. Moshiri, R.K. Bartlett, G.R. Brown, R.J. Eilers, C. Dart, Y. Qi, S. Flasinski and S.J. Franklin. 2009. Dicamba monooxygenase: Structural insights into a dynamic Rieske oxygenase that catalyzes an exocyclic monooxygenation. Journal of Molecular Biology 392:481-497.

Darrouzet, E., J.W. Cooley and F. Daldal. 2004. The cytochrome bc_1 complex and its homologue the b_6f complex: Similarities and differences. Photosynthesis Research 79:25-44.

Della-Cioppa, G., S.C. Bauer, B.K. Klein, D.M. Shah, R.T. Fraley and G.M. Kishore. 1986. Translocation of the precursor of 5-*enol*pyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants *in vitro*. Proceedings of the National Academy of Sciences of the United States of America 83:6873-6877.

Denton, M. and K.G. Kerr. 1998. Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clinical Microbiology Reviews 11:57-80.

Denton, M., N.J. Todd, K.G. Kerr, P.M. Hawkey and J.M. Littlewood. 1998. Molecular epidemiology of *Stenotrophomonas maltophilia* isolated from clinical specimens from patients with cystic fibrosis and associated environmental samples. Journal of Clinical Microbiology 36:1953-1958.

Depicker, A., S. Stachel, P. Dhaese, P. Zambryski and H.M. Goodman. 1982. Nopaline synthase: Transcript mapping and DNA sequence. Journal of Molecular and Applied Genetics 1:561-573.

Duke, S.O. and S.B. Powles. 2009. Glyphosate-resistant crops and weeds: Now and in the future. AgBioForum 12:346-357.

Dumitru, R., W.Z. Jiang, D.P. Weeks and M.A. Wilson. 2009. Crystal structure of dicamba monooxygenase: A Rieske nonheme oxygenase that catalyzes oxidative demethylation. Journal of Molecular Biology 392:498-510.

Duncan, D.R. 2010. Cotton transformation. Pages 65-77 in Cotton: Biotechnological Advances. Volume 65. U.B. Zehr (ed.). Springer-Verlag, Berlin, Germany.

[redacted] 2011. Methods for inducing cotton embryogenic callus. Patent 7,947,869, U.S. Patent Office, Washington, D.C.

Echemendia, Y. 2010. Microorganism of the month: *Stenotrophomonas maltophilia*. Environmental Microbiology Laboratory, Inc., Cherry Hill, New Jersey. http://www.emlab.com/s/sampling/env-report-07-2007.html [Accessed August 10, 2010].

FARRP. 2011. Allergen database. University of Nebraska, Food Allergy Research and Resource Program, Lincoln, Nebraska. http://www.allergenonline.org/databasebrowse.shtml [Accessed February 14, 2011].

Ferraro, D.J., L. Gakhar and S. Ramaswamy. 2005. Rieske business: Structure-function of Rieske non-heme oxygenases. Biochemical and Biophysical Research and Communications 338:175-190.

Figley, K.D. 1949. Sensitivity to edible vegetable oils. The Journal of Allergy 20:198-206.

Fling, M.E., J. Kopf and C. Richards. 1985. Nucleotide sequence of the transposon Tn*7* gene encoding an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase. Nucleic Acids Research 13:7095-7106.

Fraley, R.T., S.G. Rogers, R.B. Horsch, P.R. Sanders, J.S. Flick, S.P. Adams, M.L. Bittner, L.A. Brand, C.L. Fink, J.S. Fry, G.R. Galluppi, S.B. Goldberg, N.L. Hoffmann and S.C. Woo. 1983. Expression of bacterial genes in plant cells.

Proceedings of the National Academy of Sciences of the United States of America 80:4803-4807.

Fu, T.-J., U.R. Abbott and C. Hatzos. 2002. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid - A comparative study. Journal of Agricultural and Food Chemistry 50:7154-7160.

Giza, P.E. and R.C.C. Huang. 1989. A self-inducing runaway-replication plasmid expression system utilizing the Rop protein. Gene 78:73-84.

Goodfellow, M. and S.T. Williams. 1983. Ecology of actinomycetes. Annual Review of Microbology 37:189-216.

Gray, J., E. Wardzala, M. Yang, S. Reinbothe, S. Haller and F. Pauli. 2004. A small family of LLS1-related non-heme oxygenases in plants with an origin amongst oxygenic photosynthesizers. Plant Molecular Biology 54:39-54.

Hamilton, K.A., P.D. Pyla, M. Breeze, T. Olson, M. Li, E. Robinson, S.P. Gallagher, R. Sorbet and Y. Chen. 2004. Bollgard II cotton: Compositional analysis and feeding studies of cottonseed from insect-protected cotton (*Gossypium hirsutum* L.) producing the Cry1Ac and Cry2Ab2 proteins. Journal of Agricultural and Food Chemistry 52:6969-6976.

Hammond, B.G. and R.L. Fuchs. 1998. Safety evaluation for new varieties of food crops developed through biotechnology. Pages 61-79 in Biotechnology and Safety Assessment, Second Edition. J.A. Thomas (ed.). Taylor & Francis, Philadelphia, Pennsylvania.

Hammond, B.G. and J.M. Jez. 2011. Impact of food processing on the safety assessment for proteins introduced into biotechnology-derived soybean and corn crops. Food and Chemical Toxicology 49:711-721.

Harayama, S., M. Kok and E.L. Neidle. 1992. Functional and evolutionary relationships among diverse oxygenases. Annual Review of Microbiology 46:565-601.

Harrigan, G.G., D. Lundry, S. Drury, K. Berman, S.G. Riordan, M.A. Nemeth, W.P. Ridley and K.C. Glenn. 2010. Natural variation in crop composition and the impact of transgenesis. Nature Biotechnology 28:402-404.

Harris, W.D. 1981. Cottonseed. Pages 375-391 in Encyclopedia of Chemical Processing and Design. Volume 12. J.J. McKetta and W.A. Cunningham (eds.). Marcel Dekker, Inc., New York, New York.

Herman, P.L., M. Behrens, S. Chakraborty, B.M. Chrastil, J. Barycki and D.P. Weeks. 2005. A three-component dicamba *O*-demethylase from *Pseudomonas maltophilia*, strain DI-6: Gene isolation, characterization, and heterologous expression. Journal of Biological Chemistry 280:24759-24767.

Hérouet, C., D.J. Esdaile, B.A. Mallyon, E. Debruyne, A. Schulz, T. Currier, K. Hendrickx, R.-J. van der Klis and D. Rouan. 2005. Safety evaluation of the phosphinothricin acetyltransferase proteins encoded by the *pat* and *bar* sequences that confer tolerance to glufosinate-ammonium herbicide in transgenic plants. Regulatory Toxicology and Pharmacology 41:134-149.

Herrmann, K.M. 1995. The shikimate pathway: Early steps in the biosynthesis of aromatic compounds. Plant Cell 7:907-919.

Hibino, T., R. Waditee, E. Araki, H. Ishikawa, K. Aoki, Y. Tanaka and T. Takabe. 2002. Functional characterization of choline monooxygenase, an enzyme for betaine synthesis in plants. Journal of Biological Chemistry 277:41352-41360.

Hileman, R.E., A. Silvanovich, R.E. Goodman, E.A. Rice, G. Holleschak, J.D. Astwood and S.L. Hefle. 2002. Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. International Archives of Allergy and Immunology 128:280-291.

Hochuli, E., W. Bannwarth, H. Döbeli, R. Gentz and D. Stüber. 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. Nature Biotechnology 6:1321-1325.

Hutjens. 2007. Herd management: Managing high-priced corn in your ration. Jersey Journal, Reynoldsburg, Ohio. http://jerseyjournal.usjersey.com/Article%20Archive/Management/2007/AlternativeFeedsArticle.pdf [Accessed October 14, 2011].

Hutjens, M. 2003. An update on fuzzy cottonseed. University of Illinois Extension, Urbana-Champaign, Illinois. http://www.livestocktrail.uiuc.edu/dairynet/paperDisplay.cfm?ContentID=1191 [Accessed October 13, 2011].

Illergård, K., D.H. Ardell and A. Elofsson. 2009. Structure is three to ten times more conserved than sequence - A study of structural response in protein cores. Proteins 77:499-508.

ILSI-CERA. 2011. A review of the environmental safety of the PAT protein. International Life Sciences Institute, Center for Environmental Risk Assessment, Washington, D.C. http://cera-gmc.org/docs/cera_publications/pub_05_2011.pdf.

ILSI. 2011. Crop Composition Database, Version 4.2. International Life Sciences Institute, Washington, D.C. http://www.cropcomposition.org/.

Janas, K.M., M. Cvikrová, A. Paągiewicz and J. Eder. 2000. Alterations in phenylpropanoid content in soybean roots during low temperature acclimation. Plant Physiology and Biochemistry 38:587-593.

John, M.E. 1996. Structural characterization of genes corresponding to cotton fiber mRNA, E6: Reduced E6 protein in transgenic plants by antisense gene. Plant Molecular Biology 30:297-306.

Jonas, D.A., I. Elmadfa, K.-H. Engel, K.J. Heller, G. Kozianowski, A. König, D. Müller, J.F. Narbonne, W. Wackernagel and J. Kleiner. 2001. Safety considerations of DNA in food. Annals of Nutrition and Metabolism 45:235-254.

Juhnke, M.E. and E. des Jardin. 1989. Selective medium for isolation of *Xanthomonas maltophilia* from soil and rhizosphere environments. Applied and Environmental Microbiology 55:747-750.

Juhnke, M.E., D.E. Mathre and D.C. Sands. 1987. Identification and characterization of rhizosphere-competent bacteria of wheat. Applied and Environmental Microbiology 53:2793-2799.

Kämpfer, P. 2006. The family *Streptomycetaceae*, Part I: Taxonomy. Pages 538-604 in The Prokaryotes. A Handbook on the Biology of Bacteria: Archaea. Bacteria: Firmicutes, Actinomycetes. Volume 3. M.Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (eds.). Springer+ Business Media, LLC., New York, New York.

Kay, R., A. Chan, M. Daly and J. McPherson. 1987. Duplication of CaMV 35*S* promoter sequences creates a strong enhancer for plant genes. Science 236:1299-1302.

Klee, H.J., Y.M. Muskopf and C.S. Gasser. 1987. Cloning of an *Arabidopsis thaliana* gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: Sequence analysis and manipulation to obtain glyphosate-tolerant plants. Molecular and General Genetics 210:437-442.

[redacted] and [redacted]. 1984. Cotton. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, Wisconsin.

Krause, E., H. Wenschuh and P.R. Jungblut. 1999. The dominance of arginine-containing peptides in MALDI-derived tryptic mass fingerprints of proteins. Analytical Chemistry 71:4160-4165.

Krueger, J.P., R.G. Butz, Y.H. Atallah and D.J. Cork. 1989. Isolation and identification of microorganisms for the degradation of dicamba. Journal of Agricultural and Food Chemistry 37:534-538.

Kutzner, H.J. 1981. The family streptomycetaceae. Pages 2028-2090 in The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Volume 2. M.P. Starr, H. Stolp, H.G. Trüper, A. Balows, and H.G. Schlegel (eds.). Springer-Verlag, Berlin, Germany.

Ladics, G.S., G.A. Bannon, A. Silvanovich and R.F. Cressman. 2007. Comparison of conventional FASTA identity searches with the 80 amino acid sliding window FASTA search for the elucidation of potential identities to known allergens. Molecular Nutrition and Food Research 51:985-998.

Lambert, B., F. Leyns, L. Van Rooyen, F. Gosselé, Y. Papon and J. Swings. 1987. Rhizobacteria of maize and their antifungal activities. Applied and Environmental Microbiology 53:1866-1871.

Lawhon, J.T., C.M. Cater and K.F. Mattil. 1977. Evaluation of the food use potential of sixteen varieties of cottonseed. Journal of the American Oil Chemists' Society 54:75-80.

Lee, J.A. 1984. Cotton as a world crop. Pages 1-26 in Cotton. R.J. Kohel and C.F. Lewis (eds.). American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, Wisconsin.

Lege, K.E., J.T. Cothren and C.W. Smith. 1995. Phenolic-acid and condensed tannin concentrations of six cotton genotypes. Environmental and Experimental Botany 35:241-249.

Liener, I.E. 1994. Implications of antinutritional components in soybean foods. Critical Reviews in Food Science and Nutrition 34:31-67.

Locci, R. 1989. Streptomycetes and related genera. Pages 2451-2508 in Bergey's Manual of Systematic Bacteriology. Volume 4. S.T. Williams and M.E. Sharpe (eds.). Williams & Wilkins, Baltimore, Maryland.

Lordelo, M.M., M.C. Calhoun, N.M. Dale, M.K. Dowd and A.J. Davis. 2007. Relative toxicity of gossypol enantiomers in laying and broiler breeder hens. Poultry Science 86:582-590.

Loveless, M.H. 1950. Cottonseed protein vs. cottonseed oil sensitivity. IV. An objective approach to the diagnosis of food-allergy as applied to cottonseed atopy. Annals of Allergy 8:15-22.

Maiti, I.B. and R.J. Shepherd. 1998. Isolation and expression analysis of peanut chlorotic streak caulimovirus (PCISV) full-length transcript (FLt) promoter in transgenic plants. Biochemical and Biophysical Research Communications 244:440-444.

Manderscheid, R. and A. Wild. 1986. Studies on the mechanism of inhibition by Phosphinothricin of glutamine synthetase isolated from *Triticum aestivum* L. Journal of Plant Physiology 123:135-142.

Metcalfe, D.D., J.D. Astwood, R. Townsend, H.A. Sampson, S.L. Taylor and R.L. Fuchs. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Critical Reviews in Food Science and Nutrition 36:S165-S186.

Moreno, F.J., F.A. Mellon, M.S.J. Wickham, A.R. Bottrill and E.N.C. Mills. 2005. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physciologically relevant *in vitro* gastrointestinal digestion. FEBS Journal 272:341-352.

Nam, J.-W., H. Nojiri, T. Yoshida, H. Habe, H. Yamane and T. Omori. 2001. New classification system for oxygenase components involved in ring-hydroxylating oxygenations. Bioscience, Biotechnology, and Biochemistry 65:254-263.

NCPA. 1993. Cottonseed oil. [redacted] (eds.). National Cottonseed Products Association, Inc. and The Cotton Foundation, Memphis, Tennessee.

NCPA. 2002a. Cottonseed and its products. National Cottonseed Products Association, Cordova, Tennessee. http://www.cottonseed.com/publications/cottonseedanditsproducts.asp [Accessed October 17, 2011].

NCPA. 2002b. Gossypol effects in animal feeding can be controlled. National Cottonseed Products Association, Cordova, Tennessee. http://www.cottonseed.com/publications/Gossypol%20Effects%20in%20Animal%20Feeding%20can%20be%20Controlled.pdf [Accessed October 10, 2011].

Nida, D.L., S. Patzer, P. Harvey, R. Stipanovic, R. Wood and R.L. Fuchs. 1996. Glyphosate-tolerant cotton: The composition of the cottonseed is equivalent to that of conventional cottonseed. Journal of Agricultural and Food Chemistry 44:1967-1974.

Niepel, M. and D.R. Gallie. 1999. Identification and characterization of the functional elements within the tobacco etch virus 5' leader required for cap-independent translation. Journal of Virology 73:9080-9088.

NRC. 1994. Nutrient requirements of chickens. Pages 19-34 in Nutrient Requirements of Poultry. Ninth Revised Edition. National Research Council, National Academy Press, Washington, D.C.

NRC. 2001. Nutrient requirement tables. Pages 258-280 in Nutrient Requirements of Dairy Cattle. Seventh Revised Edition. National Research Council, National Academy Press, Washington, D.C.

Nunes, F.V. and I.S. de Melo. 2006. Isolation and characterization of endophytic bacteria of coffee plants and their potential in caffeine degradation. Environmental Toxicology 10:293-297.

Odell, J.T., F. Nagy and N.-H. Chua. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810-812.

OECD. 1999. Consensus document on general information concerning the genes and their enzymes that confer tolerance to glyphosate herbicide. ENV/JM/MONO(99)9 Series on Harmonization of Regulatory Oversight in Biotechnology No.10. Organisation for Economic Co-operation and Development, Paris, France.

OECD. 2002a. Series on harmonization of regulatory oversight in biotechnology No. 25 Module II: Herbicide biochemistry, herbicide metabolism and the residues in glufosinate-ammonium (Phosphinothricin)-tolentant transgenic plants. ENV/JM/MONO(2002)14. Organisation for Economic Co-operation and Development, Paris, France.

OECD. 2002b. Report of the OECD workshop on the toxicological and nutritional testing of novel foods. SG/ICGB(1998)1/FINAL. Organisation for Economic Co-operation and Development, Paris, France.

OECD. 2003. Considerations for the safety assessment of animal feedstuffs derived from genetically modified plants. ENV/JM/MONO(2003)10. Series on the Safety of Novel Foods and Feeds No. 9. Organisation for Economic Co-operation and Development, Paris, France.

OECD. 2004. Consensus document on the compositional considerations for new varities of cotton (*Gossypium hirsutum* and *Gossypium barbadense*): Key food and feed nutrients and anti-nutrients. ENV/JM/MONO(2004)16. Organisation for Economic Co-operation and Development, Paris, France.

OECD. 2008. Consensus document on the biology of cotton (*Gossypium* spp.). ENV/JM/MONO(2008)33. Organisation for Economic Co-operation and Development, Paris, France.

OECD. 2009a. Guidance document on overview of residue chemistry studies. ENV/JM/MONO(2009)31. Organisation for Economic Co-operation and Development, Paris, France.

OECD. 2009b. Consensus document on the compositional considerations for new varities of cotton (*Gossypium hirsutum* and *Gossypium barbadense*): Key food and feed nutrients and anti-nutrients. ENV/JM/MONO(2004)16. Organisation for Economic Co-Operation and Development, Paris, France.

OGTR. 2008. The biology of *Gossypium hirsutum* L. and *Gossypium barbadense* L. (cotton). Australian Government, Department of Health and Ageing, Office of the Gene Technology Regulator, Canberra, Australia.

Palleroni, N.J. and J.F. Bradbury. 1993. *Stenotrophomonas*, a new bacterial genus for *Xanthomonas maltophilia* (Hugh 1980) Swings et al. 1983. International Journal of Systematic Bacteriology 43:606-609.

Pariza, M.W. and E.A. Johnson. 2001. Evaluating the safety of microbial enzyme preparations used in food processing: Update for a new century. Regulatory Toxicology and Pharmacology 33:173-186.

Percival, A.E., J.F. Wendel and J.M. Stewart. 1999. Taxonomy and germplasm resources. Pages 33-63 in Cotton: Origin, History, Technology, and Production. W.C. Smith and J.T. Cothren (eds.). John Wiles & Sons, Inc., New York, New York.

Polevoda, B. and F. Sherman. 2000. N^{α}-terminal acetylation of eukaryotic proteins. Journal of Biological Chemistry 275:36479-36482.

Qureshi, A., L. Mooney, M. Denton and K.G. Kerr. 2005. *Stenotrophomonas maltophilia* in salad. Emerging Infectious Diseases 11:1157-1158.

Rademacher, T.W., R.B. Parekh and R.A. Dwek. 1988. Glycobiology. Annual Review of Biochemistry 57:785-838.

Rathinasabapathi, B., M. Burnet, B.L. Russell, D.A. Gage, P.-C. Liao, G.J. Nye, P. Scott, J.H. Golbeck and A.D. Hanson. 1997. Choline monooxygenase, an unusual iron-sulfur enzyme catalyzing the first step of *Glycine betaine* synthesis in plants: Prosthetic group characterization and cDNA cloning. Proceedings of the National Academy of Sciences of the United States of America 94:3454-3458.

Reeves, J.B. and J.C. Weihrauch. 1979. Composition of foods. Agricultural Handbook 8-4. U.S. Department of Agriculture, Washington, D.C.

Rensing, S.A. and U.-G. Maier. 1994. Phylogenetic analysis of the stress-70 protein family. Journal of Molecular Evolution 39:80-86.

Reynolds, T.L., M.A. Nemeth, K.C. Glenn, W.P. Ridley and J.D. Astwood. 2005. Natural variability of metabolites in maize grain: Differences due to genetic background. Journal of Agricultural and Food Chemistry 53:10061-10067.

[redacted] and [redacted]. 2007. Cotton growth and development. University of Georgia Cooperative Extension, Athens, Georgia.

Rodoni, S., W. Mühlecker, M. Anderl, B. Kräutler, D. Moser, H. Thomas, P. Matile and S. Hörtensteiner. 1997. Chlorophyll breakdown in senescent chloroplasts (cleavage of pheophorbide *a* in two enzymic steps). Plant Physiology 115:669-676.

Rolph, C.E., R.S. Moreton and J.L. Harwood. 1990. Control of acyl lipid desaturation in the yeast *Rhodotorula gracilis* via the use of the cyclopropenoid fatty acid, sterculate. Applied Microbiology and Biotechnology 34:91-96.

Rosche, B., B. Tshisuaka, B. Hauer, F. Lingens and S. Fetzner. 1997. 2-oxo-1,2-dihydroquinoline 8-monooxygenase: Phylogenetic relationship to other multicomponent nonheme iron oxygenases. Journal of Bacteriology 179:3549-3554.

Russell, B.L., B. Rathinasabapathi and A.D. Hanson. 1998. Osmotic stress induces expression of choline monooxygenase in sugar beet and amaranth. Plant Physiology 116:859-865.

Ryan, R.P., S. Monchy, M. Cardinale, S. Taghavi, L. Crossman, M.B. Avison, G. Berg, D. van der Lelie and J.M. Dow. 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nature Reviews Microbiology 7:514-525.

Salomon, S. and H. Puchta. 1998. Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. EMBO Journal 17:6086-6095.

Salvaggio, J.E., C.E. O'Neil and B.T. Butcher. 1986. Immunologic responses to inhaled cotton dust. Environmental Health Perspectives 66:17-23.

Schmelz, E.A., J. Engelberth, H.T. Alborn, P. O'Donnell, M. Sammons, H. Toshima and J.H. Tumlinson. 2003. Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. Proceedings of the National Academy of Sciences of the United States of America 100:10552-10557.

Schmidt, C.L. and L. Shaw. 2001. A comprehensive phylogenetic analysis of Rieske and Rieske-type iron-sulfur proteins. Journal of Bioenergetics and Biomembranes 33:9-26.

Shaver, R. 2008. By-product feedstuffs in dairy cattle diets in the upper midwest. University of Wisconsin Extension, Madison, Wisconsin. http://www.uwex.edu/ces/dairynutrition/documents/byproductfeedsrevised2008.pdf [Accessed October 10, 2011].

Silvanovich, A., M.A. Nemeth, P. Song, R. Herman, L.Tagliani and G.A. Bannon. 2006. The value of short amino acid sequence matches for prediction of protein allergenicity. Toxicological Sciences 90:252-258.

Sjoblad, R.D., J.T. McClintock and R. Engler. 1992. Toxicological considerations for protein components of biological pesticide products. Regulatory Toxicology and Pharmacology 15:3-9.

Smith, C.W. and J.T. Cothren. 1999. Cotton: Origin, history, technology, and production. John Wiley and Sons, Inc., Hoboken, New Jersey.

Smith, C.W. and R.A. Creelman. 2001. Vitamin E concentrations in upland cotton seeds. Crop Science 41:577-579.

Smith, K.J. 1970. Practical significance of gossypol in feed formulation. Journal of the American Oil Chemists' Society 47:448-450.

Stalker, D.M., C.M. Thomas and D.R. Helinski. 1981. Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Molecular and General Genetics 181:8-12.

Sutcliffe, J.G. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Pages 77-90 in Cold Spring Harbor Symposia on Quantitative Biology, Cold Spring Harbor, New York.

Swings, J., P. De Vos, M. Van den Mooter and J. De Ley. 1983. Transfer of *Pseudomonas maltophilia* Hugh 1981 to the genus *Xanthomonas* as *Xanthomonas maltophilia* (Hugh 1981) comb. nov. International Journal of Systematic Bacteriology 33:409-413.

Tanaka, A., H. Ito, R. Tanaka, N.K. Tanaka, K. Yoshida and K. Okada. 1998. Chlorophyll *a* oxygenase (*CAO*) is involved in chlorophyll *b* formation from chlorophyll *a*. Proceedings of the National Academy of Sciences of the United States of America 95:12719-12723.

Thomas, K., M. Aalbers, G.A. Bannon, M. Bartels, R.J. Dearman, D.J. Esdaile, T.J. Fu, C.M. Glatt, N. Hadfield, C. Hatzos, S.L. Hefle, J.R. Heylings, R.E. Goodman, B. Henry, C. Herouet, M. Holsapple, G.S. Ladics, T.D. Landry, S.C. MacIntosh, E.A. Rice, L.S. Privalle, H.Y. Steiner, R. Teshima, R. van Ree, M. Woolhiser and J. Zawodny. 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.

Thomas, K., G. Bannon, S. Hefle, C. Herouet, M. Holsapple, G. Ladics, S. MacIntosh and L. Privalle. 2005. In silico methods for evaluating human allergenicity to novel proteins: International Bioinformatics Workshop Meeting Report, 23-24 February 2005. Toxicological Sciences 88:307-310.

Thompson, C.J., N.R. Movva, R. Tizard, R. Crameri, J.E. Davies, M. Lauwereys and J. Botterman. 1987. Characterization of the herbicide-resistance gene bar from *Streptomyces hygroscopicus*. EMBO Journal 6:2519-2524.

U.S. EPA. 1996. Residue chemistry test guidelines - Background. OPPTS 860.1000. U.S. Environmental Protection Agency, Office of Prevention, Pesticides, and Toxic Substances, Washington, D.C.

U.S. EPA. 1997. Phosphinothricin acetyltransferase and the genetic material necessary for its production in all plants; Exemption from the requirement of a tolerance on all raw agricultural commodities. Federal Register 62:17717-17720.

U.S. EPA. 2003. Memorandum: Glufosinate ammonium (PC code 128850). Section 3 registrations for transgenic cotton and cotton (ID# - 0F06140), transgenic rice (ID# - 0F06210), and bushberry (ID# - 2E06404). Human health risk assessment. DP barcode: D290086. Case number: 293386. Submission: S635308. 40 CFR 180.473. U.S. Environmental Protection Agency, Washington, D.C.

U.S. EPA. 2009. Correction to the amendments to the dicamba RED. U.S. Environmental Protection Agency, Office of Prevention, Pesticides, and Toxic Substances, Washington, D.C.

U.S. FDA. 1992. Statement of policy: Foods derived from new plant varieties. Federal Register 57: 22984-23005.

U.S. FDA. 1995a. AgrEvo BNF No. 23: Oilseed rape: Tolerance to the herbicide glufosinate-ammonium. HCN92. U.S. Food and Drug Administration, Washington, D.C. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=bioListing&id=8 [Accessed April 2, 2012].

U.S. FDA. 1995b. Dekalb Genetics BNF No. 28: Corn: Tolerance to the herbicide glufosinate-ammonium. DLL25. U.S. Food and Drug Administration, Washington, D.C. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=bioListing&id=16 [Accessed April 2, 2012].

U.S. FDA. 1995c. AgrEvo BNF No. 29: Corn: Tolerance to the herbicide glufosinate-ammonium. T14, T25. U.S. Food and Drug Administration, Washington, D.C. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=bioListing&id=15 [Accessed April 2, 2012].

U.S. FDA. 1997. AgrEvo BNF No. 46: Canola: Tolerance to the herbicide glufosinate-ammonium. T45. U.S. Food and Drug Administration, Washington, D.C. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=bioListing&id=30 [Accessed April 2, 2012].

U.S. FDA. 1998a. AgrEvo BNF No. 55: Soybean: Tolerance to the herbicide glufosinate-ammonium. A2704-12, A5547-127. U.S. Food and Drug Administration, Washington, D.C. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=bioListing&id=38 [Accessed April 2, 2012].

U.S. FDA. 1998b. AgrEvo BNF No. 38: Sugar beet: Tolerance to the herbicide glufosinate-ammonium. T120-7. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=bioListing&id=43 [Accessed April 2, 2012].

U.S. FDA. 1999. Aventis BNF No. 63: Rice: Tolerance to the herbicide glufosinate-ammonium. LLRICE E06, LLRICE E62. U.S. Food and Drug Administration, Washington, D.C. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=bioListing&id=50 [Accessed April 2, 2012].

U.S. FDA. 2002. Bayer CropScience BNF No. 86: Cotton: Tolerance to the herbicide glufosinate-ammonium. LLCotton25. U.S. Food and Drug Administration, Washington, D.C. http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=bioListing&id=56 [Accessed April 2, 2012].

USDA-ERS. 2010. Cotton and wool yearbook 2010: U.S. cotton supply and use, 1965/66-2010/11. U.S. Department of Agriculture, Economic Research Service, Washington, D.C. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1282 [Accessed October 13, 2011].

USDA-ERS. 2011. Oil crops yearbook 2011: Cottonseed: Acreage planted, harvested, yield, production, and value, U.S. 1980-2010. U.S. Department of Agriculture, Economic Research Service, Washington D.C. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1290 [Accessed October 13, 2011].

USDA-FAS. 2005. Oilseeds: World markets and trade. Circular Series FOP 7 - 05. U.S. Department of Agriculture, Foreign Agricultural Service, Washington, D.C.

USDA-NASS. 2011. Crop production: 2010 summary, January 2011. U.S. Department of Agriculture, National Agricultural Statistics Service, Washington, D.C.

USDA-NASS. 2012. Quick stats: Cotton, acres planted, pima - Acres planted, pima - Price received, upland acres. U.S. Department of Agriculture, National Agricultural Statistics Service, Washington, D.C. http://quickstats.nass.usda.gov/data/printable/2550EF99-0495-3D73-97B4-C71FFC453437 [Accessed April 4, 2012].

USDA. 2001. The classification of cotton. Agricultural Handbook 566. U.S. Department of Agriculture, Washington, D.C.

USDA. 2011. Oil crops outlook: U.S. soybean yield forecast is revised higher. U.S. Department of Agriculture, Washington D.C. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1288 [Accessed October 13, 2011].

Vassilopoulou, E., N. Rigby, F.J. Moreno, L. Zuidmeer, J. Akkerdaas, I. Tassios, N.G. Papadopoulos, P. Saxoni-Papageorgiou, R. van Ree and C. Mills. 2006. Effect of *in vitro* gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. Journal of Allergy and Clinical Immunology 118:473-480.

Vieths, S., J. Reindl, U. Müller, A. Hoffmann and D. Haustein. 1999. Digestibility of peanut and hazelnut allergens investigated by a simple in vitro procedure. European Food Research and Technology 209:379-388.

Waksman, S.A. and A.T. Henrici. 1943. The nomenclature and classification of the actinomycetes. Journal of Bacteriology 46:337-341.

Wang, X.-Z., B. Li, P.L. Herman and D.P. Weeks. 1997. A three-component enzyme system catalyzes the O demethylation of the herbicide dicamba in *Pseudomonas maltophilia* DI-6. Applied and Environmental Microbiology 63:1623-1626.

Wehrmann, A., A.V. Vliet, C. Opsomer, J. Botterman and A. Schulz. 1996. The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. Nature Biotechnology 14:1274-1278.

Werlen, C., H.-P.E. Kohler and J.R. van der Meer. 1996. The broad substrate chlorobenzene dioxygenase and *cis*-chlorobenzene dihydrodiol dehydrogenase of

Pseudomonas **sp. strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation. Journal of Biological Chemistry 271:4009-4016.**

Wild, A. and R. Manderscheid. 1984. The effect of phosphinothricin on the assimilation of ammonia in plants. Zeitschrift füer Naturforschung 39:500-504.

Winter, J., R. Wright, N. Duck, C. Gasser, R. Fraley and D. Shah. 1988. The inhibition of petunia hsp70 mRNA processing during $CdCl_2$ stress. Molecular and General Genetics 211:315-319.

Wishart, D.S. 2010. Human metabolome database. www.hmdb.ca [Accessed June 2, 2010].

Wishart, D.S., C. Knox, A.C. Guo, R. Eisner, N. Young, B. Gautam, D.D. Hau, N. Psychogios, E. Dong, S. Bouatra, R. Mandal, I. Sinelnikov, J. Xia, L. Jia, J.A. Cruz, E. Lim, C.A. Sobsey, S. Shrivastava, P. Huang, P. Liu, L. Fang, J. Peng, R. Fradette, D. Cheng, D. Tzur, M. Clements, A. Lewis, A. De Souza, A. Zuniga, M. Dawe, Y. Xiong, D. Clive, R. Greiner, A. Nazyrova, R. Shaykhutdinov, L. Li, H.J. Vogel and I. Forsythe. 2009. HMDB: A knowledgebase for the human metabolome. Nucleic Acids Research 37:D603-D610.

Wohllenben, W., W. Arnold, I. Broer, D. Hillemann, E. Strauch and A. Pühler. 1988. Nucleotide sequence of the phosphinothricin *N*-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Nicotiana tabacum*. Gene 70:25-37.

Yang, M., E. Wardzala, G.S. Johal and J. Gray. 2004. The wound-inducible *Lls1* gene from maize is an orthologue of the *Arabidopsis Acd1* gene, and the LLS1 protein is present in non-photosynthetic tissues. Plant Molecular Biology 54:175-191.

Zambryski, P., A. Depicker, K. Kruger and H.M. Goodman. 1982. Tumor induction by *Agrobacterium tumefaciens*: Analysis of the boundaries of T-DNA. Journal of Molecular and Applied Genetics 1:361-370.

APPENDICES

Appendix A: Materials and Methods Used for Molecular Analyses of MON 88701

A.1. Materials

The genomic DNA used in molecular analyses was isolated from leaf tissue of the R_3 generation of MON 88701 and the conventional control (Coker 130). The leaf tissue was harvested from a greenhouse production in 2010. For generational stability analysis, genomic DNA was extracted from leaf tissue of the R_2, R_3, R_4, R_5 and R_6 generations of MON 88701. The leaf tissue was harvested from production plan PPN-10-113. The reference substance, PV–GHHT6997 (Figure IV-1), was used as a positive hybridization control in Southern blot analyses. Probe templates generated from PV–GHHT6997 were used as additional positive hybridization controls. As additional reference standards, the 1 Kb DNA Extension Ladder and λ DNA/*Hind* III Fragments from Invitrogen (Carlsbad, CA) were used for size estimations on agarose gels and subsequent Southern blots. The 1 Kb DNA Ladder from Invitrogen was used for size estimations on agarose gels for PCR analyses.

A.2. Characterization of the Materials

The identity of the source materials was verified by methods used in molecular characterization to confirm the presence or absence of MON 88701. The stability of the genomic DNA was confirmed by observation of interpretable signals from digested DNA samples on ethidium bromide stained agarose gels and/or specific PCR products, and the samples did not appear visibly degraded on the ethidium bromide stained gels.

A.3. DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA was isolated from MON 88701 leaf tissue using a hexadecyltrimethylammonium bromide (CTAB) based method. Briefly, 20 ml of CTAB buffer (1.5% w/v CTAB, 75 mM Tris HCl, 100 mM EDTA, 1.05 M NaCl, and 0.75% w/v PVP) and 10 mg RNase A were added to approximately 4 ml of ground leaf tissue and incubated at 60-70 °C for 40-50 min with intermittent mixing. Twenty milliliters of chloroform was added to the samples and mixed by hand for 2-3 min, then centrifuged at 10,300 × g for 8-10 min. The upper aqueous phase was put into a clean tube and the chloroform step was repeated twice. After the last chloroform step, the aqueous phase was put into a clean tube and the DNA was precipitated with 20 ml of 100% ethanol. The sample was centrifuged for one minute to condense the pellet, and then the precipitated DNA was hooked out and put into a tube with 4-6 ml of 70% ethanol to wash the DNA pellet. The samples were centrifuged at 5,100 × g for 5 min to pellet the DNA. DNA pellets were air dried, then resuspended in 250 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). All extracted DNA was stored in a -20 C freezer.

A.4. Quantification of Genomic DNA

Genomic DNA was quantified using a Qubit™ Fluorometer (Invitrogen, Carlsbad, CA).

A.5. Restriction Enzyme Digestion of Genomic DNA

Approximately 10 µg of genomic DNA extracted from MON 88701 and conventional control were digested with restriction enzyme *Bcl* I (New England Biolabs, Inc. Beverly, MA) or with restriction enzyme *Ssp* I- HF (New England Biolabs, Inc.). All *Bcl* I digests were conducted in 10X NEBuffer 3 buffer at 50 °C in a total volume of ~500 µl with ~50 units of restriction enzyme. All *Ssp* I-HF digests were conducted in 10X NEBuffer 4 at 37 °C in a total volume of ~500 µl with ~100 units of restriction enzyme. For the purpose of running positive hybridization controls, ~10 µg of genomic DNA extracted from the conventional control was digested with the restriction enzyme *Bcl* I and the appropriate positive hybridization control(s) were added to these digests prior to loading the agarose gel.

A.6. Agarose Gel Electrophoresis

Digested DNA was resolved on ~0.8% (w/v) agarose gels. For T-DNA insert/copy number and plasmid vector backbone analyses, individual digests containing ~10 µg each of MON 88701 and conventional control genomic DNA were loaded on the same gel in a long run/short run format. The long run allows for greater resolution of large molecular weight DNA, whereas the short run allows for retaining the small molecular weight DNA on the gel. The positive hybridization controls were only run in the short run format. For the insert stability analysis, individual digests of ~10 µg each of genomic DNA extracted from five leaf samples from multiple generations of MON 88701 and the conventional control along with the positive hybridization controls were loaded on the agarose gel in a single run format.

A.7. DNA Probe Preparation for Southern Blot Analyses

Probe templates were prepared by PCR amplification using the PV–GHHT6997 DNA as template. The PCR products were separated on an agarose gel by electrophoresis and purified from the gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. The probe templates were designed based on the nucleotide composition (%GC) of the sequence in order to optimize the detection of DNA sequences during hybridization. When possible, probes possessing similar melting temperature (Tm) were combined in the same Southern blot hybridization. Approximately 25 ng of each probe template were radiolabeled with either [α-^{32}P] deoxycytidine triphosphate (dCTP) (6000 Ci/mmol) or [α-^{32}P] deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA) according to manufacturer's instruction.

A.8. Southern Blot Analyses of Genomic DNA

Genomic DNA isolated from MON 88701 and the conventional control was digested and evaluated using Southern blot analyses (Southern, 1975). The PV–GHHT6997 DNA, previously digested with the restriction enzyme *Pci* I was added to conventional control genomic DNA digested with *Bcl* I to serve as positive hybridization control on each

Southern blot. When multiple probes were hybridized simultaneously to one Southern blot, the probe templates were spiked in the digested conventional control genomic DNA to serve as additional positive hybridization controls on the Southern blot. The DNA was then separated by agarose gel electrophoresis and transferred onto a nylon membrane. Southern blots were hybridized and washed at 55 °C, 60 °C, or 65 °C, depending on the calculated melting temperature (Tm) of the probes that were used. Table A-1 lists the radiolabeling conditions and hybridization temperatures of the probes used in this study. Multiple exposures of each blot were then generated using Kodak Biomax MS film (Eastman Kodak, Rochester, NY) in conjunction with one Kodak Biomax MS intensifying screen in a -80 °C freezer.

Table A-1. Hybridization Conditions of Utilized Probes

Probe	Labeling Method	Probe labeled with dNTP (^{32}P)	Hybridization/ Wash Temperature (°C)
1	RadPrime	dATP	55
2	RadPrime	dCTP	65
3	RadPrime	dATP	55
4	RadPrime	dCTP	65
5	RadPrime	dATP	55
6	RadPrime	dCTP	60
7	RadPrime	dCTP	60
8	RadPrime	dCTP	60

References for Appendix A

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology 98: 503-517.

Appendix B: Materials and Methods for Characterization of MON 88701 DMO and PAT (*bar*) Proteins Produced in MON 88701

B.1. Forms of DMO

Various forms of the DMO protein (Figure B-1) were used to establish enzyme structure, activity, substrate specificity and safety of the proteins in MON 88701. The wild-type DMO was first isolated and characterized from *Stenotrophomonas maltophilia* (Herman et al., 2005). The MON 88701 DMO protein present in MON 88701 is identical to the wild-type DMO except for an additional leucine at position two (Figure B-1) and an additional nine amino acids at the N-terminus from the chloroplast transit peptide, CTP2. The two *E. coli*-produced forms of DMO, identical to the wild-type DMO, but with one having a histidine-tag on the N-terminus and the other having an additional alanine at position two and a histidine-tag on the C-terminus (Figure B-1) were used for crystallography and specificity experiments. The differences in the amino acid sequence or the addition of N-terminal or C-terminal histidine tags did not appear to have an effect on mode-of-action, structure, functional activity, or specificity of DMO, as these changes are sterically distant from the catalytic domain centers involved in electron transport (Rieske and non-heme iron centers) and the catalytic centers for the dicamba substrate (D'Ordine et al., 2009; Dumitru et al., 2009).

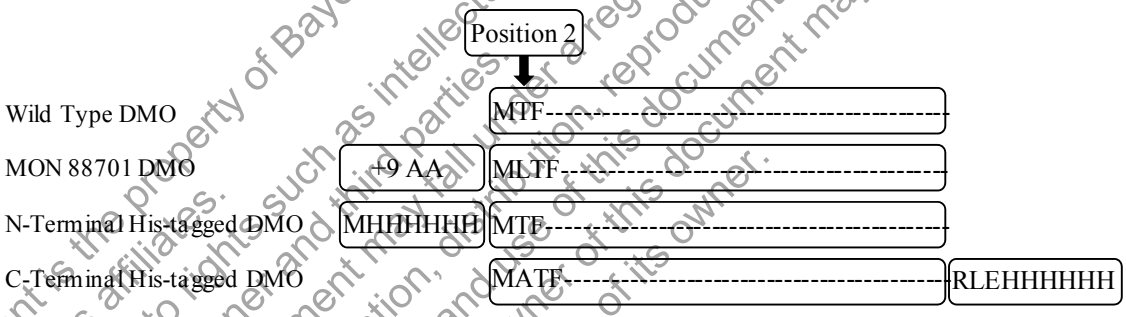


Figure B-1. Forms of DMO Protein and Their Relation to the Wild-Type DMO Protein

The diagram represents the various DMO forms described in this dossier. The wild-type DMO form isolated from *S. maltophilia* was the first form sequenced (Herman et al., 2005). The MON 88701 DMO protein has an insertion of a leucine at position 2, and there is addition of 9 amino acids from CTP2 at the N-terminus. MON 88701 DMO was purified from cottonseed of MON 88701. The N-terminal histidine-tagged DMO was produced in *E. coli* and was used for *in vitro* specificity studies (Section V1.A.3.). The C-terminal histidine-tagged DMO was produced in *E. coli* and was used for crystallography studies (D'Ordine et al., 2009; Dumitru et al., 2009).

B.2. Materials

The MON 88701 DMO protein (lot 11299151) was purified from cottonseed of MON 88701 (lot 11287350). The MON 88701 DMO protein was stored in a -80 ºC

freezer in a buffer solution containing 50 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine HCl, 0.1 M sodium chloride and 10% glycerol.

The *E. coli*-produced MON 88701 DMO protein (lot 11300031) was used as the reference substance. The DMO protein reference substance was generated from cell paste produced by large-scale fermentation of *E. coli* containing the pMON136400 expression plasmid. The coding sequence for *dmo* contained on the expression plasmid (pMON136400) was confirmed prior to and after fermentation. The *E. coli*-produced MON 88701 DMO protein was previously characterized.

The MON 88701-produced PAT (*bar*) protein (lot 11295997) was purified from cottonseed of MON 88701 (lot 11287350). The MON 88701-produced PAT (*bar*) protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 0.16 M sodium chloride and 20% glycerol.

The *E. coli*-produced PAT (*bar*) protein (lot 11270310) was used as the reference substance. The PAT (*bar*) protein reference substance was generated from cell paste produced by large-scale fermentation of *E. coli* containing the pMON106653 expression plasmid. The coding sequence for *bar* contained on the expression plasmid (pMON106653) was confirmed prior to and after fermentation. The *E. coli*-produced PAT (*bar*) protein was previously characterized.

B.3. Description of Assay Controls

Protein MW standards (Precision Plus Protein Standards Dual color; Bio-Rad, Hercules, CA) were used to calibrate some SDS-PAGE gels and verify protein transfer to polyvinylidene difluoride (PVDF) and nitrocellulose membranes. Broad Range SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA) were used to generate a standard curve for the apparent MW estimation. Bovine serum albumin (BSA) and α-aminobutyric acid (AAbA) were used as hydrolysis control and internal calibration standard for amino acid analysis. The *E. coli*-produced MON 88701 DMO reference standard was used to construct a standard curve for the estimation of total protein concentration using a Bio-Rad protein assay. A phenylthiohydantoin (PTH) amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to calibrate the Applied Biosystems 494 Procise Sequencing System for each analysis. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis. Transferrin (Sigma-Aldrich, St. Louis, MO) was used as positive control for glycosylation analysis.

B.4. Protein Purification

B.4.1. MON 88701 DMO Protein

The MON 88701 DMO was purified from cottonseed of MON 88701. The purification procedure was not performed under a GLP plan; however, all procedures were

documented on worksheets and, where applicable, SOPs were followed. The MON 88701 DMO protein was purified from an extract of ground cottonseed using a combination of ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, mixed mode ion exchange chromatography and size exclusion chromatography. The purification procedure is briefly described below.

Approximately 1 kg of MON 88701 cottonseed expressing the DMO protein was mixed with ~1 kg of dry ice and ground to fine powder using a laboratory mill (model 3100, Perten Instruments). The ground powder was suspended in two liters of hexane (EMD Chemicals Inc., Gibbstown, NJ) and filtered. This process was repeated four times in order to completely defat the powder. After drying overnight, the powder was ready for further processing. All grinding and defatting steps were done in a fume hood at room temperature.

The ground powder was mixed with extraction buffer (50 mM Tris, pH 8.0, 2.0 M deionized urea, 0.2 M boric acid, 1.0 mM dithiothreitol (DTT), 1.0 mM benzamidine-HCl, 1.0 µm bestatin, 1.0 µM E-64 and Complete EDTA free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)) to a final volume of 8 liters and incubated for 2 h at room temperature. The slurry was centrifuged at 15000 × g for 30 min at 4 °C. The supernatant was collected and brought to 0.05% polyethyleneimine (PEI). The solution was stirred at ~4 °C for 30 min and then centrifuged at 15000 × g for 30 min. The supernatant was collected and ~2.2 kg of ammonium sulfate was slowly added to bring the solution to 50% ammonium sulfate saturation. This solution was stirred at ~4 °C for 2 h and the pellet was collected by centrifugation at 15000 × g for 30 min. The pellet was resuspended in 10 liters of the resuspension buffer (50 mM Tris-HCl, pH 8.0, 0.35 M ammonium sulfate, 10 mM DTT, 1.0 mM benzamidine-HCl, 1.0 µM bestatin, 1.0 µM E-64 and Complete EDTA free protease inhibitor cocktail). The solution was stirred in the cold room overnight and then centrifuged at 30,000 × g for 1 h. Supernatant was collected and loaded onto a 1 liter butyl sepharose column (GE Healthcare) equilibrated with butyl sepharose equilibration (BSE) buffer (50 mM Tris-HCl, pH 8.0, 0.35 M ammonium sulfate, 1 mM DTT, 1.0 mM benzamidine-HCl, 1.0 µM bestatin, 1.0 µM E-64 and Complete EDTA-free protease inhibitor cocktail). All column steps were run at room temperature. The column was washed with 5 liters BSE buffer. Proteins were eluted with 1 liter of buffer containing 25 mM Triethanolamine, pH 8.0, 100 µM dicamba, 1.0 mM DTT, 1.0 mM benzamidine-HCl, 1.0 µM bestatin, 1.0 µM E-64 and Complete EDTA free protease inhibitor cocktail (BSEL buffer). After eluting the proteins with 1 liter of BSEL buffer , the flow was stopped for one hour and then elution was continued with additional 1 liter of BSEL buffer. Both elutions were pooled and loaded onto a 25 ml DEAE macroprep column (Bio Rad) equilibrated with DEAE macroprep equilibration (DME) buffer (50 mM Tris-HCl, pH 8.0, 100 µM dicamba, 1.0 mM DTT, 1.0 mM benzamidine-HCl, 1.0 µM bestatin, 1.0 µM E-64 and Complete EDTA free protease inhibitor cocktail). All steps associated with DEAE macroprep were performed at ~4 °C. The column was washed with 125 ml DME buffer and proteins were eluted with 75 ml DME buffer containing 70 mM NaCl and then with a

linear gradient that increased from 70 mM to 350 mM NaCl over 500 ml. Fractions containing MON 88701 DMO were pooled and loaded onto a 2.5 ml ceramic hydroxyapatite (CHT) column (Bio-Rad) equilibrated with CHT equilibration buffer (50 mM Tris-HCl, pH 8.0, 100 µM dicamba,1.0 mM DTT, 1.0 mM benzamidine-HCl, 1.0 µM bestatin, 1.0 µM E-64 and Complete EDTA free protease inhibitor cocktail). All steps associated with CHT were performed at ~4 °C. Most of the MON 88701 DMO was found in the flow through and wash fractions. Flow through and wash fractions from CHT were pooled separately (Pooled FT and Pooled Wash, respectively) and reloaded on two separate CHT columns as follows.

The Pooled FT was loaded onto a ~10 ml CHT column (CHT3). The column was washed with 50 ml of CHT equilibration buffer and step eluted using the CHT equilibration buffer containing 1 mM, 2 mM and 3 mM potassium phosphate, pH 8.0. The Pooled Wash was loaded onto a ~3 ml CHT column (CHT2). CHT2 was washed with ~45 ml of CHT equilibration buffer and step eluted using the CHT equilibration buffer containing 1 mM, 2 mM and 3 mM potassium phosphate, pH 8.0.

Wash fractions from both the CHT2 and CHT3 chromatography runs that contained MON 88701 DMO were pooled and loaded onto a ~1 ml DEAE macroprep column equilibrated with DME buffer for concentration. The column was washed with 10 ml of the DME buffer and eluted with DME buffer containing 500 mM NaCl. The MON 88701 DMO containing fractions were pooled and loaded onto a Hi-Prep Sephacryl S 100 size exclusion column equilibrated at ~4 °C with 50 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 0.1 M NaCl and 10% (v/v) glycerol. Fractions containing MON 88701 DMO were pooled and concentrated with aquacide (EMD Biosciences, Inc., La Jolla, CA) at ~4 °C to a final volume of 750 µl.

Elution fractions (1-3 mM potassium phosphate, pH 8.0 fractions) from both the CHT2 and CHT3 that contained MON 88701 DMO were pooled and concentrated using a Amicon ultra spin concentrator (Millipore, Bedford, MA) with a 10K MWCO. The centriprep concentrated pool was then loaded onto a Hi Prep Sephacryl S 100 size exclusion column equilibrated at ~4 °C with 50 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 0.1 mM NaCl and 10% (v/v) glycerol. Fractions containing MON 88701 DMO were pooled and concentrated with aquacide at ~4 °C to a final volume of 750 µl.

Both aquacide concentrated samples were pooled to a final volume of 1.5 ml. The final buffer composition of the purified MON 88701 DMO protein was 50 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 100 mM sodium chloride and 10% (v/v) glycerol. This MON 88701 DMO purified from the cottonseed of MON 88701 was aliquoted and stored in a -80 °C freezer.

B.4.2. PAT (*bar*) Protein

The plant-produced PAT (*bar*) protein was purified from cottonseed of MON 88701. The purification procedure was not performed under a GLP plan; however, all procedures

were documented on worksheets and, where applicable, SOPs were followed. The plant-produced PAT (*bar*) was purified from an extract of ground cottonseed using a combination of dye affinity chromatography and anionic exchange chromatography. The purification procedure is briefly described below.

Approximately 1 kg of cottonseed of MON 88701 was ground to fine powder using a laboratory mill (model 3100, Perten Instruments). The ground powder was suspended in 4 liters of hexane (EMD Chemicals Inc., Gibbstown, NJ) and filtered 3 times in order to defat the powder. After drying overnight, the powder was ready for further processing. All grinding and defatting steps were done in a fume hood at room temperature.

A portion (200 g) of the defatted powder was extracted with 2 liters of 20 mM Tris-HCl, pH 7.5, and the solids were removed by centrifugation at 25,000 × g for 20 min. The decanted solution was treated with 15 ml of 1 M CaCl2 solution to precipitate some proteins and centrifuged at 25,000 × g for 20 min to remove the precipitated proteins. The soluble portion (~1450 ml), containing the PAT (*bar*) protein, was batch absorbed onto 20 ml of reactive brown 10 agarose (Sigma-Aldrich, St. Louis, MO) equilibrated with 200 ml of 20 mM Tris-HCl, pH 7.5. The reactive brown 10 agarose was centrifuged at 1000 × g for 2 min and the resin, after decanting the supernatant, was transferred to a column. To remove unbound proteins, reactive brown 10 agarose was washed with 80 ml of 20 mM Tris-HCl buffer, pH 7.5, followed by 120 ml of 20 mM Tris-HCl buffer, pH 7.5, 1.5 M NaCl. Finally, the column was rinsed with 120 ml of 20 mM Tris-HCl buffer, pH 7.5. The PAT (*bar*) protein was then eluted, with 80 ml of 1 mM acetyl CoA in 20 mM Tris-HCl, pH 7.5. The eluted PAT (*bar*) protein was loaded onto a 1 ml Q Sepharose Fast Flow (GE Healthcare) column, equilibrated with 10 ml of 20 mM Tris-HCl, pH 7.5, using an automated chromatography system (AKTA, GE Healthcare). The Q Sepharose Fast flow column was washed with 20 ml of 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl and consecutive step wise elution using 0.2 M and 0.5 M NaCl in 20 mM Tris-HCl, pH 7.5 to a total volume of 23 ml was conducted. Fractions containing PAT (*bar*) protein were pooled (8 ml) and concentrated to a volume of 1170 µl using a centrifugal filter (Ultracel 10K; Millipore, Billerica, MA; Molecular Weight Cutoff (MWCO) of 10 kDa). Buffer was added to the concentrated sample to bring the final volume to 2 ml and the final buffer composition to 50 mM Tris-HCl, pH 7.5, 0.16 M NaCl, and 20% (v/v) glycerol. This PAT (*bar*) protein purified from the cottonseed of MON 88701 was aliquoted and stored in a -80 °C freezer.

B.5. Methods for Characterization

B.5.1. N-Terminal Sequencing

B.5.1.1. MON 88701 DMO Protein

N-terminal sequencing by automated Edman degradation chemistry was carried out in an attempt to confirm the identity of MON 88701 DMO.

MON 88701 DMO was separated by SDS-PAGE and transferred to PVDF membrane. The blot was stained using Coomassie Blue R-250. The major band at ~39 kDa containing MON 88701 DMO was excised from the blot and was used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller et al., 1983) using an Applied Biosystems 494 Procise Sequencing System equipped with 140C Microgradient system a Perkin Elmer Series 200 UV/VIS Absorbance Detector with Procise™ Control Software (version 2.1) for amino acid detection after each cycle. Chromatographic data were collected using SequencePro (version 2.1) software. A control protein, β-lactoglobulin, (Applied Biosystems, Foster City, CA) was analyzed before and after the sequence analysis of the MON 88701 DMO protein to verify that the sequencer met performance criteria for repetitive yield and sequence identity. Identity was established if ≥ 8 amino acids, consistent with the predicted sequence of the N-terminus of the MON 88701 DMO, were observed during analysis.

B.5.1.2. PAT (*bar*) Protein

N-terminal sequencing, carried out by automated Edman degradation chemistry, was used to confirm the identity of the MON 88701-produced PAT (*bar*).

One aliquot of MON 88701-produced PAT (*bar*) was used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller et al., 1983) using an Applied Biosystems 494 Procise Sequencing System equipped with 140C Microgradient system a Perkin Elmer Series 200 UV/VIS Absorbance Detector with Procise™ Control Software (version 2.1) for amino acid detection after each cycle. Chromatographic data were collected using SequencePro (version 2.1) software. A control protein, β-lactoglobulin, (Applied Biosystems, Foster City, CA) was analyzed before and after the sequence analysis of the MON 88701-produced PAT (*bar*) protein to verify that the sequencer met performance criteria for repetitive yield and sequence identity. Identity was established if ≥ 8 amino acids, consistent with the predicted sequence of the N-terminus of the MON 88701-produced PAT (*bar*), were observed during analysis.

B.5.2. MALDI-TOF Tryptic Mass Map Analysis

B.5.2.1. MON 88701 DMO Protein

MALDI-TOF tryptic mass fingerprint analysis was used to confirm the identity of the MON 88701 DMO protein. MON 88701 DMO protein (~15 µg) was chilled in a -20 °C freezer for at least 10 min. The chilled protein was precipitated with 200 µl of 95% acetone in a -20 °C freezer overnight. Precipitated protein sample was pelleted in a refrigerated centrifuge for at least 45 min at more than 13,000 × g. The supernatant was carefully removed and discarded. The protein pellet was washed twice with 200 µl of chilled ethanol to remove residual supernatant. The pellet was dried to completion using a Speed Vac concentrator and resuspended in 30 µl of 40% 2,2,2,-trifluoroethanol (TFE) in 25 mM ammonium bicarbonate. The resuspended protein was vortexed vigorously

and then sonicated for 5 min in a water bath. The sample was incubated at ~37 °C for 1 h to denature the proteins. Denatured protein sample was reduced with ~5 mM tris(2-carboxyethyl)phosphine (TCEP) for 1 h at ~37 °C. Reduced protein sample was then alkylated in the dark for 30 min at room temperature with ~10 mM iodoacetic acid. Additional TCEP was added to ~5 mM and the sample was incubated for 10 min at room temperature. The reduced and denatured test substance was mixed with 67 µl of 25 mM ammonium bicarbonate and 2.5 µl of trypsin solution (0.2 µg/µl in 25 mM ammonium bicarbonate). The tryptic digestion was allowed to proceed for 15 h at 37 °C followed by quenching with 1 µl of formic acid. Proteolytic peptides were dried to completion using Speed Vac concentrator. To solubilize the dried peptides, a solution of 50% acetonitrile, 0.1% TFA was added and sonicated for 5 min. Aliquots from the digest were spotted to three wells on an analysis plate. For each spot, 0.75 µl of 2,5 dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α-Cyano), or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Thermo Fisher Scientific Inc.) was added to one of the spots. The sample in DHB matrix was analyzed in the 300 to 5000 Da range. Samples in α-Cyano and Sinapinic acid were analyzed in the 500 to 5000 Da and 500 to 7000 Da range, respectively. The analysis was performed using a Voyager™ DE Pro Biospectrometry™ workstation (Applied Biosystems) using Voyager Instrument Control Panel software (version 5.10.2) and Data Explorer data analysis software (version 4.0.0.0). Protonated peptide masses were monoisotopically resolved in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). CalMix 2 was used as the external calibrant (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) for the analysis. GPMAW32 software (Lighthouse Data, Odense M, Denmark) was used to generate a theoretical trypsin digest of the MON 88701 DMO protein sequence. Masses within 1 Da of a monosiotopic mass were matched against the theoretical digest of the MON 88701 DMO sequence. All matching masses were tallied and a coverage map was generated for the mass fingerprint. The tryptic mass fingerprint coverage was considered acceptable if ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments (Biron et al., 2006, Krause et al., 1999).

B.5.2.2. PAT (*bar*) Protein

MALDI-TOF tryptic mass fingerprint analysis was used to confirm the identity of the MON 88701-produced PAT (*bar*) protein. MON 88701-produced PAT (*bar*) protein was subjected to SDS-PAGE and the gel was stained using Brilliant Blue G Colloidal stain. Each ~25 kDa band was excised and transferred to a microcentrifuge tube. The gel slices were destained with 40% (v/v) methanol/ 10% (v/v) acetic acid and washed in 100 mM ammonium bicarbonate and then, to reduce the protein in each, gel slices were incubated in 100 µl of 10 mM DTT at ~37 °C for 1 h. The protein was then alkylated in the dark for 20 min with 100 µl of 20 mM iodoacetic acid and washed three times for 15-20 min each with 200 µl of 25 mM ammonium bicarbonate. Gel slices were dried with a Speed-Vac® concentrator (Thermo Fisher Scientific, Waltham, MA) and then rehydrated with 20 µl of trypsin solution (20 µg/ml). After 1.25 h, excess liquid was removed and the gel was incubated overnight at ~37.5 °C in 40 µl of 10% acetonitrile in 25 mM

ammonium bicarbonate. Gel slices were sonicated for 5 min to further elute proteolytic fragments. The resulting extracts were transferred to new microcentrifuge tubes labeled Extract 1 and dried using Speed-Vac concentrator. The gel slices were re-extracted twice with 30 µl of a 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% octyl-β-glucopyranoside solution and sonicated for 5 min. Both extracts were pooled into a new tube labeled Extract 2 and dried with a Speed-Vac concentrator. A solution (20 µl) of 0.1% trifluoroacetic acid (TFA) was added to all Extract 1 and 2 tubes and the samples were dried to completion via vacuum centrifugation. To solubilize the extracts, 5 µl of 50% acetonitrile, 0.1% trifluoroacetic acid was added to Extract 1 tube and 10 µl of 50% acetonitrile, 0.1% trifluoroacetic acid was added to Extract 2 tube and all were sonicated for 5 min. Each extract was spotted to three wells on an analysis plate. For each extract, 0.75 µl of 2, 5 dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α-Cyano), or 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Thermo Fisher Scientific Inc.) was added to one of the spots. The samples in DHB matrix were analyzed in the 300 to 5000 Da range. Samples in α-Cyano and Sinapinic acid were analyzed in the 500 to 5000 Da and 500 to 7500 Da range, respectively. The analysis was performed using a Voyager™ DE Pro Biospectrometry™ workstation (Applied Biosystems) using Voyager Instrument Control Panel software (version 5.10.2) and Data Explorer data analysis software (version 4.0.0.0). Protonated peptide masses were monoisotopically resolved in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). CalMix 2 was used as the external calibrant (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) for the analysis. GPMAW32 software (Lighthouse Data, Odense M, Denmark) was used to generate a theoretical trypsin digest of the PAT (*bar*) protein sequence. Masses within 1 Da of a monosiotopic mass were matched against the theoretical digest of the PAT (*bar*) sequence. All matching masses were tallied and a coverage map was generated for the mass fingerprint. The tryptic mass fingerprint coverage was considered acceptable if ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments (Biron et al., 2006, Krause et al., 1999).

B.5.3. Western Blot Analysis-Immunoreactivity

B.5.3.1. MON 88701 DMO Protein

Western blot analysis was performed to confirm the identity of the MON 88701 DMO protein purified from cottonseed of MON 88701 and to compare the immunoreactivity of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins.

The MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were analyzed concurrently on the same gel using three loadings of 0.5, 2 and 6 ng. Loadings of the three concentrations were made in duplicate on the gel. Aliquots of each protein were diluted in water and 5X Laemmli buffer (LB) containing 312 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) SDS, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8), heated at 101 °C for 3 min, and applied to a 15 well pre-cast Tris-glycine 4-20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). Pre-stained molecular weight markers (Precision Plus Protein Standards Dual color; Bio-Rad,

Hercules, CA) were loaded in parallel to verify electrotransfer of the proteins to the membrane and to estimate the size of the immunoreactive bands observed. Electrophoresis was performed at a constant voltage of 150 V for 90 min. Electrotransfer to a 0.45 µm nitrocellulose membrane (Invitrogen, Carlsbad, CA) was performed for 105 min at a constant voltage of 25 V. After electrotransfer, the membrane was stored overnight with 1× phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST) at 4 °C. The membrane was blocked for 1 h with 5% (w/v) NFDM in PBST at room temperature. The membrane was then probed with a 1:5000 dilution of goat anti-DMO antibody (lot 11223358) in 2% NFDM in PBST for 1 h at room temperature. Excess antibody was removed using two 1 min washes followed by three 5 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase (HRP)-conjugated horse anti-goat IgG (Thermo, Rockford, IL) at a dilution of 1:10,000 in 2% NFDM in PBST for 1 h at room temperature. Excess HRP-conjugate was removed using two 1 min washes and three 5 min washes with PBST. All washes were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (GE, Healthcare, Piscataway, NJ) with exposure to Amersham Hyperfilm ECL (GE, Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

Quantification of the bands on the blot was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA) using the band location and volume tool. The signal intensities of the immunoreactive bands observed for the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins migrating at the expected position on the blot film were quantified as "adjusted volume" values. The raw data was exported to a Microsoft Excel (2007) file. The immunoreactivity of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were reported as the mean signal intensity at each amount of protein analyzed. The immunoreactivity of the MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were considered equivalent if the overall mean of the immunoreactive signal of the MON 88701 DMO protein was within ± 35% of the overall mean of immunoreactive signal of the *E. coli*-produced MON 88701 DMO protein.

B.5.3.2. PAT (*bar*) Protein

Western blot analysis was performed to confirm the identity of the PAT (*bar*) protein purified from cottonseed of MON 88701 and to compare the immunoreactivity of the MON 88701- and *E. coli*-produced proteins.

The MON 88701- and *E. coli*-produced PAT (*bar*) proteins were analyzed concurrently on the same gel using three loadings of 2, 4 and 6 ng. Loadings of the three concentrations were made in duplicate on the gel. Aliquots of each protein were diluted in water and 5X Laemmli buffer (LB) containing 312 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) SDS, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8), heated at ~96 °C for 4 min, and applied to a 15 well pre-cast Tris-glycine 4-20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). Pre-stained molecular weight markers (Precision Plus Protein Standards Dual color; Bio-Rad,

Hercules, CA) were loaded in parallel to verify electrotransfer of the proteins to the membrane and to estimate the size of the immunoreactive bands observed. Electrophoresis was performed at a constant voltage of 150 V for 85 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 60 min at a constant current of 200 mA. After electrotransfer, the membrane was blocked for 1 h with 10% (w/v) non-fat dried milk (NFDM) in 1X phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST). The membrane was then probed with a 1:2000 dilution of goat anti-PAT (*bar*) antibody (lot G863803) in 5% NFDM in PBST for 1 h at room temperature. Excess antibody was removed using three 10 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase (HRP)-conjugated horse anti-goat IgG (Thermo, Rockford, IL) at a dilution of 1:10,000 in 5% NFDM in PBST for 1 h at room temperature. Excess HRP-conjugate was removed using three 10 min washes with PBST. All washes were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (GE, Healthcare, Piscataway, NJ) with exposure to Amersham Hyperfilm ECL (GE, Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

Quantification of the bands on the blot was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA) using the lane selection and contour tool. The signal intensities of the immunoreactive bands observed for the MON 88701- and *E. coli*-produced proteins migrating at the expected position on the blot film were quantified as "contour quantity" values. The raw data was exported to a Microsoft Excel (2007) file. The immunoreactivity of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were reported as the mean signal intensity at each amount of protein analyzed. The immunoreactivity of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were considered equivalent if the overall mean of the immunoreactive signal of the MON 88701-produced protein was within ± 35% of the overall mean of immunoreactive signal of the *E. coli*-produced PAT (*bar*) protein.

B.5.4. Molecular Weight and Purity Estimation SDS-PAGE

B.5.4.1. MON 88701 DMO Protein

MON 88701 DMO and *E. coli*-produced MON 88701 DMO proteins were mixed with 5X LB and diluted with water to a final total protein concentration of 0.1 µg/µl. Molecular Weight Standards, Bio-Rad broad range (Hercules, CA) were diluted to a final total protein concentration of 0.9 µg/µl. The MON 88701 DMO was analyzed in duplicate at 0.5, 1, and 1.5 µg protein per lane. The *E. coli*-produced MON 88701 DMO reference standard was analyzed at 0.5 µg total protein in a single lane. The samples were loaded onto a 10-well pre-cast Tris glycine 4-20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA) and electrophoresis was performed at a constant voltage of 125 V for 90 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) acetic acid for 25 min, stained for ~16 h with Brilliant Blue G Colloidal stain (Sigma-Aldrich, St. Louis, MO). Gels were destained once for 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and with

25% (v/v) methanol for a total of 6 h. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). The apparent MW of each observed band was estimated from a standard curve generated by the Quantity One software which was based on the MWs of the markers and their migration distance on the gel. To determine purity, all visible bands within each lane were quantified using Quantity One software. Apparent MW and purity were reported as an average of all six lanes containing the MON 88701 DMO.

B.5.4.2. PAT (*bar*) Protein

MON 88701- and *E. coli*-produced PAT (*bar*) proteins were mixed with 5X LB and diluted with water to a final total protein concentration of 0.136 µg/µl. Molecular Weight Standards, Bio-Rad broad range (Hercules, CA) were diluted to a final total protein concentration of 0.9 µg/µl. The MON 88701-produced PAT (*bar*) was analyzed in duplicate at 1, 2, and 3 µg protein per lane. The *E. coli*-produced PAT (*bar*) reference standard was analyzed at 1 µg total protein in a single lane. The samples were loaded onto a 10-well pre-cast Tris glycine 4-20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA) and electrophoresis was performed at a constant voltage of 150 V for 95 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) acetic acid for 30 min, stained for 16.25 h with Brilliant Blue G Colloidal stain (Sigma-Aldrich, St. Louis, MO). Gels were destained once for 30 to 45 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and four times for 2 h each (for a total of 8 h) with 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). The apparent MW of each observed band was estimated from a standard curve generated by the Quantity One software which was based on the MWs of the markers and their migration distance on the gel. To determine purity, all visible bands within each lane were quantified using Quantity One software. Apparent MW and purity were reported as an average of all six lanes containing the MON 88701-produced PAT (*bar*).

B.5.5. Glycosylation Analysis

B.5.5.1. MON 88701 DMO Protein

Glycosylation analysis was used to determine whether the MON 88701 DMO was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 88701 DMO protein, the *E. coli*-produced MON 88701 DMO (negative control) and the positive control, transferrin (Sigma-Aldrich, St. Louis, MO), were each diluted with water and brought to 1X LB. These samples were heated at ~101 °C for 3 min. The MON 88701 DMO, the *E. coli*- produced MON 88701 DMO and transferrin were loaded at approximately 50 and 100 ng per lane on a Tris-glycine 10 well 4-20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA). Precision Plus Protein Dual color Standards (Bio-Rad, Hercules, CA) were also loaded to verify electrotransfer of the proteins to the membrane and as markers for molecular weight. Electrophoresis was performed at a constant voltage of 150 V for 90 min. Electrotransfer to a 0.45 µm PVDF

membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 25 V.

Carbohydrate detection was performed directly on the PVDF membrane at room temperature using the Amersham ECL glycoprotein Detection Module (GE, Healthcare, Piscataway, NJ). With this module, carbohydrate moieties of proteins were oxidized with sodium metaperiodate and then biotinylated with biotin-X-hydrazide. The biotinylated proteins can be detected on the blot by addition of streptavidin conjugated to HRP for luminol-based detection using ECL reagents (GE, Healthcare, Piscataway, NJ) and with subsequent exposure to Amersham Hyperfilm (GE, Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

An identical blot run in parallel to that used for the glycosylation analysis was stained to visualize the proteins present on the membrane. Proteins were stained for 30 sec to 2 min using Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, Hercules, CA) and then destained with 1X Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad) for 5 min. After washing with water, the blot was dried and scanned using Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0).

B.5.5.2. PAT (*bar*) Protein

Glycosylation analysis was used to determine whether the MON 88701-produced PAT (*bar*) was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 88701-produced PAT (*bar*) protein, the *E. coli*-produced PAT (*bar*) (negative control) and the positive control, transferrin (Sigma-Aldrich, St. Louis, MO), were each diluted with water and brought to 1X LB. These samples were heated at ~102 °C for 4 min. The MON 88701-produced PAT (*bar*), the *E. coli*-produced PAT (*bar*) and transferrin were loaded at approximately 50 and 100 ng per lane on a Tris-glycine 10 well 4-20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA). Precision Plus Protein Dual color Standards (Bio-Rad, Hercules, CA) were also loaded to verify electrotransfer of the proteins to the membrane and as markers for molecular weight. Electrophoresis was performed at a constant voltage of 150 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 200 mA.

Carbohydrate detection was performed directly on the PVDF membrane at room temperature using the Amersham ECL glycoprotein Detection Module (GE, Healthcare, Piscataway, NJ). With this module, carbohydrate moieties of proteins were oxidized with sodium metaperiodate and then biotinylated with biotin-X-hydrazide. The biotinylated proteins can be detected on the blot by addition of streptavidin conjugated to HRP for luminol-based detection using ECL reagents (GE, Healthcare, Piscataway, NJ) and with subsequent exposure to Amersham Hyperfilm (GE, Healthcare). The film was developed using a Konica SRX-101A automated film processor (Konica Minolta Medical & Graphic, Inc., Tokyo, Japan).

An identical blot run in parallel to that used for the glycosylation analysis was stained to visualize the proteins present on the membrane. Proteins were stained for 30 sec to 2 min using Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, Hercules, CA) and then destained with 1X Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad) for more than 5 min. After washing with water, the blot was dried and scanned using Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0).

B.5.6. Functional Activity

B.5.6.1. MON 88701 DMO Protein

The specific activity of MON 88701 DMO and *E. coli*-produced MON 88701 DMO was determined by quantifying the conversion of 3,6-dichloro-2-methoxybenzoic acid (dicamba) to 3,6-dichlorosalicylic acid (DCSA) via HPLC (Agilent Technologies 1100 series, Santa Clara, CA) separation and fluorescence detection (Agilent Technologies 1200 series, G1321A). Each assay reaction contained 25 mM potassium phosphate, pH 7.2, 3.4 µg ferredoxin, 3.4 µg reductase, 0.5 mM $FeSO_4$, 10 mM $MgCl_2$, 0.7 mM NADH, 0.3 mM dicamba, 2 µl (42.48 U/ml) of formaldehyde dehydrogenase and either 2.9 µg MON 88701 DMO or 3 µg his-DMO as an assay positive control. The reactions were performed in PCR tubes (Sorenson, Salt Lake City, UT) and incubated at 30 °C for 15 min. Reactions (200 µl) were initiated by the addition of dicamba and quenched with the addition of 50 µl of 5% H_2SO_4. Reactions were then filtered using Whatman Anotop 10 filters (0.2 µm, GE healthcare), and 40 µl was transferred to a HPLC sample vial (200 µl, Agilent) for analysis. Twenty-five microliters of the filtered reaction was injected onto a Phenomenex® Synergi 4 µm C18/ODS Hydro-RP column (150 × 4.6 mm ID, Torrance, CA). The mobile phase consisted of solvent A (21.5 mM phosphoric acid) and solvent B (100% acetonitrile) running at 1.5 ml/min. DCSA was eluted from the column using a linear gradient from 90% to 40% solvent A for the first 14 min, followed by a step to 10% solvent A for 1 min and then re-equilibration at 90% solvent A for 10 min before the next injection. DCSA was monitored by the detection of fluorescent emission at 424 nm (excitation 306 nm) and quantified relative to a standard curve of DCSA generated using 0.1, 0.3, 0.6, 0.9, 1.2, 2.4, and 4.8 nmol/250 µl. Chromatographic data were collected using AtlasTM 2003 software (Thermo Fisher Scientific Inc). The specific activity was calculated based on the amount of purity corrected MON 88701 DMO protein added to the reaction mixture and expressed as nmol of DCSA produced per minute per mg of MON 88701 DMO protein (nmol × min^{-1} × mg^{-1}).

B.5.6.2. PAT (*bar*) Protein

PAT (*bar*) catalyzes the reaction of phosphinothricin (PPT) with acetyl CoA to form acetyl PPT and free CoA. To assess functional activity of PAT (*bar*), the amount of CoA released during the reaction can be monitored using the reduction of 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) by CoA to form the colorimetric reagent 5-thio-nitrobenzoate (TNB) (Wehrmann et al., 1996).

Prior to functional activity analysis, both MON 88701- and *E. coli*-produced PAT (*bar*) proteins were diluted to a purity corrected concentration of 1 ng/µl with a 50 mM Tris, pH 7.5, and 0.5 mM EDTA buffer. Assays for both proteins were conducted using five replicates. The reaction mixtures containing 2 mM acetyl CoA, 1 mM DTNB, 50 mM Tris, pH 7.8, and 0.5 mM EDTA with or without 1 mM phosphinothricin were pre incubated at ~30 °C for 10-60 min. The reactions were then initiated by the addition of 10 ng of PAT (*bar*) enzyme. The reaction rate was monitored in each well at 412 nm and ~30 °C using a plate reader in one minute intervals for 30 min. A response curve was prepared using 3.9 µM to 250 µM β-mercaptoethanol in 1 mM DTNB, 50 mM Tris, pH 7.8, and 0.5 mM EDTA. The response curve was generated only to verify assay conditions and instrument performance. The initial assay results are reported as the mean velocity of the reaction of PAT (*bar*) (generated by the KC4 software, Power Wave Xi, Bio Tek, Richmond, VA) and expressed as min^{-1}. The specific activities of the MON 88701- and *E. coli*-produced PAT (*bar*) proteins were then calculated using the molar absorptivity of product released during the assay, TNB (13,600 $M^{-1} \times cm^{-1}$ or 13.6 $\mu mol^{-1} \times ml$). Specific activity is expressed as µmol of TNB released per minute per mg of PAT (*bar*) ($\mu mol \times min^{-1} \times mg^{-1}$). Calculations of the specific activities were performed using Microsoft Excel (2007).

B.5.7. Prediction Intervals as Acceptance Criteria

Acceptance criteria (acceptance limits) based on prediction intervals were used to assess the equivalence of the MON 88701-produced and *E. coli*-produced proteins for apparent MW and functional activity. A prediction interval is an estimate of an interval in which a randomly selected future observation from a population will fall, with a certain degree of confidence, given what has already been observed (Hahn & Meeker, 1991a; b); *i.e.*, prediction intervals are generated based on the statistical analysis of the existing data. Data obtained from multiple assays of *E. coli*-produced protein conducted under GLP guidelines were used for this purpose.

To generate the 95% prediction interval (PI), the mean and standard deviation of the data from several assays were calculated. The number of assays used to calculate the mean and the number of future assays (one for equivalence studies) were used in the following formula to generate the PI:

$$\overline{X} \pm r(1-\alpha;\ m,n) \quad (s)$$

$r_{(1-\alpha;\, m,\, n)}$ is estimated using the formula given below:

$$r_{(1-\alpha;m,n)} \cong t_{(1-.05/(2m);\, n-1)} \sqrt{1+\frac{1}{n}}$$

Where $\bar{X}$ is mean of the replicate assays; s is standard deviation of the replicates; $1-\alpha$ is the level of confidence; n is the number of assays used to generate the mean; and m is the number of future assays (one for equivalence studies). The t-value is the $100(1-.05/(2m))^{th}$ percentile from Student's t-distribution with n-1 degrees of freedom. With 95% confidence, all m future values of the assay will fall within this interval (Hahn & Meeker, 1991a; b). If the assay means do not appear to have been derived from a normal distribution, but the logarithms of the raw values do follow a normal distribution, then prediction intervals may be applied to the logarithms of the raw values (Hahn & Meeker, 1991a; b).

References for Appendix B

Aebersold, R. 1993. Mass spectrometry of proteins and peptides in biotechnology. Current Opinion in Biotechnology 4:412-419.

Billeci, T.M. and J.T. Stults. 1993. Tryptic mapping of recombinant proteins by matrix-assisted laser desorption/ionization mass spectrometry. Analytical Chemistry 65:1709-1716.

Biron, D.G., C. Brun, T. Lefevre, C. Lebarbenchon, H.D. Loxdale, F. Chevenet, J.-P. Brizard and F. Thomas. 2006. The pitfalls of proteomics experiments without the correct use of bioinformatics tools. Proteomics 6:5577-5596.

D'Ordine, R.L., T.J. Rydel, M.J. Storek, E.J. Sturman, F. Moshiri, R.K. Bartlett, G.R. Brown, R.J. Eilers, C. Dart, Y. Qi, S. Flasinski and S.J. Franklin. 2009. Dicamba monooxygenase: Structural insights into a dynamic Rieske oxygenase that catalyzes an exocyclic monooxygenation. Journal of Molecular Biology 392:481-497.

Dumitru, R., W.Z. Jiang, D.P. Weeks and M.A. Wilson. 2009. Crystal structure of dicamba monooxygenase: A Rieske nonheme oxygenase that catalyzes oxidative demethylation. Journal of Molecular Biology 392:498-510.

Hahn, G.J. and W.Q. Meeker. 1991. Methods for calculating statistical intervals for a normal distribution. Pages 53-74 in Statistical Intervals: A Guide for Practitioners. John Wiley and Sons, Inc., Hoboken, New Jersey.

Hahn, G.J. and W.Q. Meeker. 1991. Overview of different types of statistical intervals. Pages 27-40 in Statistical Intervals: A Guide for Practitioners. John Wiley and Sons, Inc., Hoboken, New Jersey.

Herman, P.L., M. Behrens, S. Chakraborty, B.M. Chrastil, J. Barycki and D.P. Weeks. 2005. A three-component dicamba *O*-demethylase from *Pseudomonas maltophilia*, strain DI-6: Gene isolation, characterization, and heterologous expression. Journal of Biological Chemistry 280:24759-24767.

Hunkapiller, M.W., R.M. Hewick, L.E. Hood and W.J. Dreyer. 1983. High-sensitivity sequencing with gas-phase sequenator. Methods in Enzymology 91:399-413.

Krause, E., H. Wenschuh and P.R. Jungblut. 1999. The dominance of arginine-containing peptides in MALDI-derived tryptic mass fingerprints of proteins. Analytical Chemistry 71:4160-4165.

Appendix C: Materials and Methods Used for the Analysis of the Levels of MON 88701 DMO and PAT (*bar*) Proteins in MON 88701

C.1. Materials

Seed, over season leaf (OSL-1-4), root, and pollen tissue samples from dicamba- and glufosinate-treated MON 88701 were harvested from eight field sites in the U.S. during the 2010 growing season from starting seed lot 11268129, with the exception of OSL-1 (7 sites) and OSL-4 (7 sites). MON 88701 plots were treated at the 3-5 leaf stage with glufosinate herbicide at the label rate (0.5 lb a.e./acre) and at the 6-10 leaf stage with dicamba herbicide at the label rate (0.5 lb a.e./acre). *E. coli*-produced MON 88701 DMO (lot 11293429) and PAT (*bar*) protein (lot 11270310) were used as the analytical reference standards.

C.2. Characterization of the Materials

The identity of MON 88701 was confirmed by conducting MON 88701 event specific polymerase chain reaction (PCR) analyses on the harvested seed from each site. Any seed sample and its associated tissues for which three or more pools out of four tested unexpectedly during PCR verification were not analyzed in this study.

C.3. Field Design and Tissue Collection

Field trials were initiated during the 2010 planting season to generate MON 88701 seed, OSL-1-4, root, and pollen samples at various cotton growing locations in the U.S. The tissue samples from the following field sites were analyzed: Arkansas (ARTI), Georgia (GACH), Kansas (KSLA), Louisiana (LACH), North Carolina (NCBD), New Mexico (NMLC), South Carolina (SCEK) and Texas (TXPL). These field sites were representative of cotton producing regions suitable for commercial production. At each site, four replicated plots of plants containing MON 88701 were planted using a randomized complete block field design. Seed, over season leaf (OSL-1-4), root, and pollen samples were collected from each replicated plot at all field sites, except OSL-1 at site TXPL and OSL-4 at site LACH. See Tables VI-12 and VI-13 for detailed descriptions of when the samples were collected.

C.4. Tissue Processing and Protein Extraction

Tissue samples were shipped to Monsanto, St. Louis and were prepared by the Monsanto Sample Management Team. The prepared tissue samples were stored in a -80° C freezer until transferred on dry ice to the analytical facility.

C.4.1. MON 88701 DMO Protein Extraction

MON 88701 DMO protein was extracted from tissue samples as described in Table C-1. MON 88701 DMO was extracted from over season leaf (OSL-1-4) and root tissues samples with the appropriate amount of Tris borate buffer with 0.5% (w/v) bovine serum

albumin (1 × TB + 0.5% BSA) [0.1 M Tris, 0.1 M $Na_2B_4O_7$, 0.05 M $MgCl_2$, 0.05% (v/v) Tween 20 at pH 7.8, 0.5% (w/v) BSA]. MON 88701 DMO was extracted from pollen and seed tissues with the appropriate amount of phosphate buffered saline (PBS) with Tween 20 (1 × PBST). Extractions were done using 8 1/4″ chrome-steel beads, and shaking in a Harbil mixer (Fluid Management, Wheeling, Illinois). Insoluble material was removed from all tissue extracts using a serum filter (Fisher Scientific, Pittsburgh, PA) The extracts were aliquoted and stored frozen in a -80°C freezer until ELISA analysis.

Table C-1. MON 88701 DMO Protein Extraction Methods for Tissue Samples

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf[1]	1:100	1 × TB + 0.5% BSA
Root	1:100	1 × TB + 0.5% BSA
Pollen	1:100	1 × PBST
Seed	1:100	1 × PBST

[1]Over- season leaf (OSL-1, OSL-2, OSL-3, and OSL-4).

C.4.2. PAT Protein Extraction

PAT (*bar*) protein was extracted from tissue samples as described in Table C-2. PAT (*bar*) was extracted from over season leaf (OSL-1-4) and root tissues samples with the appropriate amount of Tris borate buffer with L-ascorbic acid (1× TBA) [0.1 M Tris, 0.1 M $Na_2B_4O_7$, 0.05 M $MgCl_2$, 0.05% (v/v) Tween 20 at pH 7.8, 0.2% (w/v) L-ascorbic acid]. PAT (*bar*) was extracted from pollen and seed tissues with the appropriate amount of 1 × PBST. Extractions were done using 8 1/4″ chrome-steel beads, and shaking in a Harbil mixer. Insoluble material was removed from all tissue extracts using a serum filter. The extracts were aliquoted and stored frozen in a -80°C freezer until ELISA analysis.

Table C-2. PAT (*bar*) Protein Extraction Methods for Tissue Samples

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf[1]	1:100	1× TBA
Root	1:100	1× TBA
Pollen	1:100	1× PBST
Seed	1:100	1× PBST

[1]Over- season leaf (OSL-1, OSL-2, OSL-3, and OSL-4.

C.5. Protein Antibodies

C.5.1. DMO Protein Antibodies

Goat polyclonal antibodies specific for the DMO protein were purified using Protein G affinity chromatography. The concentration of the purified IgG was determined to be 8.1 mg/ml by spectrophotometric methods. The purified antibody was stored in 1× PBS.

Protein G-affinity purified goat polyclonal anti-DMO antibodies were coupled with biotin (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. The detection reagent was NeutrAvidin (Thermo Fisher Scientific, Rockford, IL) conjugated to horseradish peroxidase (HRP).

C.5.2. PAT (*bar*) Protein Antibodies

Goat polyclonal PAT (*bar*)-specific IgG was purified by Protein G-affinity chromatography followed by PAT (*bar*) antigen affinity chromatography. The concentration of the purified IgG was determined to be 3.6 mg/ml by spectrophotometric methods. The purified antibody was stored in 1× PBS.

Protein G-affinity purified goat polyclonal anti-PAT (*bar*) antibodies were coupled with biotin (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. The detection reagent was NeutrAvidin (Thermo Fisher Scientific, Rockford, IL) conjugated to horseradish peroxidase (HRP).

C.6. Protein ELISA Methods

C.6.1. DMO Protein ELISA

Goat anti-DMO antibodies were diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl) to a final concentration of 5 µg/ml and immobilized onto 96 well microtiter plates followed by incubation in a 4° C refrigerator for >8 hours. Prior to each step in the assay, plates were washed with 1× PBST. Plates were blocked with the addition of 200 µl per well of blocking buffer, Blocker Casein (Thermo Fisher Scientific, Rockford, IL) in Tris Buffered Saline (TBS) for 60 to 70 minutes at room temperature (RT). DMO protein standard or sample extract was added at 100 µl per well and incubated for 60 to 65 minutes at 37° C. Biotinylated goat anti-DMO antibodies prepared in 1× Tris-borate buffer with 10% Blocker Casein in TBS were added at 100 µl per well and incubated for 60 to 65 minutes at 37° C. NeutrAvidin HRP conjugate was added at 100 µl per well and incubated for 30 to 35 minutes at 37° C. Plates were developed by adding 100 µl per well of 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 DMO protein was accomplished by interpolation from a DMO protein standard curve that ranged from 0.313 – 10 ng/ml.

C.6.2. PAT Protein ELISA

Affinity purified goat anti PAT (*bar*) antibodies were diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM sodium chloride) to a final concentration of 4 µg/ml and immobilized onto 96 well microtiter plates, followed by incubation in a 4° C refrigerator for >12 h. Prior to each step in the assay, plates were washed with 1× PBST. Plates were blocked with the addition of 200 µl per well of blocking buffer (1× PBST+1% BSA) for 60 to 70 minutes at 37° C. PAT (*bar*) protein standard or sample extract was added at 100 µl per well and incubated for 60 to 70 minutes at 37° C. Biotinylated goat anti-PAT (*bar*) antibodies diluted in 1 × PBST + 0.1% BSA were added at 100 µl per well and incubated for 60 to 70 minutes at 37° C. NeutrAvidin HRP conjugate was added at 100 µl per well and incubated for 60 to 70 minutes at 37° C. Plates were developed by adding 100 µl per well of TMB substrate. The enzymatic reaction was terminated by the addition of 100 µl per well of 3 M H_3PO_4. Quantification of the PAT (*bar*) protein was accomplished by interpolation from a PAT (*bar*) protein standard curve that ranged from 0.625 – 20 ng/ml.

C.7. Moisture Analysis

Tissue moisture content was determined using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO). A homogeneous tissue-specific site pool (TSSP) was prepared consisting of samples of a given tissue type grown at a given site. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$\text{DWCF} = 1 - \left(\frac{\text{Mean\% TSSP Moisture}}{100}\right)$$

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

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

Due to a limited amount of tissue, pollen was not analyzed for moisture content. Therefore, no dry weight calculation was performed and pollen was reported on a µg/g fresh weight (fw) basis only.

The protein levels (ng/ml) 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.

C.8. Data Analyses

All MON 88701 DMO and PAT (*bar*) ELISA plates were analyzed on a 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 software. 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 converted to a µg/g fw basis for data that were greater than or equal to the LOQ. This conversion utilized a sample dilution factor, and tissue-to-buffer ratio. The protein values in µg/g fw were also converted to µg/g dw by applying the DWCF (except pollen). Microsoft Excel 2007 (Microsoft, Redmond, WA) was used to calculate the protein levels in all cotton tissues. The sample means, standard deviations, and ranges were also calculated by Microsoft Excel 2007. All protein expression levels were rounded to two significant figures.

Any MON 88701 sample extracts that resulted in an unexpectedly negative result by ELISA analysis was re extracted twice for the protein of interest and re analyzed by ELISA to confirm the results. Samples with confirmed unexpected results were omitted from all calculations.

Appendix D: Western Blot Analysis of MON 88701 DMO and PAT (*bar*) Proteins in MON 88701 Cotton Tissue across Multiple Generations

D.1. Materials

Leaf tissues of MON 88701 were collected from plants of multiple breeding generations grown in a U.S. greenhouse (St. Louis, MO).

D.2. MON 88701 Materials

A summary of the MON 88701 leaf samples and the starting seed lot numbers are listed in the table below. The breeding tree for MON 88701 is found in Figure V-8.

Generation	Lot Number	Starting Seed Lot Number
R_2	11283923	11275782
R_3	11272996	11265489
R_4	11283927	11275781
R_5	11283931	11268129
R_6	11295869	11293269

D.3. Control Material

The negative control substance was leaf tissue of a conventional cotton variety (Coker 130) with a similar genetic background to MON 88701. The conventional control does not contain the MON 88701 DMO or PAT (*bar*) protein.

Description	Lot Number	Starting Seed Lot Number
Conventional Control	11272987	11266506

D.4. Characterization of MON 88701 and Control Materials

The identity of MON 88701 was confirmed by verifying the chain of custody documentation prior to analysis. A molecular fingerprint of MON 88701 was generated, further confirming the identity of the test substance, as well as the absence of the test substance in the control substance (Section V).

D.5. Reference Material

D.5.1. MON 88701 DMO Protein

The *E. coli*-produced MON 88701 DMO (lot 11287544) protein was used as the analytical reference standard for the western blot analysis.

D.5.2. PAT (*bar*) Protein

The *E. coli*-produced PAT (*bar*) (lot 11270310) protein was used as the analytical reference standard for the western blot analysis.

D.6. Methods

Cotton leaf tissue samples from MON 88701 collected across multiple generations were analyzed by western blot to demonstrate the presence of both MON 88701 DMO and PAT (*bar*) protein in all generations. Leaf tissue from the conventional control substance was analyzed by western blot to confirm the expected absence of the MON 88701 DMO and PAT (*bar*) proteins.

D.7. MON 88701 Tissue Processing

All samples were processed by the Monsanto Sample Management Team in St. Louis, Missouri. The processed tissue samples were stored in a -80° C freezer until transferred on dry ice to the analytical facility.

D.8. Extraction

D.8.1. MON 88701 DMO Protein

The MON 88701 DMO protein was extracted from processed leaf samples in 1 × Tris-Borate with L-Ascorbic Acid (TBA). All processed tissues were kept on dry ice during extract preparation. The MON 88701 DMO protein was extracted from the tissue by adding the appropriate volume of extraction buffer, and shaking in a Harbil mixer (Fluid Management, Wheeling, Illinois). Insoluble material was removed from the extracts by using a serum filter (Fisher Scientific, Pittsburgh, PA). The extracts were aliquoted and stored in a -80° C freezer until analysis.

D.8.2. PAT (*bar*) Protein

The PAT (*bar*) protein was extracted from processed leaf samples in 1 × TBA. All processed tissues were kept on dry ice during extract preparation. The PAT (*bar*) protein was extracted from the tissue by adding the appropriate volume of extraction buffer, and shaking in a Harbil mixer. Insoluble material was removed from the extracts by using a serum filter. The extracts were aliquoted and stored in a -80° C freezer until analysis.

D.9. SDS-PAGE

D.9.2. MON 88701 DMO Protein

Prior to analysis by SDS-PAGE and immunoblotting, all MON 88701 DMO and control sample extracts were diluted 1:10 (v/v) in Phosphate Buffered Saline w/ 0.05% Tween and 0.1% Bovine Serum Albumin (1× PBST + 0.1% BSA) then 1:2 using 2× Laemmli Buffer (Bio Rad, Hercules, CA) with β-mercaptoethanol for a final dilution of 1:20.

Standards were prepared by diluting the *E. coli*-produced MON 88701 DMO protein reference standard (lot 11287544) in 1× PBST + 0.1% BSA, in order to estimate the molecular weight of the purified protein. Sample extracts and 1 ng of protein reference standard were loaded on Novex 4-20% Tris-Glycine gradient gels (Invitrogen), along with the Precision Plus molecular weight marker (Bio Rad) to demonstrate the transfer of protein to membrane. Electrophoresis was conducted at 125 V for 90 min in 1× Novex Tris-Glycine SDS running buffer (Invitrogen) until the dye front reached the end of the gel.

D.9.1. PAT (*bar*) Protein

Prior to analysis by SDS-PAGE and immunoblotting, all PAT (*bar*) and control sample extracts were diluted 1:2 using 2× Laemmli Buffer with β-mercaptoethanol. Standards were prepared by diluting the *E. coli*-produced PAT (*bar*) protein reference standard (lot 11270310) 1× PBST + 0.1% BSA, in order to estimate show the molecular weight of the purified protein. Sample extracts and 0.5 ng of protein reference standards were loaded on Novex 4-20% Tris-Glycine gradient gels, along with the Precision Plus molecular weight marker to demonstrate the transfer of protein to membrane. Electrophoresis was conducted at 125 V for 90 min in 1× Novex Tris-Glycine SDS running buffer until the dye front reached the end of the gel.

D.10. Western Blot Analysis (Immunoblotting)

D.10.1. MON 88701 DMO Protein

Proteins separated by SDS-PAGE were electrophoretically transferred to a 0.45 µm nitrocellulose membrane (Bio Rad) using 1× Novex Tris-Glycine transfer buffer (Invitrogen) containing 20% methanol. After transfer, non specific sites on the membrane were blocked using 5% (w/v) non fat dried milk (NFDM, Bio Rad) in 1× PBST. The MON 88701 DMO specific membrane was probed for the presence of the MON 88701 DMO protein with a 1:1000 dilution of purified goat anti DMO antibody (lot G-844411) in 1× PBST with 1% (w/v) NFDM. The membrane was washed three times for 5 min each in 1× PBST to remove unbound antibody. Bound antibody was probed with a 1:5000 dilution of anti-goat IgG antibody conjugated to horseradish peroxidase (lot G-821342) in 1× PBST with 1% (w/v) NFDM. The membrane was washed three times for 5 min each in 1× PBST. The SuperSignal West Dura Extended Duration Substrate (Thermo Scientific Rockford, IL) was added to the membrane according to the manufacturer's instructions. Each membrane was exposed to Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) to generate an image of the immunoreactive bands.

D.10.2. PAT (*bar*) Protein

Proteins separated by SDS-PAGE were electrophoretically transferred to a 0.45 µm nitrocellulose membrane (Bio Rad) using 1× Novex Tris-Glycine transfer buffer (Invitrogen) containing 20% methanol. After transfer, non specific sites on the

membrane were blocked using 5% (w/v) NFDM, in 1× PBST. The PAT (*bar*) specific membrane was probed for the presence of the PAT (*bar*) protein with a 1:1000 dilution of purified goat anti PAT (*bar*) antibody (lot G-858911) in 1× PBST with 1% (w/v) NFDM. The membrane was washed three times for 5 min each in 1× PBST to remove unbound antibody. Bound antibody was probed with a 1:5000 dilution of anti-goat IgG antibody conjugated to horseradish peroxidase (lot G-821342) in 1× PBST with 1% (w/v) NFDM. The membrane was washed three times for 5 min each in 1× PBST. The SuperSignal West Dura Extended Duration Substrate was added to the membrane according to the manufacturer's instructions. Each membrane was exposed to Hyperfilm ECL to generate an image of the immunoreactive bands.

Appendix E: Bioinformatics Evaluation of MON 88701

E.1. Bioinformatics Evaluation of the MON 88701 DMO and PAT (*bar*) Proteins in MON 88701

E.1.1. Sequence Database Preparation

The allergen, gliadin, and glutenin sequence database (AD_2011) was obtained from FARRP (FARRP, 2011) and was used as provided. The AD_2011 database contains 1,491 sequences.

GenBank protein database, release 181.0 was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2011 database and contains 20,807,555 sequences.

The toxin database is a subset of sequences derived from the PRT_2011 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. It is referred to herein as the TOX_2011 database and contains 10,570 sequences.

E.1.2. Sequence Database Searches

FASTA analyses using the AD_2011, PRT_2011 and TOX_2011 databases were performed on a virtual machine loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The structural similarity of the translated protein sequences to sequences in each database (AD_2011, TOX_2011 and PRT_2011) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (k-tuple), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation 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 will need to have an *E*-score of 1×10^{-5} or less to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50%

identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of the MON 88701 DMO and PAT (*bar*) protein sequence to allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2011). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Goodman et al., 2002; Hileman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive

proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

E.1.3. Significance of the Alignment

An *E*-score of 1×10^{-5} was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than 1×10^{-5} was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2011 database were inspected to determine whether they exceeded the CODEX threshold of 35% or greater identity in 80 or greater amino acids amino acids.

E.2. Bioinformatics Evaluation of the Transfer DNA Insert in MON 88701

E.2.1. Methods

E.2.1.1. Database Assembly

The allergen, gliadin, and glutenin sequence database (AD_2011) was obtained from FARRP (FARRP, 2011) and was used as provided. The AD_2011 database contains 1,491 sequences.

GenBank protein database, release 181.0 was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2011 database and contains 20,807,555 sequences.

The toxin database is a subset of sequences derived from the PRT_2011 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. It is referred to herein as the TOX_2011 database and contains 10,570 sequences.

E.2.1.2. Translation of Query Sequences

The DNA insert sequence was translated beginning with nucleotide 1, 2 or 3 through the final nucleotide to yield frames 1, 2 or 3, respectively. Likewise, the reverse complement (anti-sense) strand of the above described sequence was translated beginning with nucleotide 1, 2 or 3 through the final nucleotide to yields frame 4, 5, or 6, respectively. All sequences were translated using standard genetic code with DNAStar, version 8.0.2 (13), 412. The resultant amino acid sequences were used to search the AD_2011, PRT_2011 and TOX_2011 databases.

E.2.1.3. Sequence Database Searches

FASTA analyses using the AD_2011, PRT_2011 and TOX_2011 databases were performed on a virtual machine loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The structural similarity of the translated

protein sequences to sequences in each database (AD_2011, TOX_2011 and PRT_2011) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

The structural similarity of the translated protein sequences to sequences in each database (AD_2011, TOX_2011 and PRT_2011) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (k-tuple), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation 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 will need to have an *E*-score of 1×10^{-5} or less to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of each putative polypeptide to allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2011). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Goodman et al., 2002; Hileman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

E.2.1.4. Significance of the Alignment

An *E*-score of 1×10^{-5} was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than 1×10^{-5} was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2011 database were inspected to determine whether they exceeded the CODEX threshold of 35% or greater identity in 80 or greater amino acids amino acids.

E.2.2. Bioinformatics Evaluation of DNA Sequences Flanking the 5′ and 3′ Junctions of Inserted DNA in MON 88701: Assessment of Putative Polypeptides

E.2.2.1. Sequence Database Preparation

The allergen, gliadin, and glutenin sequence database (AD_2011) was obtained from FARRP (FARRP, 2011)and was used as provided. The AD_2011 database contains 1,491 sequences.

GenBank protein database, release 181.0 was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2011 database and contains 20,807,555 sequences.

The toxin database is a subset of sequences derived from the PRT_2011 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. It is referred to herein as the TOX_2011 database and contains 10,570 sequences.

E.2.2.2. Translation of Putative Polypeptides

DNA sequence spanning the 5′ and 3′ junctions of the MON 88701 insertion site were analyzed for translational stop codons (TGA, TAG, TAA). All six reading frames originating or terminating within the MON 88701 T-DNA insert were translated using the standard genetic code from stop codon to stop codon using DNAStar, version 8.0.2 (13), 412. A total of nine unique sequences of eight amino acids or greater that spanned the 5′ and 3′ junctions were analyzed.

E.2.2.3. Sequence Database Searches

FASTA analyses using the AD_2011, PRT_2011 and TOX_2011 databases were performed on a desktop PC loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The DNA sequence was translated to the amino acid sequence with DNAStar, version 8.0.2 (13), 412 or SeqBuilder 8.0.2 (13). The structural similarity of the translated protein sequences to sequences in each database (AD_2011, TOX_2011, and PRT_2011) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (k-tuple), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation 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 will need to have an *E*-score of 1×10^{-5} or less to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992)was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50%

identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of each putative polypeptide to known allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2011). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Goodman et al., 2002; Hileman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight

contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

E.2.2.4. Significance of the Alignment

An *E*-score of 1×10^{-5} was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than 1×10^{-5} was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2011 database were inspected to determine whether they exceeded the CODEX threshold of 35% or greater identity in 80 or greater amino acids.

References for Appendix E

Aalberse, R.C. 2000. Structural biology of allergens. Journal of Allergy and Clinical Immunology 106:228-238.

Aalberse, R.C., J. Akkerdaas and R. van Ree. 2001. Cross-reactivity of IgE antibodies to allergens. Journal of Allergy and Clinical Immunology 56:478-490.

FARRP. 2011. Allergen database. University of Nebraska, Food Allergy Research and Resource Program, Lincoln, Nebraska. http://www.allergenonline.org/databasebrowse.shtml [Accessed February 14, 2011].

Gendel, S.M. 1998. The use of amino acid sequence alignments to assess potential allergenicity of proteins used in genetically modified foods. Advances in Food and Nurtition Research 42:45-62.

Goodman, R.E., A. Silvanovich, R.E. Hileman, G.A. Bannon, E.A. Rice and J.D. Astwood. 2002. Bioinformatic methods for identifying known or potential allergens in the safety assessment of genetically modified crops. Comments on Toxicology 8:251-269.

Henikoff, S. and J.G. Henikoff. 1992. Amino acid substitution matrices from protein blocks. Proceedings of the National Academy of Sciences of the United States of America 89:10915-10919.

Hileman, R.E., A. Silvanovich, R.E. Goodman, E.A. Rice, G. Holleschak, J.D. Astwood and S.L. Hefle. 2002. Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. International Archives of Allergy and Immunology 128:280-291.

Kleter, G.A. and A.A.C.M. Peijnenburg. 2002. Screening of transgenic proteins expressed in transgenic food crops for the presence of short amino acid sequences identical to potential, IgE-binding linear epitopes of allergens. BMC Structural Biology 2:8-18.

Lipman, D.J. and W.R. Pearson. 1985. Rapid and sensitive protein similarity searches. Science 227:1435-1441.

Metcalfe, D.D., J.D. Astwood, R. Townsend, H.A. Sampson, S.L. Taylor and R.L. Fuchs. 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Critical Reviews in Food Science and Nutrition 36:S165-S186.

Pearson, W.R. 2000. Flexible sequence similarity searching with the FASTA3 program package. Methods in Molecular Biology 132:185-219.

Pearson, W.R. and D.J. Lipman. 1988. Improved tools for biological sequence comparison. Proceedings of the National Academy of Sciences of the United States of America 85:2444-2448.

Silvanovich, A., M.A. Nemeth, P. Song, R. Herman, L.Tagliani and G.A. Bannon. 2006. The value of short amino acid sequence matches for prediction of protein allergenicity. Toxicological Sciences 90:252-258.

Stadler, M.B. and B.M. Stadler. 2003. Allergenicity prediction by protein sequence. FASEB Journal 17:1141-1143.

Thomas, K., G. Bannon, S. Hefle, C. Herouet, M. Holsapple, G. Ladics, S. MacIntosh and L. Privalle. 2005. In silico methods for evaluating human allergenicity to novel proteins: International Bioinformatics Workshop Meeting Report, 23-24 February 2005. Toxicological Sciences 88:307-310.

Appendix F: Methods Used in Assessing Stability of MON 88701 DMO and PAT (*bar*) Proteins in Simulated Digestive Fluids

F.1. Materials

F.1.1. MON 88701 DMO Protein

Purified *E. coli*-produced MON 88701 DMO protein (Lot 11300031) was used as the test substance. The *E. coli*-produced MON 88701 DMO protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM potassium phosphate, pH 8.0, 100 mM sodium chloride, 1 mM benzamidine-HCl, 1 mM DTT and 5% (v/v) Glycerol. The *E. coli*-produced MON 88701 protein has a purity of 88% and a total protein concentration of 0.82 mg/ml.

Simulated gastric fluid (SGF) contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH of 1.2. The SGF was prepared using a highly purified form of pepsin (catalog number P 6887, Sigma Company, St. Louis, MO).

F.1.2. PAT (*bar*) Protein

Purified *E. coli*-produced PAT (*bar*) protein (Lot 11270310) was used as the test substance. The *E. coli*-produced PAT (*bar*) protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 0.16 M sodium chloride, 20% (v/v) glycerol. The *E. coli*-produced PAT (*bar*) protein has a purity of 99% and a concentration of 1.0 mg/ml.

Simulated gastric fluid (SGF) contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH of 1.2. The SGF was prepared using a highly purified form of pepsin (catalog number P 6887, Sigma, St. Louis, MO).

F.2. Digestion of MON 88710 DMO and PAT (*bar*) Proteins in Simulated Gastric Fluid (SGF) Method

F.2.1. MON 88701 DMO Protein

Digestions were initiated by addition of *E. coli*-produced MON 88701 DMO to tubes containing SGF, where 10 units of pepsin activity were used per 1 µg of total protein. Digestions were incubated at ~37 °C in separate tubes for various durations, and the reactions were quenched by addition of a sodium carbonate solution to each tube. The zero incubation time point (T = 0) was quenched by addition of sodium carbonate solution to SGF prior to addition of the *E. coli*-produced MON 88701 DMO. The SGF was assayed before conducting the timed incubations to demonstrate that pepsin was active. Experimental controls were prepared to demonstrate the stability of *E. coli*-produced MON 88701 DMO in the system without pepsin. These controls were incubated for 0 and 60 min and were designated with the letter "P" (SGF P0 and SGF P7, respectively). Additionally, experimental controls to characterize the system without *E. coli*-produced MON 88701 DMO were also included. These experimental controls

were prepared by substituting buffer (50 mM potassium phosphate, pH 8.0, 100 mM sodium chloride, 1 mM benzamidine-HCl, 1 mM DTT and 5% (v/v) glycerol) for *E. coli*-produced MON 88701 DMO. These controls were incubated for 0 and 60 min and were designated with the letter "N" (SGF N0 and SGF N7).

All quenched specimens were heated to 99 °C for 5 min, frozen on dry ice and stored in a –80 ºC freezer until analyzed. The digestibility of *E. coli*-produced MON 88701 DMO in SGF was assessed using SDS-PAGE gel followed by Brilliant Blue G Colloidal dye (Sigma, St. Louis, MO) staining and immunoblotting. Limits of detection (LOD) were determined for the gel staining and immunoblot methods.

F.2.2. PAT (*bar*) Protein

Digestions were initiated by addition of *E. coli*-produced PAT (*bar*) to tubes containing simulated gastric fluid (SGF), where 10 units of pepsin activity were used per 1 µg of total protein. Digestions were incubated at ~37 °C in separate tubes for various durations, and the reactions were quenched by addition of a sodium carbonate solution to each tube. The zero incubation time point (T = 0) was quenched by addition of sodium carbonate solution to SGF prior to addition of the *E. coli*-produced PAT (*bar*). The SGF was assayed before conducting the timed incubations to demonstrate that pepsin was active. Experimental controls were prepared to demonstrate the stability of *E. coli*-produced PAT (*bar*) in the system without pepsin. These controls were incubated for 0 and 60 min and were designated with the letter "P" (SGF P0 and SGF P7, respectively). Additionally, experimental controls to characterize the system without *E. coli*-produced PAT (*bar*) were also included. These experimental controls were prepared by substituting buffer (50 mM Tris-HCl, pH 7.5, 0.16 M sodium chloride, 20% (v/v) glycerol) for *E. coli*-produced PAT (*bar*). These controls were incubated for 0 and 60 min and were designated with the letter "N" (SGF N0 and SGF N7).

All quenched specimens were heated to 95-100 °C for 5-10 min and stored in a –80 ºC freezer until analyzed. The digestibility of *E. coli*-produced PAT (*bar*) in SGF was assessed using SDS-PAGE gel followed by Brilliant Blue G Colloidal dye (Sigma, St. Louis, MO) staining and immunoblotting. Limits of detection (LOD) were determined for the gel staining and immunoblot methods.

F.3. Digestion of MON 88701 DMO and PAT (*bar*) Proteins in Simulated Intestinal Fluid (SIF) Method

F.3.1. MON 88701 DMO Protein

Digestions were initiated by addition of *E. coli*-produced MON 88701 DMO to tubes containing SIF, where 55.3 µg of pancreatin were used per 1 µg of total protein. Digestions were incubated at ~37°C in separate tubes for various durations, and the reactions were quenched by addition of 5× LB, heated to 95-100 ºC for 5-10 min, and frozen on dry ice. Zero incubation time points (T = 0) were quenched by addition of 5× LB, heated to 95-100 ºC for 5-10 min to SIF prior to addition of *E. coli*-produced

MON 88701 DMO. The SIF was assayed before conducting the timed incubations to demonstrate that pancreatin was active.

Experimental controls were prepared to demonstrate the stability of *E. coli*-produced MON 88701 DMO in the system without pancreatin. These controls were incubated for 0 min and 24 h and were designated with the letter "P" (SIF P0 and SIF P8). Additionally, experimental controls to characterize the system without *E. coli*-produced MON 88701 DMO were also included. These experimental controls were prepared by substituting buffer (50 mM potassium phosphate, pH 8.0, 100 mM sodium chloride, 1 mM benzamidine-HCl, 1 mM DTT 5% Glycerol) for *E. coli*-produced MON 88701 DMO, and were designated with the letter "N" (SIF N0 and SIF N8).

All quenched specimens, were heated to 95-100 ºC for 5-10 min, frozen on dry ice, and stored in a -80 ºC freezer until analyzed. The digestibility of purified *E. coli*-produced MON 88701 DMO in SIF was assessed using western blotting. Limits of detection (LOD) were determined for the western blot methods.

F.3.2. PAT (*bar*) Protein

Digestions were initiated by addition of *E. coli*-produced PAT (*bar*) to tubes containing simulated intestinal fluid (SIF), where 55.3 µg of pancreatin were used per 1 µg of total protein. Digestions were incubated at ~37°C in separate tubes for various durations, and the reactions were quenched by addition of 5× LB, heated to 95-100 ºC for 5-10 min, and frozen on dry ice. Zero incubation time points (T = 0) were quenched by addition of 5× LB, heated to 95-100 ºC for 5-10 min to SIF prior to addition of *E. coli*-produced PAT (*bar*). The SIF was assayed before conducting the timed incubations to demonstrate that pancreatin was active

Experimental controls were prepared to demonstrate the stability of *E. coli*-produced PAT (*bar*) in the system without pancreatin. These controls were incubated for 0 min and 24 h and were designated with the letter "P" (SIF P0 and SIF P8). Additionally, experimental controls to characterize the system without *E. coli*-produced PAT (*bar*) were also included. These experimental controls were prepared by substituting buffer (50 mM Tris, pH 7.5, 0.16 M sodium chloride, 20% (v/v) glycerol) for *E. coli*-produced PAT (*bar*), and were designated with the letter "N" (SIF N0 and SIF N8).

All quenched specimens, were heated to 95-100 ºC for 5-10 min, frozen on dry ice, and stored in a -80 ºC freezer until analyzed. The digestibility of purified *E. coli*-produced PAT (*bar*) in SIF was assessed using western blotting. Limits of detection (LOD) were determined for the western blot methods.

Appendix G: Heat Stability of MON 88701 DMO and PAT (*bar*) Protein

The previously characterized *E. coli*-produced proteins were used as both the test substance and reference protein. As reference protein, *E. coli*-produced proteins were maintained at -80 °C until the heat treatment samples were ready for analysis. The reference protein was evaluated along with the heat treatment samples in the functional assay and the SDS-PAGE analysis.

G.1. Heat Treatment

G.1.1. MON 88701 DMO Protein

The *E. coli*-produced MON 88701 DMO protein (lot 11300031) was thawed on wet ice and diluted in 50 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl 100 mM sodium chloride, 5% glycerol to a final total protein concentration of 0.82 mg/ml. Aliquots of 100 µl of the diluted DMO protein were transferred to eleven tubes. The eleven aliquots in tubes were maintained on wet ice until the heat treatments were initiated.

Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95 °C, each ± 2 °C) and incubated for 15 ± 1 min. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95 °C, each ± 2 °C) and incubated for 30 ± 1 min. The eleventh tube, a control treatment, were maintained on wet ice throughout the heat treatment incubation period. All temperature-treated samples were returned immediately to wet ice following the incubation period.

Following the heat treatments, 40 µl of each temperature treated sample (including the control treatment) was transferred to a clean tube and mixed with 10 µl of 5X LB (0.312 M Tris HCl, pH 6.8, 10% SDS, 50% glycerol, 3.6 M 2-mercaptoethanol, 0.025% Bromophenol Blue) in preparation for SDS-PAGE analysis. The 50 µl samples were heated at 95 ± 5 °C for 3-5 min, quick frozen by placement on dry ice, and stored at -80 °C until analysis. The remainder of each temperature-treated sample (approximately 60 µl each) was maintained on wet ice and used for functional activity assessment.

G.1.2. PAT (*bar*) Protein

The *E. coli*-produced PAT (*bar*) protein (lot 11270310) was thawed on wet ice and diluted in 50 mM Tris HCl, pH 7.5, 0.16 M sodium chloride, 20% glycerol to a final total protein concentration of 1.0 mg/ml. Aliquots of 100 µl of the diluted PAT (*bar*) protein were transferred to eleven tubes. The eleven aliquots in tubes were maintained on wet ice until the heat treatments were initiated. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95 °C, each ± 2 °C) and incubated for 15 ± 1 min. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95 °C, each ± 2 °C) and incubated for 30 ± 1 min. The eleventh tube, a control treatment, were maintained on wet ice throughout the heat treatment incubation period. All

temperature-treated samples were returned immediately to wet ice following the incubation period.

Following the heat treatments, 40 µl of each temperature treated sample (including the control treatment) was transferred to a clean tube and mixed with 10 µl of 5X LB (0.312 M Tris HCl, pH 6.8, 10% SDS, 50% glycerol, 3.6 M 2-mercaptoethanol, 0.025% Bromophenol Blue) in preparation for SDS-PAGE analysis. The 50 µl samples were heated at 95 ± 5 °C for 3-5 min, quick frozen by placement on dry ice, and stored at -80 °C until analysis. The remainder of each temperature-treated sample (approximately 60 µl each) was maintained on wet ice and used for functional activity assessment.

G.2. Functional Activity Assay

G.2.1. MON 88701 DMO Protein

The DMO functional activity of the heat treated samples, the control treatment sample, and the reference protein were determined using the functional activity assay described in Appendix B, Section B.5.6.1. All samples were at a total protein concentration of 0.82 mg/ml in 50 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl 100 mM sodium chloride, 5% glycerol prior to analysis. Three replicates of each diluted protein sample were used for the analysis.

G.2.2. PAT (*bar*) Protein

The PAT (*bar*) functional activity of the heat treated samples, the control treatment sample, and the reference protein were determined using the functional activity assay described in Appendix B, Section B.5.6.2. All samples were diluted to a total protein concentration of 1 ng/µl in 50 mM Tris, pH 7.5, and 0.5 mM EDTA prior to analysis. Three replicates of each diluted protein sample were used for the analysis.

G.3. SDS-PAGE

G.3.1. MON 88701 DMO Protein

The samples prepared above (Section G.1.1) for SDS-PAGE analysis, were thawed, heated at 95 ± 5 °C for 3-5 min, and loaded on one 4-20% polyacrylamide gradient gel at 3.3 µg total protein per lane. The reference protein was loaded on the same gel at 3.3 µg total protein per lane (100% reference protein equivalent) and at 0.33 µg total protein per lane (10% reference protein equivalent). Following electrophoresis, gels were stained with Brilliant Blue G Colloidal (Sigma, St. Louis, MO).

After staining, the stability of DMO at each heat treatment was evaluated qualitatively. The intensity of the major protein band at ~38 kDa in the heat treatment lanes was compared visually to the same band in the lanes with the control treatment, 100% reference protein equivalent, and 10% reference protein equivalent.

G.3.2. PAT (*bar*) Protein

The samples prepared above (Section G.1.2) for SDS-PAGE analysis, were thawed, heated at 95 ± 5 °C for 3-5 min, and loaded on one 4-20% polyacrylamide gradient gel at 3 µg total protein per lane. The reference protein was loaded on the same gel at 3 µg total protein per lane (100% reference protein equivalent) and at 0.3 µg total protein per lane (10% reference protein equivalent). Following electrophoresis, gels were stained with Brilliant Blue G Colloidal (Sigma, St. Louis, MO).

After staining, the stability of PAT (*bar*) at each heat treatment was evaluated qualitatively. The intensity of the major protein band at ~25 kDa in the heat treatment lanes was compared visually to the same band in the lanes with the control treatment, 100% reference protein equivalent, and 10% reference protein equivalent.

Appendix H: Materials, Methods, and Individual Site Results for Compositional Analysis of MON 88701 Cottonseed

H.1. Materials

Cottonseed from MON 88701 (Seed Lot Number 11268129) and the conventional control (Seed Lot Number 11268128) was evaluated. The conventional control has background genetics similar to that of MON 88701 but does not contain either the dicamba mono-oxygenase (DMO) or phosphinothricin N-acetyltransferase (PAT) proteins. The commercial reference varieties were nine conventional cotton varieties (Table H-1).

Table H-1. Commercial Reference Varieties

Material Name	Seed Lot No.	Field Sites[1]
SG 125	11266155	ARTI, SCEK, NCBD, TXPL
DP 435	11266762	ARTI, NCBD, TXPL
DP 5415	11266157	ARTI, LACH, KSLA
FM 989	10001810	ARTI, LACH, GACH
Delta Opal	11266158	SCEK, GACH, NCBD
Atlas	11266765	SCEK, TXPL, KSLA, NMLC
ST 474	11266156	GACH, LACH, NCBD, NMLC, SCEK
DP 565	11266764	GACH, LACH, KSLA, NMLC
NM 1517-99	11268233	TXPL, KSLA

[1]Field sites described in Section VII.A.

H.2. Characterization of the Materials

The identities of MON 88701, the conventional control, and commercial reference varieties were confirmed by verifying the chain of custody documentation prior to analysis. To further confirm the identities of MON 88701, the conventional control, and commercial reference varieties, event-specific polymerase chain reaction (PCR) analyses were conducted on the harvested, acid-delinted cottonseed from each site to confirm the presence or absence of the MON 88701 event.

H.3. Field Production of the Samples

Cottonseed samples were collected from MON 88701 and the conventional control Coker 130 grown in a 2010 U.S. field production. Four different conventional cotton varieties, known as reference substances, were included at each site of the field production to provide data on natural variability of each compositional component analyzed. The field production was conducted at eight sites: Arkansas (ARTI), Georgia (GACH), Kansas (KSLA), Louisiana (LACH), North Carolina (NCBD), New Mexico (NMLC), South Carolina (SCEK) and Texas (TXPL). The sites were planted in a

randomized complete block design with four blocks per site. MON 88701 plots were treated at the 3-5 leaf stage with glufosinate herbicide at the label rate (0.5 lb a.e. /acre) and at the 6-10 leaf stage with dicamba herbicide at the label rate (0.5 lb a.e./acre). T/C/R substances were grown under normal agronomic field conditions for their respective geographic regions. Cottonseed samples were harvested and ginned from all plots and shipped at ambient temperature to Monsanto Company (St. Louis, Missouri). The samples were acid-delinted and a subsample was obtained from each for compositional analyses. These subsamples were ground and stored in a freezer set to maintain -20°C until their shipment on dry ice to Covance Laboratories Inc. (Madison, Wisconsin) for analysis. The label on the samples shipped listed the protocol (study) number, tissue type, material name, storage conditions, and a unique sample ID number.

H.4. Summary of Analytical Methods

Harvested, acid-delinted cottonseed samples were analyzed by Covance Laboratories Inc. Upon receipt, the samples were stored in a freezer set to maintain -20 °C until their use. Nutrients assessed in this analysis included proximates (ash, fat, moisture, protein, and carbohydrates and calories by calculation), acid detergent fiber (ADF), neutral detergent fiber (NDF), crude fiber (CF), total dietary fiber (TDF), amino acids (AA), fatty acids (C8-C22), minerals (Ca, Cu, Fe, Mg, Mn, P, K, Na, Zn) and vitamin E (α-tocopherol). Anti-nutrients analyzed included gossypol and cyclopropenoid fatty acids (CPFA).

H.4.1. Acid Detergent Fiber

The ANKOM2000 Fiber Analyzer automated the process of removal of proteins, carbohydrates, and ash. Fats and pigments were removed with an acetone wash prior to analysis. The fibrous residue that is primarily cellulose, lignin, and insoluble protein complexes remained in the Ankom filter bag, and were determined gravimetrically. (Komarek, et al., 1993; USDA, 1970). The results were reported on fresh weight basis. The limit of quantitation was 0.100%.

H.4.2. Amino Acid Composition

The following 18 amino acids were analyzed:

Total threonine	Total aspartic acid (including asparagine)
Total serine	Total tyrosine
Total phenylalanine	Total glutamic acid (including glutamine)
Total proline	Total histidine
Total glycine	Total lysine
Total alanine	Total arginine
Total valine	Total tryptophan
Total isoleucine	Total methionine
Total leucine	Total cystine (including cysteine)

The samples were hydrolyzed in 6N hydrochloric acid for approximately 24 hours at approximately 106-110ºC. Phenol was added to the 6N hydrochloric acid to prevent halogenation of tyrosine. Cystine and cysteine are converted to S-2-carboxyethylthiocysteine by the addition of dithiodipropionic acid. Tryptophan was hydrolyzed from proteins by heating at approximately 110ºC in 4.2N sodium hydroxide for 20 hours. The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids are derivatized with fluorenylmethyl chloroformate (FMOC) before injection. (AOAC, 2011a; Barkholt and Jensen, 1989; [redacted] and Brooks, 2010., [redacted] et al, 2000; Schuster, 1988). The results were reported on fresh weight basis. The limit of quantitation was 0.100 mg/g.

Reference Standards:

Component	Manufacturer	Lot No.	Purity (%)
L-Alanine	Sigma-Aldrich	1440397	99.9
L-Arginine Monohydrochloride	Sigma-Aldrich	1361811	100
L-Aspartic Acid	Sigma-Aldrich	BCBB9274	100.6
L-Cystine	Sigma-Aldrich	1418036	99.9
L-Glutamic Acid	Sigma-Aldrich	1423805	100.2
Glycine	Sigma-Aldrich	1119375	100
L-Histidine Monohydrochloride Monohydrate	Sigma-Aldrich	BCBB1348	99.9
L-Isoleucine	Sigma-Aldrich	1423806	100
L-Leucine	Sigma-Aldrich	BCBB1733	98.6
L-Lysine Monohydrochloride	Sigma-Aldrich	1362380	100.2
L-Methionine	Sigma-Aldrich	1423807	99.9
L-Phenylalanine	Sigma-Aldrich	BCBB9200	100
L-Proline	Sigma-Aldrich	1414414	99.7
L-Serine	Sigma-Aldrich	1336081	99.9
L-Threonine	Sigma-Aldrich	1402329	100
L-Tryptophan	Sigma-Aldrich	BCBB1284	99.8
L-Tyrosine	Sigma-Aldrich	BCBB5393	99.5
L-Valine	Sigma-Aldrich	1352709	100

H.4.3. Ash

The sample was placed in an electric furnace at 550 °C and ignited. The nonvolatile matter remaining was quantified gravimetrically and calculated to determine percent ash (AOAC, 2011b). The limit of quantitation was 0.100%.

H.4.4. Calories

Calories were calculated using the Atwater factors with the fresh weight-derived data and the following equation:

$$\text{calories (Kcal/100g)} = (4 \times \% \text{ protein}) + (9 \times \% \text{ fat}) + (4 \times \% \text{ carbohydrates})$$

The limit of quantitation was calculated as 2.00 Kcalories/100g on a fresh weight basis (Code of Federal Regulation, Title 21, Part 101.9, pp. 24-25).

H.4.5. Carbohydrates

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation:

$$\% \text{ carbohydrates} = 100\% - (\% \text{ protein} + \% \text{ fat} + \% \text{ moisture} + \% \text{ ash})$$

The results were reported on fresh weight basis (USDA, 1973). The limit of quantitation was 0.100%.

H.4.6. Crude Fiber

Crude fiber was quantitated as the loss on ignition of dried residue remaining after digestion of the sample with 1.25% sulfuric acid and 1.25% sodium hydroxide solutions under specific conditions (AOAC, 2011c). The results were reported on fresh weight basis. The limit of quantitation was 0.100%.

H.4.7. Fat by Soxhlet Extraction

The sample was weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was then evaporated, dried, and weighed (AOAC, 2011d; e). The results were reported on fresh weight basis. The limit of quantitation was 0.100%.

H.4.8. Cyclopropenoid Fatty Acids

The total lipid fraction was extracted from the sample using chloroform and methanol. A portion of the lipid fraction was then saponified with a mild alkaline hydrolysis. The free fatty acids were extracted with ethyl ether and hexane. The free fatty acids were then converted to their phenacyl derivatives with 2-bromoacetophenone. The derivatives were quantitated on a high-performance liquid chromatography system equipped with an ultraviolet detector. The amount of malvalic, sterculic and dihydrosterculic acids were determined by comparison to an external calibration curve of similarly derivatized reference standards (Wood, 1986). The results were expressed on a fresh weight basis. The limit of quantitation was 50.0 µg/g.

Reference Standards:

- Monsanto, Malvalic Acid, 100%, Lot Number GLP-0208-12964-A
- Monsanto, Sterculic Acid, 99%, Lot Number GLP-0208-12963-A
- Monsanto, Dihydrosterculic, 98%, Lot Number GLP-0311-14467-A

H.4.9. Fatty Acids

The lipid was extracted and saponified with 0.5 N methanolic sodium hydroxide, and methylated with 14% boron trifluoride in methanol. The resulting methyl esters of the fatty acids were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation (AOCS, 1997; 2001; 2009a; c). The results were reported on fresh weight basis. The limit of quantitation was 0.0200%.

Reference Standards:

Component	Lot Number	Component	Weight (%)	Purity (%)
Nu-Chek Prep GLC Reference Standard Hazelton No. 1	JY20-U	Methyl Octanoate	16.66	99.6
		Methyl Decanoate	16.66	99.6
		Methyl Laurate	16.66	99.8
		Methyl Myristate	16.66	99.8
		Methyl Palmitoleate	16.66	99.7
		Methyl Linolenate	16.66	99.5
Nu-Chek Prep GLC Reference Standard Hazelton No. 2	AU16-U	Methyl Arachidate	33.33	99.6
		Methyl 11-Eicosenoate	33.33	99.5
		Methyl Arachidonate	33.33	99.6
Nu-Chek Prep GLC Reference Standard Hazelton No. 3	J28-U	Methyl Myristoleate	12.5	99.5
		Methyl Pentadecanoate	12.5	99.6
		Methyl 10-Pentadecenoate	12.5	99.5
		Methyl Heptadecanoate	12.5	99.6
		Methyl 10-Heptadecenoate	12.5	99.5
		Methyl 11-14 Eicosadienoate	12.5	99.6
		Methyl Behenate	12.5	99.8
		Methyl 11-14-17 Eicosatrienoate	12.5	99.5
Nu-Chek Prep GLC Reference Standard Hazelton No. 3	F15-V	Methyl Myristoleate	12.5	99.5
		Methyl Pentadecanoate	12.5	99.6
		Methyl 10-Pentadecenoate	12.5	99.5
		Methyl Heptadecanoate	12.5	99.6
		Methyl 10-Heptadecenoate	12.5	99.5
		Methyl 11-14 Eicosadienoate	12.5	99.6
		Methyl Behenate	12.5	99.8
		Methyl 11-14-17 Eicosatrienoate	12.5	99.5
Nu-Chek Prep GLC Reference Standard Hazelton No. 4	MA30-U	Methyl Palmitate	27.0	99.6
		Methyl Stearate	19.0	99.5
		Methyl Oleate	27.0	99.8
		Methyl Linoleate	27.0	99.8
Nu-Chek Prep GLC Reference Standard Hazelton No. 4	JA31-V	Methyl Palmitate	27.0	99.7
		Methyl Stearate	19.0	99.7
		Methyl Oleate	27.0	99.8
		Methyl Linoleate	27.0	99.8
Nu-Chek Prep Methyl Gamma Linolenate	U-63M-M18-U	Not applicable	Not applicable	>99
Nu-Chek Prep Methyl Gamma Linolenate	U-63M-N2-U	Not applicable	Not applicable	>99

Nu-Chek Prep Methyl Tridecanoate	N-13M-F16-V	Not applicable	Not applicable	>99
Nu-Chek Prep Methyl Tridecanoate	N-13M-MA25-T	Not applicable	Not applicable	>99

H.4.10. Free and Total Gossypol

For free gossypol, the sample was extracted with aqueous acetone. The solution was then filtered and the free gossypol was reacted with aniline. For total gossypol analysis, the sample was extracted using a complexing reagent containing acetic acid, 3-amino-1-propanol, and dimethylformamide. The solution was then filtered and the total gossypol was reacted with aniline. For both analyses, the dianilinogossypol was quantitated spectrophotometrically using a standard curve (AOCS, 2011a; b) The results were reported on fresh weight basis. The limit of quantitation was 0.00200%.

Reference Standard:

- Sigma-Aldrich, Gossypol, 97.7%, Lot Number 059K4046

H.4.11. ICP Emission Spectrometry

The sample was dried, precharred, and ashed overnight in a muffle furnace set to maintain 500 °C. The ashed sample was re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions (AOAC, 2011f; g) The results were reported on fresh weight basis.

Inorganic Ventures Reference Standards and Limits of Quantitation:

Reference Standards:

Mineral	Lot Numbers	Concentration (µg/mL)	Limit of Quantitation (ppm)
Calcium	E2-MEB360079MCA, E2-MEB360081	200, 1000	20.0
Copper	E2-MEB360079MCA, E2-MEB360080MCA	2.00, 10.0	0.500
Iron	E2-MEB360079MCA, E2-MEB360082	10.0, 50.0	2.00
Magnesium	E2-MEB360079MCA, E2-MEB360080MCA	50.0, 250	20.0
Manganese	E2-MEB360079MCA, E2-MEB360080MCA	2.00, 10.0	0.300
Phosphorus	E2-MEB360079MCA, E2-MEB360081	200, 1000	20.0
Potassium	E2-MEB360079MCA, E2-MEB360081	200, 1000	100
Sodium	E2-MEB360079MCA, E2-MEB360081	200, 1000	100
Zinc	E2-MEB360079MCA, E2-MEB360080MCA	10.0, 50.0	0.400

H.4.12. Moisture

The sample was dried in a vacuum oven at approximately 100 °C to a constant weight. The moisture weight loss was determined and converted to percent moisture (AOAC, 2011h, i). The results were reported on fresh weight basis. The limit of quantitation was 0.100%.

H.4.13. Neutral Detergent Fiber, Enzyme Method

The ANKOM2000 Fiber Analyzer automated the process of the removal of proteins, carbohydrates, and ash. The fats and pigments were removed with an acetone wash prior to analysis. Hemicellulose, cellulose, lignin and insoluble protein fraction were left in the filter bag and determined gravimetrically (AACC, 1999; Komarek et al., 1994; USDA, 1970). The results were reported on fresh weight basis. The limit of quantitation was 0.100%.

H.4.14. Protein

The protein and other organic nitrogen in the sample were converted to ammonia by digesting the sample with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25 (AOAC, 2011j; k; AOCS, 2009b) The results were reported on fresh weight basis. The limit of quantitation was 0.100%.

H.4.15. Total Dietary Fiber

Duplicate samples were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber. The sample was filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated using protein and ash values (AOAC, 2011l). The results were reported on fresh weight basis. The limit of quantitation was 1.00%.

H.4.16. Vitamin E

The sample was saponified to break down any fat and release vitamin E. The saponified mixture was extracted with ethyl ether and then quantified by high-performance liquid chromatography using a silica column (Cort, et al., 1983; McMurray, et al., 1980; Speek, et al., 1985). The results were reported on fresh weight basis. The limit of quantitation was 0.500 mg/100g.

Note: Alpha tocopherol is part of a mixed standard which also includes beta, delta, and gamma isomers. The reference standard material for those isomers may contain small amounts of alpha tocopherol. All reference standards that contributed to the alpha tocopherol concentration are listed below.

Reference Standards:

- USP, Alpha Tocopherol, 98.9%, Lot Number N0F068
- Acros Organics, D-gamma-Tocopherol, 99.4%, A0083534
- Sigma-Aldrich, (+)-delta-Tocopherol, 92%, 090M1916V

H.5. Data Processing and Statistical Analysis

After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Monsanto Company for review. Data were then transferred to Certus International (Chesterfield, MO) where they were converted into the appropriate units and statistically analyzed. The formulas that were used for re-expression of composition data for statistical analysis are listed in Table H-2.

Table H-2. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)	To	Formula[1]
Proximates (excluding Moisture and Calories), Fiber, Gossypol	% fw	% dw	X/d
Calories	Kcal/100g fw	Kcal/100g dw	X/d
Copper, Iron, Manganese, Zinc	ppm fw	mg/kg dw	X/d
Calcium, Magnesium, Phosphorus, Potassium, Sodium	ppm fw	% dw	$X/(10^4 d)$
Vitamin E	mg/100g fw	mg/kg dw	10(X/d)
Amino Acids (AA)	mg/g fw	% dw	X/(10d)
Sterculic, Malvalic, and Dihydrosterculic Acids[2]	µg/g fw	% fw	$X/10^4$
Fatty Acids (FA)	% fw	% Total FA	$(100)X_j/\Sigma X$, for each FA_j where ΣX is over all the FA

[1]d is the fraction of the sample that is dry matter.
[2]Sterculic Acid, Malvalic Acid and Dihydrosterculic Acid were first converted to % fw as an intermediate step for final re-expression as % Total FA.

In order to complete a statistical analysis for a compositional component in this study, at least 50% of the values for a component had to be greater than the assay limit of quantitation (LOQ). Components with more than 50% of observations below the assay LOQ were excluded from summaries and analysis. The following 13 components with more than 50% of the observations below the assay LOQ were excluded: 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma-linolenic acid, 20:1 eicosenoic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid and 20:4 arachidonic acid.

If less than 50% of the observations for a component were below the LOQ, individual analyses that were below the LOQ were assigned a value equal to one-half the LOQ. In this study 187 values for 22:0 behenic acid were assigned a value of 0.010% fw and 187 values for sodium were assigned a value of 50.00 ppm fw.

The data were assessed for potential outliers using a studentized PRESS residuals calculation. A PRESS residual is the difference between any value and its value predicted 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. One sodium value from the commercial control at the ARTI site and one sodium value from a commercial reference at the ARTI site were extreme data points that were outside the ± 6 studentized PRESS residual range and were removed from the statistical analysis.

All cottonseed components were statistically analyzed using a mixed model analysis of variance. The eight replicated field sites were analyzed individually and as a combined data set. Individual site analysis mean comparison tests were not conducted on site ARTI sodium content because only one Coker 130 replicate was available at that site.

Analyses of the combined replicated sites were performed using model (1).

(1) $Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk,}$

where Y_{ijk} = unique individual observation, U = overall mean, T_i = substance effect, L_j = random site effect, $B(L)_{jk}$ = random block within site effect, LT_{ij} = random site by substance interaction effect, and e_{ijk} = residual error.

Individual sites were also analyzed separately. Individual site analyses were performed using model (2).

(2) $Y_{ij} = U + T_i + B_j + e_{ij},$

where Y_{ij} = unique individual observation, U = overall mean, T_i = substance effect, B_j = random block effect, and e_{ij} = residual error.

For each compositional component, a range of observed values and a 99% tolerance interval were calculated. 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. The calculated tolerance intervals are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of conventional cotton. Each tolerance interval estimate was based upon the average observation for each unique reference material. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

SAS® (Version 9.2) 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.

Table H-3. Statistical Summary of Site ARTI Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.62 (0.087) (4.51 - 4.74)	4.74 (0.12) (4.49 - 5.00)	-0.13 (0.15) (-0.49 - 0.047)	-0.55, 0.29	0.440	3.42, 4.65 (3.18 - 4.68)
Calories	495.41 (3.00) (487.88 - 504.08)	488.30 (3.88) (487.70 - 494.60)	7.11 (3.90) (4.92 - 16.38)	-3.73, 17.94	0.142	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	45.08 (0.58) (43.42 - 46.31)	46.35 (0.81) (45.03 - 47.37)	-1.27 (0.96) (-3.95 - -0.33)	-3.94, 1.39	0.254	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	7.10 (0.27) (6.71 - 7.58)	7.63 (0.35) (7.32 - 7.40)	-0.53 (0.35) (-0.69 - -0.36)	-1.49, 0.43	0.197	4.79, 9.92 (6.05 - 10.50)
Protein	27.53 (0.24) (27.16 - 28.11)	27.04 (0.33) (26.97 - 27.11)	0.49 (0.41) (0.12 - 0.65)	-0.65, 1.63	0.297	22.30, 29.41 (20.58 - 29.28)
Total Fat	22.76 (0.59) (21.32 - 24.40)	21.50 (0.78) (21.15 - 22.89)	1.26 (0.81) (0.69 - 3.25)	-0.98, 3.50	0.193	15.01, 28.51 (16.58 - 25.25)

Table H-3. Statistical Summary of Site ARTI Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Acid Detergent Fiber	24.81 (0.32) (24.44 - 25.20)	27.53 (0.45) (26.57 - 28.49)	-2.71 (0.55) (-3.98 - -2.13)	-4.23, -1.20	0.007	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	18.33 (0.90) (15.97 - 20.56)	19.47 (1.20) (19.33 - 19.85)	-1.14 (1.26) (-3.36 - -0.40)	-4.64, 2.36	0.417	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	31.27 (0.79) (29.99 - 32.89)	32.89 (1.06) (30.67 - 34.42)	-1.61 (1.13) (-4.43 - 0.095)	-4.75, 1.53	0.227	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	40.85 (1.06) (39.82 - 42.13)	41.67 (1.50) (40.50 - 42.84)	-0.82 (1.83) (-0.70 - 1.09)	-5.91, 4.27	0.678	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.05 (0.015) (1.01 - 1.09)	1.02 (0.018) (0.99 - 1.05)	0.029 (0.017) (0.011 - 0.036)	-0.018, 0.077	0.161	0.86, 1.11 (0.83 - 1.22)
Arginine	3.00 (0.052) (2.86 - 3.07)	3.02 (0.073) (2.89 - 3.13)	-0.018 (0.084) (-0.098 - -0.032)	-0.25, 0.21	0.840	2.38, 3.47 (2.30 - 3.55)

Table H-3. Statistical Summary of Site ARTI Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.41 (0.045) (2.29 - 2.48)	2.32 (0.060) (2.19 - 2.42)	0.087 (0.065) (-0.031 - 0.10)	-0.093, 0.27	0.252	1.94, 2.57 (1.79 - 2.72)
Cystine	0.40 (0.010) (0.38 - 0.42)	0.37 (0.014) (0.35 - 0.39)	0.034 (0.017) (0.014 - 0.074)	-0.015, 0.082	0.124	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.82 (0.099) (4.61 - 5.07)	4.51 (0.14) (4.34 - 4.67)	0.31 (0.17) (0.11 - 0.27)	-0.15, 0.78	0.134	3.74, 5.28 (3.39 - 5.45)
Glycine	1.10 (0.020) (1.05 - 1.14)	1.08 (0.026) (1.03 - 1.11)	0.027 (0.028) (-0.015 - 0.021)	-0.052, 0.11	0.397	0.90, 1.14 (0.85 - 1.23)
Histidine	0.74 (0.016) (0.71 - 0.76)	0.74 (0.023) (0.71 - 0.77)	0.0028 (0.026) (-0.0019 - -0.00095)	-0.071, 0.076	0.919	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.89 (0.018) (0.87 - 0.92)	0.91 (0.024) (0.88 - 0.93)	-0.010 (0.025) (-0.027 - -0.0076)	-0.079, 0.058	0.696	0.75, 0.96 (0.72 - 1.03)

Table H-3. Statistical Summary of Site ARTI Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Leucine	1.53 (0.027) (1.47 - 1.55)	1.50 (0.037) (1.44 - 1.55)	0.029 (0.042) (-0.0060 - 0.032)	-0.086, 0.14	0.524	1.25, 1.62 (1.20 - 1.72)
Lysine	1.22 (0.044) (1.15 - 1.27)	1.23 (0.058) (1.19 - 1.26)	-0.0029 (0.062) (-0.041 - -0.016)	-0.18, 0.17	0.965	1.01, 1.30 (0.99 - 1.44)
Methionine	0.39 (0.015) (0.35 - 0.43)	0.35 (0.021) (0.35 - 0.36)	0.041 (0.025) (-0.014 - 0.087)	-0.029, 0.11	0.181	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.43 (0.027) (1.36 - 1.45)	1.41 (0.038) (1.34 - 1.48)	0.016 (0.045) (-0.027 - 0.022)	-0.11, 0.14	0.737	1.12, 1.58 (1.10 - 1.63)
Proline	0.97 (0.021) (0.95 - 1.00)	1.00 (0.028) (0.98 - 1.02)	-0.027 (0.030) (-0.027 - -0.024)	-0.11, 0.057	0.417	0.83, 1.08 (0.79 - 1.17)
Serine	1.11 (0.022) (1.07 - 1.19)	1.03 (0.031) (0.99 - 1.06)	0.089 (0.038) (0.011 - 0.096)	-0.016, 0.19	0.079	0.83, 1.21 (0.81 - 1.24)

Table H-3. Statistical Summary of Site ARTI Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.88 (0.012) (0.85 - 0.90)	0.85 (0.017) (0.82 - 0.88)	0.029 (0.021) (-0.0045 - 0.033)	-0.028, 0.087	0.230	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.41 (0.0062) (0.40 - 0.42)	0.42 (0.0087) (0.40 - 0.44)	-0.012 (0.011) (-0.041 - 0.0060)	-0.042, 0.017	0.306	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.82 (0.015) (0.79 - 0.84)	0.79 (0.021) (0.76 - 0.82)	0.030 (0.026) (0.0011 - 0.028)	-0.042, 0.10	0.313	0.67, 0.84 (0.63 - 0.91)
Valine	1.19 (0.021) (1.14 - 1.23)	1.21 (0.027) (1.17 - 1.24)	-0.018 (0.026) (-0.030 - -0.016)	-0.090, 0.055	0.537	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.77 (0.0085) (0.76 - 0.79)	0.78 (0.012) (0.77 - 0.78)	-0.0049 (0.013) (-0.029 - 0.0018)	-0.041, 0.031	0.723	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	25.16 (0.10) (25.01 - 25.28)	24.98 (0.14) (24.92 - 25.05)	0.18 (0.18) (0.028 - 0.094)	-0.31, 0.67	0.360	16.54, 30.55 (19.11 - 26.73)

Table H-3. Statistical Summary of Site ARTI Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.53 (0.0052) (0.52 - 0.54)	0.52 (0.0073) (0.52 - 0.52)	0.017 (0.0089) (-0.00060 - 0.017)	-0.0082, 0.041	0.137	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.68 (0.026) (2.65 - 2.72)	2.51 (0.036) (2.45 - 2.57)	0.17 (0.045) (0.083 - 0.22)	0.044, 0.29	0.019	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	14.81 (0.11) (14.46 - 15.08)	14.68 (0.15) (14.58 - 14.70)	0.12 (0.17) (-0.24 - 0.18)	-0.34, 0.59	0.501	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	54.73 (0.22) (54.24 - 55.29)	55.31 (0.31) (55.26 - 55.38)	-0.59 (0.37) (-0.45 - 0.037)	-1.62, 0.45	0.189	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.14 (0.0021) (0.14 - 0.15)	0.13 (0.0030) (0.12 - 0.14)	0.015 (0.0035) (0.0064 - 0.021)	0.0056, 0.025	0.012	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.31 (0.0079) (0.31 - 0.32)	0.29 (0.011) (0.27 - 0.31)	0.023 (0.011) (0.0032 - 0.046)	-0.0087, 0.054	0.115	0.17, 0.38 (0.20 - 0.36)

Table H-3. Statistical Summary of Site ARTI Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.14 (0.0026) (0.13 - 0.14)	0.15 (0.0033) (0.15 - 0.16)	-0.015 (0.0031) (-0.019 - -0.012)	-0.024, -0.0065	0.008	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.15 (0.0035) (0.14 - 0.16)	0.12 (0.0050) (0.12 - 0.13)	0.028 (0.0061) (0.024 - 0.035)	0.011, 0.045	0.010	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	9.66 (0.34) (9.23 - 10.15)	9.64 (0.41) (8.79 - 9.79)	0.018 (0.38) (-0.57 - 0.58)	-1.03, 1.06	0.963	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	75.27 (5.63) (72.55 - 77.65)	80.76 (7.78) (72.89 - 87.72)	-5.49 (8.79) (-15.17 - 2.25)	-29.91, 18.93	0.566	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.41 (0.0076) (0.40 - 0.42)	0.40 (0.010) (0.38 - 0.41)	0.0099 (0.011) (0.0077 - 0.016)	-0.020, 0.040	0.413	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	13.27 (0.63) (12.58 - 13.63)	11.50 (0.89) (11.34 - 11.55)	1.77 (1.06) (1.03 - 1.95)	-1.18, 4.73	0.171	9.07, 17.33 (9.07 - 17.14)

Table H-3. Statistical Summary of Site ARTI Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (% dw)	0.83 (0.012) (0.82 - 0.84)	0.84 (0.016) (0.82 - 0.87)	-0.015 (0.018) (-0.027 - 0.0054)	-0.066, 0.036	0.450	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.18 (0.029) (1.16 - 1.20)	1.11 (0.040) (1.06 - 1.15)	0.071 (0.047) (0.028 - 0.14)	-0.059, 0.20	0.204	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.027 (0.0038) (0.023 - 0.029)	 (0.013 - 0.013)	ND[6]	ND[6]	ND[6]	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	37.08 (1.65) (35.15 - 38.88)	40.81 (2.03) (38.63 - 40.71)	-3.72 (1.87) (-3.48 - -3.41)	-8.92, 1.47	0.117	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	147.20 (2.44) (144.99 - 149.40)	136.55 (3.46) (133.79 - 139.31)	10.65 (4.23) (6.47 - 15.60)	-1.10, 22.40	0.065	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.
[6]Not determined due to insufficient number of observations for the control.

Table H-4. Statistical Summary of Site ARTI Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15 (0.0073) (0.14 - 0.16)	0.15 (0.010) (0.14 - 0.15)	0.0022 (0.013) (-0.0021 - -0.00026)	-0.033, 0.037	0.867	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.37 (0.016) (0.34 - 0.39)	0.36 (0.023) (0.33 - 0.37)	0.0088 (0.025) (-0.031 - 0.032)	-0.061, 0.078	0.742	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.21 (0.014) (0.19 - 0.22)	0.20 (0.020) (0.19 - 0.20)	0.013 (0.023) (-0.0076 - 0.032)	-0.052, 0.078	0.605	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	0.91 (0.035) (0.80 - 1.02)	0.82 (0.049) (0.80 - 0.84)	0.090 (0.060) (-0.035 - 0.10)	-0.076, 0.26	0.207	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	0.97 (0.034) (0.89 - 1.04)	0.93 (0.044) (0.94 - 0.98)	0.042 (0.044) (0.0013 - 0.10)	-0.081, 0.16	0.399	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-5. Statistical Summary of Site GACH Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.53 (0.044) (4.45 - 4.57)	4.21 (0.047) (4.12 - 4.23)	0.32 (0.034) (0.31 - 0.34)	0.23, 0.41	<0.001	3.42, 4.65 (3.18 - 4.68)
Calories	497.72 (2.28) (489.91 - 504.20)	496.55 (2.63) (494.57 - 498.27)	1.16 (3.48) (-6.91 - 8.49)	-7.79, 10.12	0.751	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	44.91 (0.59) (43.42 - 46.94)	45.84 (0.68) (44.64 - 47.09)	-0.94 (0.91) (-3.23 - 1.14)	-3.27, 1.39	0.348	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	6.98 (0.15) (6.42 - 7.33)	7.23 (0.18) (6.99 - 7.48)	-0.25 (0.23) (-1.06 - 0.12)	-0.85, 0.35	0.328	4.79, 9.92 (6.05 - 10.50)
Protein	27.41 (0.33) (26.87 - 27.78)	27.30 (0.37) (26.45 - 28.21)	0.11 (0.37) (-0.43 - 0.96)	-0.83, 1.06	0.770	22.30, 29.41 (20.58 - 29.28)
Total Fat	23.14 (0.46) (21.58 - 24.46)	22.67 (0.53) (22.26 - 23.02)	0.47 (0.70) (-1.16 - 1.93)	-1.34, 2.28	0.536	15.01, 28.51 (16.58 - 25.25)

Table H-5. Statistical Summary of Site GACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Acid Detergent Fiber	25.69 (0.52) (25.04 - 26.77)	27.52 (0.60) (26.81 - 28.13)	-1.83 (0.79) (-3.09 - -0.87)	-3.86, 0.20	0.068	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	19.27 (0.68) (17.10 - 20.64)	19.92 (0.78) (18.70 - 21.18)	-0.64 (1.00) (-1.60 - -0.54)	-3.21, 1.92	0.547	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	31.47 (0.90) (29.71 - 34.04)	33.92 (1.04) (32.79 - 35.89)	-2.44 (1.38) (-5.13 - -1.40)	-5.98, 1.09	0.135	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	39.64 (0.86) (37.72 - 41.91)	41.11 (1.00) (39.89 - 42.04)	-1.46 (1.32) (-4.32 - -0.44)	-4.85, 1.93	0.318	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.04 (0.019) (1.02 - 1.05)	1.10 (0.022) (1.06 - 1.17)	-0.059 (0.025) (-0.13 - -0.016)	-0.12, 0.0050	0.064	0.86, 1.11 (0.83 - 1.22)
Arginine	2.95 (0.061) (2.87 - 3.02)	3.21 (0.067) (3.07 - 3.46)	-0.27 (0.064) (-0.47 - -0.14)	-0.43, -0.10	0.008	2.38, 3.47 (2.30 - 3.55)

Table H-5. Statistical Summary of Site GACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.31 (0.043) (2.24 - 2.36)	2.45 (0.047) (2.36 - 2.60)	-0.15 (0.044) (-0.29 - -0.054)	-0.26, -0.035	0.019	1.94, 2.57 (1.79 - 2.72)
Cystine	0.40 (0.012) (0.38 - 0.42)	0.40 (0.013) (0.38 - 0.43)	-0.0089 (0.018) (-0.052 - 0.011)	-0.054, 0.036	0.636	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.57 (0.098) (4.35 - 4.77)	4.96 (0.11) (4.77 - 5.21)	-0.39 (0.099) (-0.63 - -0.17)	-0.65, -0.14	0.010	3.74, 5.28 (3.39 - 5.45)
Glycine	1.08 (0.020) (1.06 - 1.12)	1.13 (0.023) (1.09 - 1.20)	-0.052 (0.026) (-0.13 - 0.011)	-0.12, 0.014	0.099	0.90, 1.14 (0.85 - 1.23)
Histidine	0.73 (0.013) (0.68 - 0.76)	0.76 (0.014) (0.75 - 0.78)	-0.029 (0.012) (-0.022 - -0.020)	-0.061, 0.0030	0.067	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.90 (0.010) (0.90 - 0.91)	0.94 (0.012) (0.92 - 0.97)	-0.040 (0.014) (-0.074 - -0.012)	-0.075, -0.0042	0.034	0.75, 0.96 (0.72 - 1.03)

Table H-5. Statistical Summary of Site GACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Leucine	1.51 (0.022) (1.49 - 1.54)	1.58 (0.024) (1.52 - 1.65)	-0.068 (0.021) (-0.14 - -0.030)	-0.12, -0.013	0.024	1.25, 1.62 (1.20 - 1.72)
Lysine	1.24 (0.018) (1.21 - 1.28)	1.23 (0.020) (1.22 - 1.25)	0.0073 (0.024) (0.0016 - 0.032)	-0.055, 0.069	0.775	1.01, 1.30 (0.99 - 1.44)
Methionine	0.40 (0.017) (0.36 - 0.43)	0.42 (0.019) (0.37 - 0.46)	-0.025 (0.024) (-0.057 - -0.011)	-0.086, 0.036	0.337	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.40 (0.030) (1.37 - 1.43)	1.49 (0.033) (1.41 - 1.61)	-0.088 (0.032) (-0.18 - -0.033)	-0.17, -0.0064	0.039	1.12, 1.58 (1.10 - 1.63)
Proline	0.98 (0.020) (0.97 - 0.99)	1.05 (0.022) (1.03 - 1.09)	-0.065 (0.022) (-0.097 - -0.032)	-0.12, -0.0075	0.033	0.83, 1.08 (0.79 - 1.17)
Serine	1.03 (0.031) (0.96 - 1.11)	1.12 (0.035) (1.08 - 1.20)	-0.090 (0.039) (-0.18 - 0.011)	-0.19, 0.010	0.069	0.83, 1.21 (0.81 - 1.24)

Table H-5. Statistical Summary of Site GACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.85 (0.015) (0.83 - 0.88)	0.90 (0.017) (0.87 - 0.95)	-0.046 (0.018) (-0.10 - -0.0034)	-0.092, -0.00006	0.049	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.42 (0.011) (0.39 - 0.45)	0.42 (0.013) (0.39 - 0.44)	0.0013 (0.017) (-0.044 - 0.041)	-0.043, 0.046	0.942	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.80 (0.011) (0.79 - 0.82)	0.84 (0.013) (0.81 - 0.89)	-0.036 (0.013) (-0.068 - -0.015)	-0.069, -0.0030	0.037	0.67, 0.84 (0.63 - 0.91)
Valine	1.21 (0.018) (1.19 - 1.23)	1.26 (0.019) (1.23 - 1.32)	-0.053 (0.015) (-0.090 - -0.022)	-0.091, -0.014	0.017	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.78 (0.0068) (0.76 - 0.79)	0.77 (0.0079) (0.76 - 0.78)	0.0029 (0.010) (-0.026 - 0.018)	-0.024, 0.030	0.793	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	24.40 (0.14) (24.27 - 24.66)	24.12 (0.16) (23.78 - 24.45)	0.28 (0.20) (-0.11 - 0.56)	-0.24, 0.80	0.227	16.54, 30.55 (19.11 - 26.73)

Table H-5. Statistical Summary of Site GACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.52 (0.0058) (0.51 - 0.54)	0.51 (0.0067) (0.50 - 0.52)	0.011 (0.0089) (-0.012 - 0.025)	-0.012, 0.034	0.268	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.54 (0.032) (2.45 - 2.67)	2.43 (0.037) (2.37 - 2.46)	0.11 (0.046) (0.066 - 0.21)	-0.0057, 0.23	0.058	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	14.64 (0.098) (14.39 - 14.84)	14.39 (0.11) (14.06 - 14.61)	0.25 (0.13) (-0.0098 - 0.34)	-0.078, 0.58	0.107	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	55.81 (0.24) (55.04 - 56.24)	56.59 (0.28) (56.02 - 57.32)	-0.78 (0.35) (-1.39 - 0.12)	-1.67, 0.12	0.075	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.15 (0.0066) (0.15 - 0.16)	0.15 (0.0076) (0.14 - 0.15)	0.0076 (0.010) (0.00091 - 0.013)	-0.018, 0.033	0.481	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.29 (0.0098) (0.26 - 0.31)	0.28 (0.011) (0.26 - 0.29)	0.013 (0.013) (0.0016 - 0.015)	-0.021, 0.047	0.376	0.17, 0.38 (0.20 - 0.36)

Table H-5. Statistical Summary of Site GACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.14 (0.0026) (0.14 - 0.15)	0.14 (0.0030) (0.14 - 0.15)	-0.00014 (0.0037) (-0.0017 - 0.0028)	-0.0097, 0.0095	0.971	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.13 (0.0015) (0.13 - 0.13)	0.11 (0.0018) (0.11 - 0.11)	0.019 (0.0023) (0.014 - 0.024)	0.013, 0.025	<0.001	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	8.51 (0.26) (8.02 - 9.13)	8.21 (0.30) (7.48 - 8.64)	0.30 (0.39) (-0.49 - 1.13)	-0.70, 1.31	0.473	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	75.42 (3.80) (70.35 - 79.72)	78.00 (4.39) (75.01 - 80.40)	-2.58 (5.81) (-8.31 - 4.71)	-17.51, 12.35	0.675	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.41 (0.0052) (0.40 - 0.41)	0.38 (0.0054) (0.37 - 0.39)	0.026 (0.0034) (0.021 - 0.031)	0.018, 0.035	<0.001	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	13.41 (0.31) (12.79 - 14.14)	11.51 (0.34) (10.81 - 11.75)	1.90 (0.37) (1.18 - 2.39)	0.95, 2.85	0.003	9.07, 17.33 (9.07 - 17.14)

Table H-5. Statistical Summary of Site GACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (% dw)	0.78 (0.011) (0.75 - 0.81)	0.76 (0.012) (0.75 - 0.79)	0.018 (0.0082) (0.0052 - 0.029)	-0.0028, 0.040	0.076	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.21 (0.012) (1.17 - 1.24)	1.12 (0.013) (1.10 - 1.13)	0.090 (0.010) (0.075 - 0.11)	0.064, 0.12	<0.001	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.022 (0.0045) (0.019 - 0.027)	0.017 (0.0052) (0.013 - 0.022)	0.0049 (0.0069) (-0.0020 - 0.014)	-0.013, 0.023	0.515	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	39.10 (0.67) (37.41 - 40.18)	39.55 (0.75) (38.49 - 40.84)	-0.45 (0.83) (-1.35 - 0.51)	-2.58, 1.68	0.610	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	151.03 (2.27) (148.34 - 154.95)	140.12 (2.63) (133.64 - 145.15)	10.90 (3.47) (3.19 - 18.52)	1.97, 19.83	0.025	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-6. Statistical Summary of Site GACH Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15 (0.0067) (0.14 - 0.16)	0.12 (0.0077) (0.11 - 0.13)	0.033 (0.010) (0.021 - 0.050)	0.0069, 0.059	0.022	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.37 (0.027) (0.26 - 0.45)	0.32 (0.031) (0.31 - 0.34)	0.044 (0.038) (-0.052 - 0.12)	-0.055, 0.14	0.304	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.21 (0.011) (0.18 - 0.25)	0.18 (0.013) (0.18 - 0.20)	0.030 (0.015) (-0.0018 - 0.050)	-0.0072, 0.068	0.092	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	0.85 (0.017) (0.83 - 0.88)	0.86 (0.019) (0.85 - 0.90)	-0.016 (0.020) (-0.055 - 0.028)	-0.068, 0.037	0.474	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	0.97 (0.019) (0.93 - 1.01)	0.96 (0.020) (0.90 - 0.99)	0.019 (0.017) (-0.012 - 0.033)	-0.026, 0.063	0.324	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-7. Statistical Summary of Site KSLA Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.53 (0.063) (4.25 - 4.66)	4.29 (0.071) (4.21 - 4.33)	0.24 (0.078) (0.042 - 0.34)	0.040, 0.44	0.027	3.42, 4.65 (3.18 - 4.68)
Calories	496.63 (2.75) (492.91 - 499.03)	495.83 (3.16) (492.30 - 504.10)	0.80 (3.87) (-8.23 - 6.50)	-9.15, 10.75	0.844	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	44.10 (0.71) (42.20 - 44.98)	44.23 (0.82) (42.53 - 45.14)	-0.14 (1.09) (-0.77 - 2.45)	-2.94, 2.67	0.905	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	7.02 (0.17) (6.63 - 7.36)	7.36 (0.19) (7.17 - 7.62)	-0.34 (0.20) (-0.73 - 0.19)	-0.86, 0.18	0.153	4.79, 9.92 (6.05 - 10.50)
Protein	28.42 (0.62) (26.95 - 30.82)	28.82 (0.72) (28.58 - 29.04)	-0.40 (0.93) (-1.63 - -0.87)	-2.80, 1.99	0.681	22.30, 29.41 (20.58 - 29.28)
Total Fat	22.96 (0.55) (22.34 - 23.50)	22.62 (0.64) (21.87 - 24.18)	0.34 (0.81) (-1.58 - 1.56)	-1.75, 2.43	0.692	15.01, 28.51 (16.58 - 25.25)

Table H-7. Statistical Summary of Site KSLA Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Acid Detergent Fiber	24.04 (0.55) (23.86 - 24.16)	24.01 (0.61) (22.08 - 25.22)	0.027 (0.65) (-1.08 - 1.77)	-1.64, 1.70	0.968	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	16.43 (0.24) (16.06 - 17.24)	17.67 (0.28) (17.49 - 17.88)	-1.24 (0.37) (-1.69 - -0.24)	-2.19, -0.29	0.019	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	28.04 (0.88) (25.13 - 30.18)	30.20 (1.01) (28.87 - 32.60)	-2.15 (1.21) (-2.93 - 0.63)	-5.27, 0.97	0.136	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	38.32 (0.42) (37.62 - 38.75)	40.14 (0.49) (39.32 - 41.35)	-1.83 (0.63) (-3.74 - -0.57)	-3.46, -0.19	0.034	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.04 (0.011) (1.02 - 1.05)	1.06 (0.012) (1.02 - 1.10)	-0.022 (0.014) (-0.048 - -0.00010)	-0.058, 0.014	0.175	0.86, 1.11 (0.83 - 1.22)
Arginine	3.02 (0.053) (2.95 - 3.10)	3.28 (0.060) (3.10 - 3.43)	-0.26 (0.069) (-0.34 - -0.14)	-0.43, -0.082	0.013	2.38, 3.47 (2.30 - 3.55)

Table H-7. Statistical Summary of Site KSLA Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.38 (0.037) (2.32 - 2.46)	2.50 (0.043) (2.39 - 2.64)	-0.11 (0.053) (-0.26 - -0.073)	-0.25, 0.022	0.083	1.94, 2.57 (1.79 - 2.72)
Cystine	0.43 (0.010) (0.41 - 0.44)	0.42 (0.012) (0.41 - 0.43)	0.0076 (0.016) (-0.017 - 0.015)	-0.033, 0.048	0.651	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.82 (0.12) (4.58 - 5.22)	5.07 (0.14) (4.86 - 5.43)	-0.25 (0.18) (-0.78 - -0.017)	-0.71, 0.21	0.219	3.74, 5.28 (3.39 - 5.45)
Glycine	1.08 (0.018) (1.06 - 1.12)	1.11 (0.021) (1.07 - 1.18)	-0.029 (0.027) (-0.11 - -0.0024)	-0.099, 0.040	0.330	0.90, 1.14 (0.85 - 1.23)
Histidine	0.75 (0.013) (0.73 - 0.77)	0.77 (0.014) (0.74 - 0.80)	-0.024 (0.011) (-0.045 - 0.0022)	-0.052, 0.0035	0.074	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.92 (0.014) (0.91 - 0.95)	0.95 (0.015) (0.91 - 0.98)	-0.027 (0.014) (-0.062 - 0.0079)	-0.063, 0.0091	0.112	0.75, 0.96 (0.72 - 1.03)

Table H-7. Statistical Summary of Site KSLA Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Leucine	1.54 (0.020) (1.51 - 1.57)	1.58 (0.022) (1.52 - 1.65)	-0.043 (0.021) (-0.083 - -0.0065)	-0.097, 0.011	0.098	1.25, 1.62 (1.20 - 1.72)
Lysine	1.22 (0.017) (1.21 - 1.23)	1.25 (0.019) (1.19 - 1.30)	-0.033 (0.021) (-0.073 - 0.027)	-0.087, 0.021	0.178	1.01, 1.30 (0.99 - 1.44)
Methionine	0.39 (0.015) (0.37 - 0.42)	0.39 (0.017) (0.34 - 0.44)	0.0021 (0.019) (-0.017 - 0.027)	-0.046, 0.050	0.915	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.44 (0.022) (1.40 - 1.46)	1.53 (0.025) (1.45 - 1.58)	-0.090 (0.029) (-0.13 - -0.049)	-0.16, -0.016	0.025	1.12, 1.58 (1.10 - 1.63)
Proline	1.01 (0.018) (0.98 - 1.03)	1.07 (0.020) (1.03 - 1.12)	-0.060 (0.027) (-0.12 - -0.042)	-0.13, 0.0090	0.075	0.83, 1.08 (0.79 - 1.17)
Serine	1.08 (0.029) (1.03 - 1.18)	1.11 (0.034) (1.06 - 1.20)	-0.031 (0.045) (-0.17 - 0.0023)	-0.15, 0.083	0.513	0.83, 1.21 (0.81 - 1.24)

Table H-7. Statistical Summary of Site KSLA Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.86 (0.013) (0.84 - 0.88)	0.88 (0.015) (0.83 - 0.92)	-0.022 (0.019) (-0.053 - 0.0050)	-0.071, 0.028	0.317	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.42 (0.0061) (0.42 - 0.43)	0.42 (0.0070) (0.42 - 0.43)	-0.0028 (0.0090) (-0.016 - 0.011)	-0.026, 0.020	0.771	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.80 (0.014) (0.78 - 0.83)	0.84 (0.016) (0.79 - 0.87)	-0.039 (0.019) (-0.074 - -0.015)	-0.087, 0.0096	0.094	0.67, 0.84 (0.63 - 0.91)
Valine	1.21 (0.017) (1.19 - 1.23)	1.26 (0.019) (1.21 - 1.30)	-0.048 (0.023) (-0.085 - -0.027)	-0.11, 0.010	0.087	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.68 (0.0087) (0.66 - 0.71)	0.72 (0.0096) (0.71 - 0.73)	-0.038 (0.0090) (-0.049 - -0.016)	-0.061, -0.015	0.007	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	22.61 (0.089) (22.34 - 22.84)	22.73 (0.10) (22.69 - 22.78)	-0.12 (0.13) (-0.11 - 0.093)	-0.46, 0.21	0.394	16.54, 30.55 (19.11 - 26.73)

Table H-7. Statistical Summary of Site KSLA Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.50 (0.0057) (0.48 - 0.52)	0.49 (0.0062) (0.49 - 0.50)	0.0067 (0.0059) (-0.0046 - 0.021)	-0.0086, 0.022	0.312	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.31 (0.031) (2.29 - 2.32)	2.20 (0.036) (2.15 - 2.28)	0.11 (0.047) (0.039 - 0.17)	-0.016, 0.23	0.075	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	14.69 (0.11) (14.51 - 14.88)	14.83 (0.13) (14.74 - 14.99)	-0.14 (0.17) (-0.48 - 0.14)	-0.59, 0.31	0.454	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	57.84 (0.19) (57.49 - 58.22)	57.78 (0.22) (57.65 - 57.93)	0.059 (0.29) (-0.44 - 0.19)	-0.67, 0.79	0.843	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.18 (0.0027) (0.18 - 0.19)	0.17 (0.0030) (0.17 - 0.18)	0.0066 (0.0033) (0.0051 - 0.010)	-0.0018, 0.015	0.100	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.23 (0.0029) (0.23 - 0.24)	0.24 (0.0034) (0.23 - 0.24)	-0.0021 (0.0045) (-0.0063 - 0.0087)	-0.014, 0.0094	0.651	0.17, 0.38 (0.20 - 0.36)

Table H-7. Statistical Summary of Site KSLA Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.13 (0.0030) (0.12 - 0.14)	0.13 (0.0035) (0.13 - 0.14)	-0.0051 (0.0046) (-0.0084 - 0.0036)	-0.017, 0.0067	0.318	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.20 (0.0060) (0.19 - 0.22)	0.18 (0.0065) (0.17 - 0.19)	0.026 (0.0061) (0.013 - 0.038)	0.010, 0.042	0.007	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	10.08 (0.54) (8.74 - 11.06)	11.01 (0.61) (10.09 - 11.33)	-0.93 (0.71) (-2.59 - 0.69)	-2.75, 0.89	0.246	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	78.87 (5.50) (74.42 - 82.99)	74.39 (6.35) (72.65 - 76.27)	4.48 (8.40) (3.93 - 8.73)	-17.11, 26.07	0.616	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.43 (0.0033) (0.41 - 0.43)	0.40 (0.0038) (0.39 - 0.40)	0.027 (0.0046) (0.015 - 0.041)	0.015, 0.039	0.002	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	13.34 (0.29) (12.74 - 13.60)	12.56 (0.33) (11.96 - 13.28)	0.78 (0.44) (-0.53 - 1.64)	-0.35, 1.90	0.135	9.07, 17.33 (9.07 - 17.14)

Table H-7. Statistical Summary of Site KSLA Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (% dw)	0.79 (0.0090) (0.75 - 0.82)	0.78 (0.010) (0.77 - 0.79)	0.0090 (0.011) (-0.024 - 0.029)	-0.019, 0.037	0.440	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.09 (0.010) (1.08 - 1.11)	1.08 (0.012) (1.05 - 1.10)	0.018 (0.015) (-0.0048 - 0.063)	-0.020, 0.056	0.281	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.022 (0.0028) (0.019 - 0.025)	0.0080 (0.0032) (0.0054 - 0.013)	0.014 (0.0042) (0.0098 - 0.016)	0.0033, 0.025	0.020	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	40.79 (1.18) (37.59 - 43.87)	42.00 (1.37) (40.59 - 43.50)	-1.21 (1.81) (-5.91 - 3.27)	-5.85, 3.44	0.533	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	93.11 (2.67) (86.23 - 100.03)	92.34 (2.91) (91.78 - 95.85)	0.76 (2.71) (-6.54 - 4.18)	-6.20, 7.72	0.789	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-8. Statistical Summary of Site KSLA Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15 (0.010) (0.11 - 0.18)	0.12 (0.012) (0.12 - 0.13)	0.026 (0.014) (-0.0087 - 0.054)	-0.011, 0.063	0.129	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.45 (0.030) (0.34 - 0.55)	0.37 (0.034) (0.33 - 0.39)	0.083 (0.039) (-0.024 - 0.15)	-0.018, 0.18	0.088	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.23 (0.014) (0.18 - 0.28)	0.20 (0.016) (0.19 - 0.21)	0.031 (0.019) (-0.023 - 0.078)	-0.017, 0.079	0.159	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	1.07 (0.027) (1.03 - 1.10)	0.95 (0.030) (0.86 - 1.05)	0.12 (0.033) (0.051 - 0.20)	0.036, 0.20	0.014	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	1.13 (0.033) (1.00 - 1.24)	1.01 (0.038) (1.00 - 1.02)	0.12 (0.047) (0.0061 - 0.23)	0.00016, 0.24	0.049	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-9. Statistical Summary of Site LACH Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.35 (0.047) (4.23 - 4.47)	4.12 (0.047) (4.06 - 4.15)	0.23 (0.066) (0.11 - 0.41)	0.066, 0.39	0.013	3.42, 4.65 (3.18 - 4.68)
Calories	494.11 (3.01) (482.46 - 501.83)	494.75 (3.01) (490.27 - 498.67)	-0.64 (4.19) (-14.30 - 4.88)	-10.89, 9.61	0.883	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	45.92 (0.79) (44.17 - 48.89)	47.84 (0.79) (46.77 - 50.30)	-1.91 (1.11) (-5.19 - 1.91)	-4.64, 0.81	0.136	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	6.70 (0.26) (6.46 - 6.94)	6.98 (0.26) (6.15 - 7.40)	-0.28 (0.36) (-0.90 - 0.79)	-1.17, 0.62	0.478	4.79, 9.92 (6.05 - 10.50)
Protein	27.44 (0.56) (27.06 - 27.92)	25.80 (0.56) (23.53 - 27.85)	1.64 (0.80) (0.074 - 3.73)	-0.31, 3.59	0.084	22.30, 29.41 (20.58 - 29.28)
Total Fat	22.27 (0.64) (19.79 - 23.86)	22.25 (0.64) (21.29 - 23.02)	0.018 (0.90) (-2.89 - 1.18)	-2.18, 2.22	0.984	15.01, 28.51 (16.58 - 25.25)

Table H-9. Statistical Summary of Site LACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Acid Detergent Fiber	25.72 (0.45) (24.16 - 27.08)	28.35 (0.45) (27.81 - 29.58)	-2.63 (0.63) (-5.42 - -0.73)	-4.18, -1.08	0.005	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	18.73 (0.66) (17.75 - 19.77)	19.62 (0.66) (18.46 - 20.54)	-0.89 (0.93) (-1.79 - 0.17)	-3.15, 1.38	0.376	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	33.12 (0.65) (32.24 - 34.42)	34.05 (0.65) (32.61 - 35.84)	-0.93 (0.42) (-1.65 - 0.23)	-1.97, 0.10	0.070	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	39.82 (0.49) (39.02 - 40.86)	43.35 (0.49) (42.33 - 44.37)	-3.53 (0.69) (-5.34 - -1.47)	-5.21, -1.85	0.002	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.07 (0.018) (1.00 - 1.11)	1.03 (0.018) (1.00 - 1.06)	0.038 (0.014) (0.00025 - 0.082)	0.0050, 0.072	0.030	0.86, 1.11 (0.83 - 1.22)
Arginine	2.96 (0.073) (2.64 - 3.13)	2.98 (0.073) (2.89 - 3.13)	-0.017 (0.084) (-0.25 - 0.15)	-0.22, 0.19	0.849	2.38, 3.47 (2.30 - 3.55)

Table H-9. Statistical Summary of Site LACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.37 (0.050) (2.17 - 2.49)	2.30 (0.050) (2.25 - 2.40)	0.074 (0.062) (-0.12 - 0.22)	-0.077, 0.22	0.278	1.94, 2.57 (1.79 - 2.72)
Cystine	0.41 (0.016) (0.38 - 0.46)	0.38 (0.016) (0.36 - 0.44)	0.026 (0.019) (-0.024 - 0.082)	-0.021, 0.074	0.222	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.78 (0.13) (4.32 - 5.26)	4.52 (0.13) (4.37 - 4.75)	0.27 (0.16) (-0.15 - 0.79)	-0.13, 0.67	0.151	3.74, 5.28 (3.39 - 5.45)
Glycine	1.11 (0.020) (1.02 - 1.18)	1.06 (0.020) (1.04 - 1.09)	0.057 (0.025) (-0.024 - 0.14)	-0.0034, 0.12	0.060	0.90, 1.14 (0.85 - 1.23)
Histidine	0.74 (0.020) (0.68 - 0.78)	0.72 (0.020) (0.67 - 0.76)	0.019 (0.014) (0.013 - 0.029)	-0.014, 0.052	0.206	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.90 (0.018) (0.84 - 0.97)	0.88 (0.018) (0.87 - 0.90)	0.020 (0.020) (-0.028 - 0.096)	-0.028, 0.068	0.355	0.75, 0.96 (0.72 - 1.03)

Table H-9. Statistical Summary of Site LACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Leucine	1.51 (0.026) (1.40 - 1.58)	1.48 (0.026) (1.44 - 1.52)	0.039 (0.028) (-0.035 - 0.12)	-0.030, 0.11	0.217	1.25, 1.62 (1.20 - 1.72)
Lysine	1.26 (0.024) (1.17 - 1.31)	1.18 (0.024) (1.12 - 1.23)	0.083 (0.027) (0.021 - 0.15)	0.016, 0.15	0.023	1.01, 1.30 (0.99 - 1.44)
Methionine	0.42 (0.017) (0.37 - 0.44)	0.38 (0.017) (0.32 - 0.42)	0.045 (0.013) (0.020 - 0.075)	0.013, 0.077	0.013	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.41 (0.027) (1.28 - 1.47)	1.39 (0.027) (1.36 - 1.43)	0.015 (0.033) (-0.077 - 0.094)	-0.066, 0.095	0.668	1.12, 1.58 (1.10 - 1.63)
Proline	1.00 (0.015) (0.94 - 1.04)	0.98 (0.015) (0.95 - 1.02)	0.017 (0.022) (-0.039 - 0.093)	-0.036, 0.071	0.459	0.83, 1.08 (0.79 - 1.17)
Serine	1.07 (0.022) (0.99 - 1.15)	1.03 (0.022) (1.01 - 1.06)	0.038 (0.030) (-0.047 - 0.12)	-0.036, 0.11	0.257	0.83, 1.21 (0.81 - 1.24)

Table H-9. Statistical Summary of Site LACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.87 (0.016) (0.80 - 0.90)	0.84 (0.016) (0.82 - 0.86)	0.027 (0.012) (-0.021 - 0.060)	-0.0036, 0.057	0.074	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.41 (0.014) (0.36 - 0.46)	0.39 (0.014) (0.38 - 0.43)	0.019 (0.018) (-0.024 - 0.078)	-0.026, 0.063	0.347	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.80 (0.018) (0.72 - 0.84)	0.79 (0.018) (0.76 - 0.81)	0.010 (0.021) (-0.045 - 0.058)	-0.042, 0.063	0.643	0.67, 0.84 (0.63 - 0.91)
Valine	1.21 (0.024) (1.11 - 1.29)	1.18 (0.024) (1.17 - 1.19)	0.026 (0.029) (-0.054 - 0.12)	-0.044, 0.096	0.397	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.74 (0.013) (0.71 - 0.76)	0.75 (0.013) (0.73 - 0.78)	-0.012 (0.018) (-0.032 - 0.0064)	-0.057, 0.032	0.523	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	24.48 (0.091) (24.37 - 24.55)	24.04 (0.091) (23.92 - 24.16)	0.44 (0.13) (0.21 - 0.61)	0.12, 0.75	0.014	16.54, 30.55 (19.11 - 26.73)

Table H-9. Statistical Summary of Site LACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.51 (0.0035) (0.50 - 0.52)	0.50 (0.0035) (0.49 - 0.51)	0.0090 (0.0049) (-0.0051 - 0.020)	-0.0030, 0.021	0.116	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.68 (0.018) (2.64 - 2.73)	2.52 (0.018) (2.49 - 2.57)	0.15 (0.026) (0.11 - 0.24)	0.089, 0.22	0.001	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	14.70 (0.095) (14.48 - 15.01)	14.29 (0.095) (14.13 - 14.53)	0.41 (0.13) (0.16 - 0.72)	0.084, 0.74	0.021	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	55.53 (0.14) (55.15 - 55.99)	56.63 (0.14) (56.52 - 56.72)	-1.09 (0.20) (-1.42 - -0.63)	-1.59, -0.60	0.001	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.15 (0.0042) (0.14 - 0.17)	0.15 (0.0042) (0.13 - 0.15)	0.0063 (0.0059) (-0.0073 - 0.019)	-0.0082, 0.021	0.327	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.31 (0.0054) (0.31 - 0.32)	0.29 (0.0054) (0.29 - 0.30)	0.020 (0.0071) (0.016 - 0.023)	0.0022, 0.037	0.033	0.17, 0.38 (0.20 - 0.36)

Table H-9. Statistical Summary of Site LACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.14 (0.0039) (0.14 - 0.15)	0.14 (0.0039) (0.14 - 0.15)	0.00029 (0.0055) (-0.0046 - 0.0027)	-0.013, 0.014	0.959	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.12 (0.0039) (0.11 - 0.13)	0.12 (0.0039) (0.12 - 0.12)	0.0047 (0.0050) (-0.012 - 0.015)	-0.0075, 0.017	0.381	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	8.48 (0.30) (7.86 - 9.49)	8.70 (0.30) (8.11 - 9.14)	-0.22 (0.42) (-1.27 - 0.99)	-1.26, 0.82	0.621	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	76.74 (4.19) (73.99 - 83.17)	68.59 (4.19) (66.28 - 70.38)	8.15 (4.72) (3.68 - 12.79)	-3.40, 19.69	0.134	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.41 (0.0065) (0.39 - 0.44)	0.39 (0.0065) (0.38 - 0.41)	0.021 (0.0093) (-0.014 - 0.054)	-0.0018, 0.043	0.065	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	13.13 (0.37) (11.92 - 13.79)	12.87 (0.37) (12.31 - 13.87)	0.26 (0.52) (-1.95 - 1.16)	-1.03, 1.54	0.642	9.07, 17.33 (9.07 - 17.14)

Table H-9. Statistical Summary of Site LACH Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (% dw)	0.75 (0.014) (0.71 - 0.80)	0.71 (0.014) (0.69 - 0.73)	0.036 (0.019) (-0.023 - 0.11)	-0.012, 0.083	0.113	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.18 (0.022) (1.16 - 1.22)	1.17 (0.022) (1.12 - 1.27)	0.0099 (0.029) (-0.087 - 0.064)	-0.061, 0.081	0.742	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.023 (0.0043) (0.021 - 0.024)	0.015 (0.0043) (0.0053 - 0.027)	0.0078 (0.0060) (-0.0031 - 0.017)	-0.0069, 0.022	0.242	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	33.97 (0.93) (31.68 - 37.84)	35.74 (0.93) (35.10 - 37.09)	-1.77 (1.32) (-5.41 - 2.55)	-4.99, 1.45	0.227	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	169.88 (2.48) (163.34 - 175.33)	149.96 (2.48) (148.96 - 152.67)	19.92 (3.48) (14.16 - 26.36)	11.40, 28.43	0.001	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-10. Statistical Summary of Site LACH Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15 (0.011) (0.13 - 0.19)	0.13 (0.011) (0.12 - 0.14)	0.019 (0.016) (-0.0082 - 0.068)	-0.019, 0.057	0.271	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.39 (0.035) (0.33 - 0.53)	0.35 (0.035) (0.31 - 0.38)	0.032 (0.049) (-0.047 - 0.16)	-0.088, 0.15	0.535	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.22 (0.016) (0.19 - 0.29)	0.20 (0.016) (0.17 - 0.21)	0.020 (0.023) (-0.011 - 0.076)	-0.037, 0.076	0.432	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	0.86 (0.026) (0.81 - 0.92)	0.81 (0.026) (0.78 - 0.84)	0.056 (0.036) (0.0028 - 0.13)	-0.033, 0.14	0.175	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	0.93 (0.025) (0.90 - 1.00)	0.90 (0.025) (0.82 - 0.94)	0.033 (0.036) (-0.015 - 0.086)	-0.055, 0.12	0.395	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-11. Statistical Summary of Site NCBD Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.34 (0.057) (4.29 - 4.40)	4.14 (0.064) (3.94 - 4.26)	0.20 (0.070) (0.13 - 0.35)	0.024, 0.39	0.033	3.42, 4.65 (3.18 - 4.68)
Calories	497.98 (1.61) (491.46 - 501.77)	491.80 (1.86) (488.93 - 494.48)	6.18 (2.46) (-3.01 - 10.35)	-0.15, 12.51	0.053	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	44.40 (0.59) (43.84 - 45.31)	44.36 (0.68) (43.65 - 45.15)	0.044 (0.87) (-1.31 - 0.79)	-2.20, 2.29	0.961	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	9.18 (0.22) (8.64 - 9.67)	8.96 (0.23) (8.59 - 9.19)	0.22 (0.19) (-0.050 - 0.48)	-0.26, 0.70	0.287	4.79, 9.92 (6.05 - 10.50)
Protein	28.24 (0.61) (26.53 - 29.33)	29.84 (0.70) (29.62 - 30.42)	-1.60 (0.85) (-1.99 - -0.53)	-3.78, 0.59	0.119	22.30, 29.41 (20.58 - 29.28)
Total Fat	23.04 (0.30) (21.89 - 23.76)	21.59 (0.34) (21.03 - 22.21)	1.45 (0.46) (-0.32 - 2.22)	0.28, 2.63	0.024	15.01, 28.51 (16.58 - 25.25)

Table H-11. Statistical Summary of Site NCBD Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Acid Detergent Fiber	25.91 (1.12) (24.26 - 27.74)	26.31 (1.29) (24.72 - 28.08)	-0.40 (1.71) (-1.88 - -0.096)	-4.80, 3.99	0.822	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	17.01 (0.29) (16.31 - 17.78)	16.93 (0.33) (16.30 - 17.90)	0.086 (0.38) (-0.12 - 0.31)	-0.90, 1.08	0.831	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	31.08 (1.03) (29.23 - 32.66)	31.14 (1.19) (30.85 - 31.49)	-0.057 (1.58) (-1.62 - 1.16)	-4.11, 4.00	0.972	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	38.51 (0.55) (36.91 - 39.40)	39.52 (0.64) (39.05 - 39.86)	-1.01 (0.85) (-0.90 - -0.088)	-3.19, 1.16	0.285	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.11 (0.020) (1.07 - 1.14)	1.10 (0.023) (1.05 - 1.14)	0.011 (0.031) (-0.0051 - 0.026)	-0.068, 0.091	0.727	0.86, 1.11 (0.83 - 1.22)
Arginine	3.08 (0.052) (2.97 - 3.20)	3.20 (0.060) (3.11 - 3.27)	-0.12 (0.080) (-0.19 - -0.031)	-0.33, 0.080	0.178	2.38, 3.47 (2.30 - 3.55)

Table H-11. Statistical Summary of Site NCBD Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.50 (0.053) (2.43 - 2.58)	2.55 (0.061) (2.46 - 2.63)	-0.045 (0.081) (-0.042 - 0.0096)	-0.25, 0.16	0.604	1.94, 2.57 (1.79 - 2.72)
Cystine	0.43 (0.018) (0.40 - 0.46)	0.42 (0.021) (0.39 - 0.46)	0.016 (0.028) (-0.051 - 0.055)	-0.056, 0.088	0.594	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.71 (0.13) (4.64 - 4.79)	5.08 (0.15) (4.85 - 5.40)	-0.37 (0.20) (-0.66 - -0.19)	-0.88, 0.14	0.120	3.74, 5.28 (3.39 - 5.45)
Glycine	1.11 (0.019) (1.06 - 1.16)	1.11 (0.022) (1.08 - 1.14)	0.0013 (0.029) (-0.015 - 0.023)	-0.072, 0.075	0.964	0.90, 1.14 (0.85 - 1.23)
Histidine	0.76 (0.013) (0.73 - 0.79)	0.76 (0.015) (0.74 - 0.79)	0 (0.019) (-0.032 - 0.052)	-0.049, 0.049	0.999	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.96 (0.018) (0.90 - 1.00)	0.96 (0.020) (0.93 - 0.97)	-0.00058 (0.020) (-0.030 - 0.040)	-0.053, 0.051	0.978	0.75, 0.96 (0.72 - 1.03)

Table H-11. Statistical Summary of Site NCBD Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Leucine	1.60 (0.023) (1.55 - 1.65)	1.60 (0.027) (1.55 - 1.63)	-0.0061 (0.035) (-0.025 - 0.023)	-0.097, 0.085	0.869	1.25, 1.62 (1.20 - 1.72)
Lysine	1.25 (0.029) (1.17 - 1.36)	1.26 (0.033) (1.22 - 1.29)	-0.010 (0.043) (-0.060 - 0.077)	-0.12, 0.10	0.819	1.01, 1.30 (0.99 - 1.44)
Methionine	0.40 (0.0093) (0.38 - 0.42)	0.41 (0.011) (0.40 - 0.42)	-0.011 (0.014) (-0.046 - 0.016)	-0.048, 0.026	0.477	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.48 (0.027) (1.44 - 1.54)	1.52 (0.031) (1.45 - 1.56)	-0.043 (0.041) (-0.058 - -0.012)	-0.15, 0.063	0.342	1.12, 1.58 (1.10 - 1.63)
Proline	1.03 (0.022) (0.98 - 1.11)	1.09 (0.025) (1.07 - 1.12)	-0.065 (0.029) (-0.094 - -0.010)	-0.14, 0.0098	0.075	0.83, 1.08 (0.79 - 1.17)
Serine	1.11 (0.027) (1.11 - 1.13)	1.13 (0.032) (1.09 - 1.19)	-0.017 (0.042) (-0.087 - 0.028)	-0.12, 0.090	0.696	0.83, 1.21 (0.81 - 1.24)

Table H-11. Statistical Summary of Site NCBD Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.90 (0.014) (0.88 - 0.92)	0.89 (0.016) (0.86 - 0.92)	0.0096 (0.021) (-0.0050 - 0.015)	-0.045, 0.064	0.669	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.41 (0.021) (0.40 - 0.44)	0.45 (0.024) (0.39 - 0.52)	-0.039 (0.032) (-0.081 - 0.024)	-0.12, 0.045	0.285	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.84 (0.012) (0.81 - 0.87)	0.84 (0.014) (0.82 - 0.87)	0.0015 (0.018) (-0.026 - 0.039)	-0.045, 0.047	0.937	0.67, 0.84 (0.63 - 0.91)
Valine	1.26 (0.025) (1.17 - 1.31)	1.30 (0.027) (1.24 - 1.32)	-0.038 (0.021) (-0.067 - -0.010)	-0.091, 0.016	0.130	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.68 (0.0074) (0.66 - 0.70)	0.75 (0.0086) (0.74 - 0.76)	-0.063 (0.011) (-0.077 - -0.044)	-0.091, -0.034	0.002	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	22.89 (0.12) (22.47 - 23.15)	23.10 (0.14) (23.07 - 23.15)	-0.21 (0.18) (-0.68 - 0.079)	-0.68, 0.25	0.286	16.54, 30.55 (19.11 - 26.73)

Table H-11. Statistical Summary of Site NCBD Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.46 (0.0047) (0.44 - 0.47)	0.48 (0.0053) (0.47 - 0.49)	-0.018 (0.0055) (-0.025 - -0.0092)	-0.033, -0.0044	0.019	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.50 (0.037) (2.39 - 2.64)	2.34 (0.043) (2.32 - 2.38)	0.16 (0.057) (0.011 - 0.22)	0.015, 0.31	0.036	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	15.04 (0.12) (14.58 - 15.26)	14.70 (0.14) (14.51 - 14.83)	0.35 (0.19) (-0.17 - 0.75)	-0.14, 0.84	0.127	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	56.95 (0.23) (56.35 - 57.88)	57.19 (0.26) (57.01 - 57.46)	-0.24 (0.35) (-1.12 - 0.80)	-1.13, 0.66	0.528	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.31 (0.012) (0.27 - 0.34)	0.29 (0.014) (0.27 - 0.30)	0.028 (0.018) (-0.0012 - 0.052)	-0.018, 0.074	0.178	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.28 (0.0065) (0.26 - 0.30)	0.28 (0.0075) (0.27 - 0.28)	0.0046 (0.0099) (-0.024 - 0.024)	-0.021, 0.030	0.663	0.17, 0.38 (0.20 - 0.36)

Table H-11. Statistical Summary of Site NCBD Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.16 (0.0056) (0.15 - 0.19)	0.15 (0.0065) (0.15 - 0.16)	0.011 (0.0086) (-0.0044 - 0.032)	-0.011, 0.033	0.258	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.15 (0.0014) (0.14 - 0.15)	0.14 (0.0017) (0.14 - 0.14)	0.0095 (0.0022) (0.0056 - 0.013)	0.0039, 0.015	0.007	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	6.82 (0.36) (5.81 - 7.58)	6.91 (0.41) (6.64 - 7.19)	-0.084 (0.54) (-1.38 - 0.53)	-1.48, 1.31	0.883	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	43.21 (1.00) (41.96 - 44.44)	48.04 (1.15) (45.03 - 50.87)	-4.83 (1.53) (-6.43 - -2.22)	-8.75, -0.90	0.025	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.41 (0.010) (0.40 - 0.43)	0.40 (0.012) (0.37 - 0.44)	0.011 (0.016) (-0.036 - 0.049)	-0.030, 0.052	0.529	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	14.12 (0.36) (13.57 - 14.81)	13.83 (0.42) (13.65 - 14.11)	0.29 (0.56) (-0.54 - 0.52)	-1.14, 1.72	0.622	9.07, 17.33 (9.07 - 17.14)

Table H-11. Statistical Summary of Site NCBD Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (% dw)	0.64 (0.020) (0.61 - 0.67)	0.66 (0.023) (0.59 - 0.71)	-0.020 (0.031) (-0.087 - 0.068)	-0.099, 0.059	0.548	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.06 (0.019) (1.04 - 1.09)	1.08 (0.022) (1.03 - 1.16)	-0.022 (0.029) (-0.12 - 0.032)	-0.097, 0.052	0.471	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.11 (0.0084) (0.068 - 0.12)	0.099 (0.0096) (0.094 - 0.10)	0.0074 (0.011) (-0.031 - 0.030)	-0.022, 0.036	0.539	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	40.79 (1.30) (40.28 - 41.37)	49.54 (1.50) (44.04 - 52.95)	-8.75 (1.98) (-11.57 - -3.76)	-13.84, -3.66	0.006	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	169.03 (3.66) (163.57 - 179.34)	156.99 (4.23) (151.55 - 162.98)	12.04 (5.60) (8.84 - 16.38)	-2.34, 26.43	0.084	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-12. Statistical Summary of Site NCBD Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.14 (0.0093) (0.13 - 0.15)	0.13 (0.011) (0.12 - 0.14)	0.012 (0.014) (0.0062 - 0.024)	-0.024, 0.048	0.422	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.36 (0.040) (0.20 - 0.43)	0.37 (0.046) (0.36 - 0.41)	-0.017 (0.056) (0.0058 - 0.056)	-0.16, 0.13	0.773	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.22 (0.024) (0.13 - 0.27)	0.22 (0.028) (0.21 - 0.23)	-0.0024 (0.037) (0.0078 - 0.040)	-0.098, 0.093	0.951	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	0.94 (0.026) (0.91 - 0.97)	0.90 (0.030) (0.81 - 0.95)	0.038 (0.039) (-0.024 - 0.097)	-0.063, 0.14	0.374	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	1.12 (0.067) (1.07 - 1.18)	1.09 (0.077) (1.08 - 1.10)	0.037 (0.10) (-0.0038 - 0.050)	-0.22, 0.30	0.731	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-13. Statistical Summary of Site NMLC Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.16 (0.087) (3.77 - 4.38)	4.27 (0.10) (4.20 - 4.39)	-0.11 (0.13) (-0.093 - 0.13)	-0.45, 0.22	0.434	3.42, 4.65 (3.18 - 4.68)
Calories	496.46 (2.64) (486.87 - 500.48)	492.81 (3.05) (490.52 - 494.31)	3.66 (4.04) (-7.44 - 9.96)	-6.73, 14.04	0.406	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	42.09 (0.46) (41.40 - 43.69)	42.60 (0.53) (42.14 - 43.05)	-0.50 (0.70) (-1.65 - 1.09)	-2.31, 1.30	0.504	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	6.59 (0.25) (5.93 - 7.28)	7.28 (0.28) (6.63 - 7.75)	-0.70 (0.32) (-1.82 - -0.34)	-1.53, 0.13	0.082	4.79, 9.92 (6.05 - 10.50)
Protein	31.15 (0.13) (30.63 - 31.47)	31.18 (0.15) (31.00 - 31.27)	-0.025 (0.19) (-0.65 - 0.46)	-0.52, 0.47	0.902	22.30, 29.41 (20.58 - 29.28)
Total Fat	22.59 (0.54) (20.62 - 23.58)	21.95 (0.62) (21.42 - 22.22)	0.64 (0.83) (-1.60 - 2.16)	-1.48, 2.77	0.471	15.01, 28.51 (16.58 - 25.25)

Table H-13. Statistical Summary of Site NMLC Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Acid Detergent Fiber	24.46 (0.51) (23.26 - 25.45)	26.40 (0.59) (25.76 - 27.10)	-1.94 (0.79) (-3.08 - -1.80)	-3.96, 0.080	0.056	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	17.90 (0.74) (17.33 - 18.57)	17.71 (0.84) (16.06 - 20.78)	0.20 (0.94) (-2.21 - 1.44)	-2.23, 2.63	0.841	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	29.73 (0.87) (27.53 - 32.00)	32.83 (1.00) (31.58 - 34.49)	-3.09 (1.32) (-6.95 - -0.41)	-6.49, 0.31	0.066	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	39.16 (0.71) (37.46 - 40.44)	41.10 (0.75) (39.09 - 43.00)	-1.94 (0.55) (-3.61 - -0.88)	-3.36, -0.53	0.016	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.11 (0.011) (1.10 - 1.13)	1.13 (0.013) (1.09 - 1.17)	-0.019 (0.014) (-0.044 - 0.0067)	-0.054, 0.015	0.212	0.86, 1.11 (0.83 - 1.22)
Arginine	3.48 (0.049) (3.42 - 3.60)	3.71 (0.053) (3.67 - 3.77)	-0.23 (0.048) (-0.34 - -0.17)	-0.35, -0.10	0.005	2.38, 3.47 (2.30 - 3.55)

Table H-13. Statistical Summary of Site NMLC Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.60 (0.033) (2.55 - 2.64)	2.66 (0.037) (2.58 - 2.74)	-0.062 (0.043) (-0.11 - -0.031)	-0.17, 0.048	0.206	1.94, 2.57 (1.79 - 2.72)
Cystine	0.43 (0.013) (0.39 - 0.47)	0.44 (0.015) (0.43 - 0.45)	-0.010 (0.020) (-0.063 - 0.038)	-0.061, 0.040	0.620	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	5.30 (0.087) (5.24 - 5.38)	5.46 (0.10) (5.29 - 5.70)	-0.16 (0.13) (-0.46 - -0.0085)	-0.50, 0.18	0.285	3.74, 5.28 (3.39 - 5.45)
Glycine	1.16 (0.014) (1.14 - 1.19)	1.18 (0.015) (1.17 - 1.20)	-0.021 (0.016) (-0.037 - -0.013)	-0.063, 0.020	0.247	0.90, 1.14 (0.85 - 1.23)
Histidine	0.82 (0.010) (0.80 - 0.85)	0.83 (0.012) (0.83 - 0.84)	-0.0065 (0.015) (-0.026 - 0.024)	-0.044, 0.031	0.675	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.97 (0.017) (0.94 - 1.01)	0.98 (0.018) (0.94 - 1.03)	-0.011 (0.012) (-0.038 - 0.021)	-0.041, 0.019	0.397	0.75, 0.96 (0.72 - 1.03)

Table H-13. Statistical Summary of Site NMLC Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Leucine	1.65 (0.021) (1.62 - 1.70)	1.66 (0.023) (1.63 - 1.69)	-0.0069 (0.022) (-0.039 - 0.0098)	-0.063, 0.049	0.763	1.25, 1.62 (1.20 - 1.72)
Lysine	1.33 (0.019) (1.26 - 1.38)	1.36 (0.022) (1.32 - 1.39)	-0.030 (0.022) (-0.058 - -0.0056)	-0.087, 0.027	0.232	1.01, 1.30 (0.99 - 1.44)
Methionine	0.43 (0.012) (0.40 - 0.46)	0.40 (0.014) (0.38 - 0.41)	0.033 (0.018) (-0.013 - 0.071)	-0.014, 0.081	0.129	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.61 (0.026) (1.56 - 1.66)	1.61 (0.028) (1.60 - 1.66)	-0.0044 (0.022) (-0.038 - 0.015)	-0.061, 0.052	0.850	1.12, 1.58 (1.10 - 1.63)
Proline	1.14 (0.027) (1.09 - 1.21)	1.18 (0.031) (1.10 - 1.25)	-0.040 (0.039) (-0.080 - -0.015)	-0.14, 0.060	0.353	0.83, 1.08 (0.79 - 1.17)
Serine	1.17 (0.026) (1.15 - 1.23)	1.20 (0.030) (1.16 - 1.24)	-0.028 (0.040) (-0.069 - -0.0042)	-0.13, 0.075	0.512	0.83, 1.21 (0.81 - 1.24)

Table H-13. Statistical Summary of Site NMLC Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.92 (0.015) (0.89 - 0.94)	0.93 (0.017) (0.91 - 0.94)	-0.0072 (0.021) (-0.024 - 0.0027)	-0.062, 0.048	0.747	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.46 (0.014) (0.43 - 0.52)	0.44 (0.016) (0.43 - 0.45)	0.019 (0.020) (-0.019 - 0.076)	-0.032, 0.071	0.382	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.87 (0.014) (0.85 - 0.92)	0.89 (0.015) (0.86 - 0.91)	-0.012 (0.014) (-0.039 - 0.0089)	-0.048, 0.024	0.440	0.67, 0.84 (0.63 - 0.91)
Valine	1.32 (0.024) (1.26 - 1.40)	1.34 (0.026) (1.31 - 1.40)	-0.021 (0.019) (-0.048 - 0.0048)	-0.070, 0.029	0.329	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.93 (0.0046) (0.92 - 0.95)	0.98 (0.0054) (0.97 - 0.98)	-0.043 (0.0071) (-0.060 - -0.037)	-0.062, -0.025	0.001	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	24.19 (0.088) (24.02 - 24.42)	24.11 (0.10) (23.89 - 24.34)	0.083 (0.13) (-0.32 - 0.33)	-0.26, 0.43	0.562	16.54, 30.55 (19.11 - 26.73)

Table H-13. Statistical Summary of Site NMLC Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.53 (0.0022) (0.52 - 0.53)	0.54 (0.0025) (0.53 - 0.54)	-0.012 (0.0033) (-0.019 - -0.0082)	-0.021, -0.0037	0.014	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.51 (0.020) (2.47 - 2.56)	2.64 (0.021) (2.61 - 2.70)	-0.14 (0.016) (-0.15 - -0.095)	-0.18, -0.094	<0.001	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	16.21 (0.067) (16.03 - 16.40)	16.21 (0.076) (16.10 - 16.35)	0.0024 (0.088) (-0.11 - 0.24)	-0.22, 0.23	0.979	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	54.32 (0.084) (54.30 - 54.33)	54.29 (0.097) (54.04 - 54.50)	0.029 (0.13) (-0.18 - 0.30)	-0.30, 0.36	0.833	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.16 (0.0019) (0.15 - 0.16)	0.14 (0.0022) (0.14 - 0.15)	0.012 (0.0029) (0.0078 - 0.014)	0.0043, 0.019	0.009	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.30 (0.0052) (0.29 - 0.30)	0.31 (0.0060) (0.28 - 0.32)	-0.011 (0.0080) (-0.025 - 0.014)	-0.031, 0.0095	0.225	0.17, 0.38 (0.20 - 0.36)

Table H-13. Statistical Summary of Site NMLC Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.16 (0.0065) (0.16 - 0.17)	0.19 (0.0075) (0.17 - 0.21)	-0.023 (0.0099) (-0.049 - -0.0086)	-0.049, 0.0021	0.065	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.15 (0.0029) (0.14 - 0.15)	0.13 (0.0034) (0.12 - 0.13)	0.021 (0.0042) (0.0081 - 0.031)	0.011, 0.032	0.003	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	11.35 (0.15) (11.11 - 11.91)	11.75 (0.17) (11.46 - 11.92)	-0.40 (0.22) (-0.76 - 0.060)	-0.97, 0.18	0.134	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	63.88 (3.33) (60.27 - 66.59)	64.62 (3.84) (63.58 - 66.45)	-0.74 (5.08) (-6.18 - 2.76)	-13.80, 12.32	0.890	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.39 (0.0053) (0.38 - 0.41)	0.37 (0.0061) (0.36 - 0.38)	0.019 (0.0081) (0.0045 - 0.036)	-0.0015, 0.040	0.062	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	12.93 (0.23) (12.73 - 13.13)	12.90 (0.26) (12.00 - 13.47)	0.029 (0.34) (-0.47 - 1.13)	-0.86, 0.92	0.936	9.07, 17.33 (9.07 - 17.14)

Table H-13. Statistical Summary of Site NMLC Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (% dw)	0.77 (0.010) (0.74 - 0.80)	0.79 (0.012) (0.78 - 0.80)	-0.020 (0.016) (-0.035 - 0.019)	-0.060, 0.021	0.264	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.10 (0.016) (1.05 - 1.14)	1.11 (0.018) (1.07 - 1.14)	-0.0086 (0.017) (-0.020 - -0.0045)	-0.052, 0.035	0.636	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.021 (0.0034) (0.019 - 0.022)	0.013 (0.0039) (0.0054 - 0.023)	0.0075 (0.0052) (-0.0044 - 0.016)	-0.0057, 0.021	0.203	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	45.63 (0.60) (44.12 - 46.74)	49.43 (0.69) (47.66 - 50.87)	-3.80 (0.92) (-5.64 - -0.92)	-6.16, -1.44	0.009	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	114.29 (2.04) (107.78 - 119.15)	112.18 (2.36) (107.02 - 115.99)	2.11 (3.12) (-5.75 - 12.13)	-5.90, 10.12	0.528	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-14. Statistical Summary of Site NMLC Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.16 (0.0079) (0.14 - 0.18)	0.14 (0.0092) (0.12 - 0.15)	0.019 (0.012) (-0.0065 - 0.041)	-0.012, 0.050	0.178	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.34 (0.023) (0.30 - 0.38)	0.29 (0.026) (0.26 - 0.31)	0.054 (0.035) (-0.0093 - 0.074)	-0.035, 0.14	0.177	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.20 (0.018) (0.18 - 0.23)	0.18 (0.020) (0.17 - 0.19)	0.018 (0.027) (-0.012 - 0.032)	-0.051, 0.087	0.531	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	0.85 (0.026) (0.83 - 0.88)	0.69 (0.030) (0.68 - 0.70)	0.15 (0.040) (0.14 - 0.18)	0.052, 0.26	0.011	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	0.92 (0.026) (0.84 - 0.97)	0.80 (0.030) (0.74 - 0.87)	0.12 (0.040) (0.060 - 0.18)	0.022, 0.23	0.026	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-15. Statistical Summary of Site SCEK Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.11 (0.095) (3.99 - 4.28)	3.74 (0.095) (3.38 - 3.98)	0.37 (0.10) (0.25 - 0.61)	0.12, 0.62	0.010	3.42, 4.65 (3.18 - 4.68)
Calories	511.69 (2.44) (505.01 - 517.46)	503.38 (2.44) (499.09 - 512.65)	8.31 (3.45) (-7.65 - 18.37)	-0.13, 16.75	0.052	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	46.56 (0.54) (45.10 - 47.48)	48.67 (0.54) (47.50 - 49.59)	-2.11 (0.75) (-3.20 - -0.23)	-3.95, -0.27	0.031	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	6.73 (0.17) (6.27 - 7.13)	7.08 (0.17) (6.63 - 7.37)	-0.35 (0.19) (-0.89 - 0.030)	-0.81, 0.12	0.119	4.79, 9.92 (6.05 - 10.50)
Protein	23.70 (0.42) (22.71 - 24.70)	23.92 (0.42) (23.56 - 24.61)	-0.22 (0.49) (-0.85 - 0.64)	-1.43, 0.98	0.669	22.30, 29.41 (20.58 - 29.28)
Total Fat	25.65 (0.44) (24.23 - 26.78)	23.65 (0.44) (22.92 - 25.20)	2.00 (0.63) (-0.97 - 3.86)	0.46, 3.54	0.019	15.01, 28.51 (16.58 - 25.25)

Table H-15. Statistical Summary of Site SCEK Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Acid Detergent Fiber	25.95 (0.44) (24.75 - 26.52)	27.04 (0.44) (26.24 - 27.74)	-1.09 (0.60) (-1.75 - -0.099)	-2.56, 0.37	0.118	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	19.72 (0.80) (17.98 - 21.66)	19.13 (0.80) (16.91 - 21.70)	0.59 (1.13) (-2.59 - 4.75)	-2.17, 3.36	0.617	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	31.34 (0.67) (29.42 - 32.89)	33.60 (0.67) (32.74 - 35.52)	-2.27 (0.95) (-6.10 - -0.44)	-4.59, 0.049	0.053	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	39.72 (0.62) (38.66 - 40.44)	41.87 (0.62) (40.16 - 43.29)	-2.14 (0.88) (-3.56 - -0.63)	-4.29, 0.0015	0.050	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	0.96 (0.022) (0.91 - 1.00)	0.94 (0.022) (0.88 - 0.97)	0.017 (0.029) (-0.020 - 0.033)	-0.055, 0.089	0.583	0.86, 1.11 (0.83 - 1.22)
Arginine	2.52 (0.088) (2.33 - 2.74)	2.59 (0.088) (2.41 - 2.71)	-0.063 (0.10) (-0.18 - 0.021)	-0.31, 0.18	0.556	2.38, 3.47 (2.30 - 3.55)

Table H-15. Statistical Summary of Site SCEK Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.08 (0.054) (1.94 - 2.20)	2.07 (0.054) (1.92 - 2.18)	0.0087 (0.070) (-0.085 - 0.096)	-0.16, 0.18	0.904	1.94, 2.57 (1.79 - 2.72)
Cystine	0.36 (0.017) (0.32 - 0.41)	0.35 (0.017) (0.31 - 0.39)	0.0082 (0.018) (-0.018 - 0.024)	-0.036, 0.052	0.666	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.14 (0.13) (3.80 - 4.40)	4.10 (0.13) (3.66 - 4.40)	0.039 (0.18) (-0.11 - 0.14)	-0.40, 0.47	0.833	3.74, 5.28 (3.39 - 5.45)
Glycine	0.99 (0.022) (0.93 - 1.04)	0.98 (0.022) (0.91 - 1.02)	0.016 (0.031) (-0.0040 - 0.033)	-0.061, 0.093	0.627	0.90, 1.14 (0.85 - 1.23)
Histidine	0.64 (0.021) (0.58 - 0.70)	0.64 (0.021) (0.61 - 0.66)	0.0012 (0.025) (-0.053 - 0.033)	-0.060, 0.062	0.961	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.81 (0.023) (0.75 - 0.88)	0.82 (0.023) (0.77 - 0.83)	-0.0053 (0.030) (-0.077 - 0.054)	-0.078, 0.068	0.865	0.75, 0.96 (0.72 - 1.03)

Table H-15. Statistical Summary of Site SCEK Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Leucine	1.36 (0.034) (1.29 - 1.46)	1.36 (0.034) (1.28 - 1.40)	0.0086 (0.046) (-0.053 - 0.064)	-0.10, 0.12	0.858	1.25, 1.62 (1.20 - 1.72)
Lysine	1.12 (0.027) (1.05 - 1.18)	1.11 (0.027) (1.06 - 1.17)	0.0024 (0.025) (-0.018 - 0.026)	-0.059, 0.064	0.925	1.01, 1.30 (0.99 - 1.44)
Methionine	0.38 (0.016) (0.35 - 0.42)	0.33 (0.016) (0.32 - 0.35)	0.041 (0.020) (0.017 - 0.077)	-0.0093, 0.091	0.093	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.22 (0.033) (1.14 - 1.31)	1.23 (0.033) (1.15 - 1.27)	-0.0097 (0.039) (-0.075 - 0.043)	-0.11, 0.086	0.811	1.12, 1.58 (1.10 - 1.63)
Proline	0.87 (0.026) (0.82 - 0.92)	0.87 (0.026) (0.81 - 0.93)	0.0028 (0.026) (-0.035 - 0.034)	-0.060, 0.066	0.916	0.83, 1.08 (0.79 - 1.17)
Serine	0.98 (0.028) (0.90 - 1.04)	0.96 (0.028) (0.86 - 1.03)	0.019 (0.033) (-0.0099 - 0.042)	-0.062, 0.10	0.587	0.83, 1.21 (0.81 - 1.24)

Table H-15. Statistical Summary of Site SCEK Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.79 (0.019) (0.74 - 0.83)	0.77 (0.019) (0.73 - 0.81)	0.016 (0.023) (0.0053 - 0.039)	-0.041, 0.073	0.518	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.35 (0.0093) (0.33 - 0.38)	0.38 (0.0093) (0.37 - 0.40)	-0.025 (0.0085) (-0.040 - -0.0010)	-0.046, -0.0048	0.023	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.72 (0.020) (0.67 - 0.78)	0.71 (0.020) (0.67 - 0.74)	0.0042 (0.025) (-0.033 - 0.040)	-0.058, 0.066	0.873	0.67, 0.84 (0.63 - 0.91)
Valine	1.07 (0.029) (1.00 - 1.14)	1.07 (0.029) (1.00 - 1.10)	0.0056 (0.039) (-0.084 - 0.049)	-0.090, 0.10	0.889	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.70 (0.011) (0.67 - 0.72)	0.73 (0.011) (0.72 - 0.75)	-0.028 (0.012) (-0.049 - 0.0062)	-0.057, 0.00046	0.052	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	24.74 (0.086) (24.59 - 24.94)	24.39 (0.086) (24.07 - 24.59)	0.35 (0.12) (0.17 - 0.61)	0.049, 0.65	0.029	16.54, 30.55 (19.11 - 26.73)

Table H-15. Statistical Summary of Site SCEK Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.48 (0.0056) (0.47 - 0.49)	0.48 (0.0056) (0.47 - 0.49)	-0.0075 (0.0076) (-0.020 - 0.018)	-0.026, 0.011	0.361	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.78 (0.036) (2.75 - 2.85)	2.67 (0.036) (2.58 - 2.76)	0.11 (0.050) (0.0076 - 0.18)	-0.015, 0.23	0.076	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	14.40 (0.11) (14.15 - 14.68)	14.46 (0.11) (14.42 - 14.49)	-0.059 (0.15) (-0.33 - 0.19)	-0.43, 0.31	0.706	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	55.54 (0.18) (55.18 - 55.96)	55.87 (0.18) (55.61 - 56.29)	-0.33 (0.25) (-1.11 - 0.13)	-0.94, 0.29	0.242	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.15 (0.0017) (0.15 - 0.16)	0.15 (0.0017) (0.14 - 0.15)	0.0044 (0.0023) (-0.00022 - 0.011)	-0.0012, 0.0099	0.103	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.29 (0.0070) (0.27 - 0.31)	0.30 (0.0070) (0.29 - 0.30)	-0.0046 (0.0099) (-0.027 - 0.019)	-0.029, 0.020	0.656	0.17, 0.38 (0.20 - 0.36)

Table H-15. Statistical Summary of Site SCEK Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.14 (0.0033) (0.13 - 0.14)	0.14 (0.0033) (0.13 - 0.14)	-0.0014 (0.0046) (-0.0030 - 0.00006)	-0.013, 0.0099	0.765	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.11 (0.0040) (0.10 - 0.11)	0.091 (0.0040) (0.081 - 0.095)	0.016 (0.0056) (0.0059 - 0.023)	0.0025, 0.030	0.027	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	5.82 (0.24) (5.22 - 6.30)	5.64 (0.24) (5.40 - 5.85)	0.18 (0.32) (-0.28 - 0.55)	-0.61, 0.97	0.593	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	63.78 (4.68) (59.75 - 67.62)	73.46 (4.68) (63.01 - 89.93)	-9.68 (4.30) (-22.31 - -1.16)	-20.21, 0.84	0.065	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.39 (0.0090) (0.37 - 0.41)	0.36 (0.0090) (0.34 - 0.37)	0.033 (0.0080) (0.014 - 0.044)	0.014, 0.053	0.005	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	11.39 (0.51) (10.88 - 11.68)	9.72 (0.51) (8.61 - 11.03)	1.67 (0.72) (0.65 - 2.27)	-0.080, 3.42	0.058	9.07, 17.33 (9.07 - 17.14)

Table H-15. Statistical Summary of Site SCEK Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (% dw)	0.67 (0.022) (0.64 - 0.71)	0.63 (0.022) (0.58 - 0.68)	0.035 (0.018) (0.0036 - 0.072)	-0.0093, 0.080	0.100	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.13 (0.031) (1.11 - 1.17)	1.02 (0.031) (0.88 - 1.08)	0.11 (0.043) (0.046 - 0.23)	0.0051, 0.22	0.042	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.022 (0.0033) (0.018 - 0.027)	0.015 (0.0033) (0.012 - 0.023)	0.0070 (0.0046) (0.0039 - 0.013)	-0.0043, 0.018	0.180	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	29.14 (0.82) (27.31 - 31.57)	30.08 (0.82) (28.22 - 31.74)	-0.94 (0.82) (-2.85 - 1.07)	-2.96, 1.08	0.297	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	162.17 (1.77) (158.92 - 165.82)	158.20 (1.77) (153.15 - 162.63)	3.97 (2.50) (2.38 - 7.55)	-2.16, 10.10	0.164	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-16. Statistical Summary of Site SCEK Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.15 (0.0087) (0.12 - 0.18)	0.15 (0.0087) (0.14 - 0.15)	0.0014 (0.012) (-0.023 - 0.043)	-0.029, 0.031	0.914	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.41 (0.029) (0.33 - 0.52)	0.43 (0.029) (0.39 - 0.46)	-0.022 (0.041) (-0.11 - 0.13)	-0.12, 0.078	0.607	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.23 (0.014) (0.19 - 0.28)	0.24 (0.014) (0.22 - 0.25)	-0.011 (0.020) (-0.060 - 0.060)	-0.060, 0.038	0.607	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	1.10 (0.029) (1.05 - 1.18)	1.13 (0.029) (1.06 - 1.20)	-0.030 (0.027) (-0.086 - 0.00085)	-0.096, 0.035	0.303	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	1.17 (0.025) (1.13 - 1.23)	1.07 (0.025) (1.05 - 1.10)	0.10 (0.031) (0.074 - 0.13)	0.026, 0.18	0.017	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-17. Statistical Summary of Site TXPL Cottonseed Nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control)			Commercial Tolerance Interval[5] (Range)
			Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	
Proximate (% dw)						
Ash	3.85 (0.051) (3.77 - 3.92)	3.46 (0.051) (3.34 - 3.61)	0.40 (0.072) (0.24 - 0.58)	0.22, 0.57	0.001	3.42, 4.65 (3.18 - 4.68)
Calories	498.01 (2.54) (489.04 - 502.78)	494.42 (2.54) (489.10 - 500.98)	3.59 (3.47) (-1.24 - 13.67)	-4.90, 12.07	0.340	457.61, 527.56 (466.09 - 509.91)
Carbohydrates	44.03 (0.48) (42.73 - 45.99)	46.39 (0.48) (45.65 - 47.07)	-2.36 (0.64) (-3.73 - -0.88)	-3.92, -0.79	0.010	40.26, 56.45 (43.28 - 54.90)
Moisture (% fw)	6.88 (0.21) (6.32 - 7.37)	7.47 (0.21) (7.11 - 7.79)	-0.59 (0.29) (-1.47 - 0.18)	-1.30, 0.13	0.090	4.79, 9.92 (6.05 - 10.50)
Protein	29.43 (0.24) (29.06 - 30.14)	28.48 (0.24) (28.09 - 28.77)	0.95 (0.29) (0.38 - 1.82)	0.24, 1.66	0.017	22.30, 29.41 (20.58 - 29.28)
Total Fat	22.71 (0.48) (20.94 - 23.59)	21.70 (0.48) (20.71 - 22.88)	1.01 (0.65) (0.15 - 2.88)	-0.58, 2.61	0.169	15.01, 28.51 (16.58 - 25.25)

Table H-17. Statistical Summary of Site TXPL Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Acid Detergent Fiber	25.53 (0.37) (25.31 - 25.83)	25.53 (0.37) (24.51 - 26.91)	0.0023 (0.41) (-1.08 - 0.91)	-1.01, 1.01	0.995	22.24, 31.96 (23.42 - 31.62)
Crude Fiber	17.93 (0.38) (17.17 - 18.84)	18.10 (0.38) (17.35 - 19.63)	-0.17 (0.45) (-1.48 - 1.07)	-1.26, 0.92	0.716	16.93, 22.68 (16.92 - 23.32)
Neutral Detergent Fiber	29.75 (0.41) (28.74 - 30.56)	32.12 (0.41) (30.49 - 33.05)	-2.38 (0.58) (-4.31 - -0.26)	-3.80, -0.96	0.006	27.03, 42.49 (29.27 - 40.63)
Total Dietary Fiber	39.54 (0.62) (38.76 - 40.86)	40.47 (0.62) (39.15 - 42.09)	-0.93 (0.72) (-3.24 - 0.56)	-2.69, 0.83	0.245	34.52, 52.58 (37.29 - 48.60)
Amino Acid (% dw)						
Alanine	1.07 (0.019) (1.05 - 1.10)	1.05 (0.019) (0.97 - 1.10)	0.022 (0.026) (-0.040 - 0.12)	-0.042, 0.086	0.438	0.86, 1.11 (0.83 - 1.22)
Arginine	3.25 (0.074) (3.15 - 3.33)	3.25 (0.074) (2.94 - 3.49)	-0.0020 (0.10) (-0.34 - 0.39)	-0.26, 0.25	0.985	2.38, 3.47 (2.30 - 3.55)

Table H-17. Statistical Summary of Site TXPL Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Aspartic Acid	2.51 (0.047) (2.43 - 2.55)	2.45 (0.047) (2.26 - 2.62)	0.060 (0.067) (-0.092 - 0.29)	-0.10, 0.22	0.407	1.94, 2.57 (1.79 - 2.72)
Cystine	0.41 (0.015) (0.39 - 0.43)	0.40 (0.015) (0.36 - 0.45)	0.0068 (0.017) (-0.030 - 0.073)	-0.034, 0.047	0.697	0.31, 0.45 (0.29 - 0.47)
Glutamic Acid	4.94 (0.13) (4.73 - 5.14)	5.02 (0.13) (4.41 - 5.32)	-0.072 (0.19) (-0.44 - 0.73)	-0.53, 0.39	0.714	3.74, 5.28 (3.39 - 5.45)
Glycine	1.12 (0.022) (1.07 - 1.15)	1.11 (0.022) (1.03 - 1.19)	0.0062 (0.031) (-0.073 - 0.12)	-0.070, 0.082	0.849	0.90, 1.14 (0.85 - 1.23)
Histidine	0.77 (0.018) (0.73 - 0.81)	0.76 (0.018) (0.71 - 0.82)	0.017 (0.026) (-0.062 - 0.091)	-0.046, 0.079	0.538	0.59, 0.81 (0.57 - 0.84)
Isoleucine	0.95 (0.013) (0.93 - 0.97)	0.93 (0.013) (0.89 - 0.97)	0.013 (0.017) (-0.023 - 0.051)	-0.027, 0.054	0.457	0.75, 0.96 (0.72 - 1.03)

Table H-17. Statistical Summary of Site TXPL Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Leucine	1.58 (0.026) (1.52 - 1.61)	1.55 (0.026) (1.45 - 1.64)	0.028 (0.037) (-0.072 - 0.16)	-0.064, 0.12	0.486	1.25, 1.62 (1.20 - 1.72)
Lysine	1.26 (0.026) (1.21 - 1.33)	1.24 (0.026) (1.19 - 1.33)	0.024 (0.037) (-0.11 - 0.13)	-0.067, 0.12	0.537	1.01, 1.30 (0.99 - 1.44)
Methionine	0.40 (0.021) (0.37 - 0.44)	0.39 (0.021) (0.32 - 0.44)	0.016 (0.029) (-0.066 - 0.12)	-0.056, 0.088	0.605	0.32, 0.38 (0.29 - 0.49)
Phenylalanine	1.51 (0.034) (1.46 - 1.55)	1.48 (0.034) (1.36 - 1.61)	0.026 (0.048) (-0.14 - 0.19)	-0.092, 0.14	0.614	1.12, 1.58 (1.10 - 1.63)
Proline	1.04 (0.024) (0.99 - 1.11)	1.04 (0.024) (1.01 - 1.11)	0.0028 (0.033) (-0.12 - 0.10)	-0.079, 0.084	0.935	0.83, 1.08 (0.79 - 1.17)
Serine	1.11 (0.031) (1.06 - 1.13)	1.11 (0.031) (0.97 - 1.17)	0.00043 (0.044) (-0.087 - 0.16)	-0.11, 0.11	0.992	0.83, 1.21 (0.81 - 1.24)

Table H-17. Statistical Summary of Site TXPL Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Threonine	0.89 (0.016) (0.86 - 0.92)	0.86 (0.016) (0.80 - 0.91)	0.032 (0.022) (-0.026 - 0.10)	-0.022, 0.086	0.197	0.72, 0.89 (0.67 - 0.96)
Tryptophan	0.42 (0.013) (0.40 - 0.44)	0.43 (0.013) (0.38 - 0.47)	-0.0094 (0.018) (-0.070 - 0.030)	-0.054, 0.035	0.626	0.34, 0.42 (0.31 - 0.46)
Tyrosine	0.84 (0.017) (0.82 - 0.86)	0.81 (0.017) (0.74 - 0.87)	0.031 (0.024) (-0.048 - 0.12)	-0.028, 0.089	0.245	0.67, 0.84 (0.63 - 0.91)
Valine	1.26 (0.030) (1.21 - 1.29)	1.23 (0.030) (1.16 - 1.29)	0.024 (0.042) (-0.046 - 0.12)	-0.079, 0.13	0.586	1.00, 1.28 (0.97 - 1.36)
Fatty Acid (% Total FA)						
14:0 Myristic	0.86 (0.010) (0.84 - 0.88)	0.84 (0.010) (0.81 - 0.87)	0.017 (0.013) (-0.010 - 0.047)	-0.016, 0.050	0.246	0.16, 1.37 (0.45 - 1.04)
16:0 Palmitic	23.15 (0.14) (22.72 - 23.62)	23.01 (0.14) (22.86 - 23.25)	0.13 (0.16) (-0.27 - 0.76)	-0.27, 0.53	0.443	16.54, 30.55 (19.11 - 26.73)

Table H-17. Statistical Summary of Site TXPL Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.48 (0.0058) (0.47 - 0.49)	0.46 (0.0058) (0.45 - 0.47)	0.016 (0.0072) (0.0040 - 0.039)	-0.0018, 0.034	0.070	0.39, 0.70 (0.44 - 0.67)
18:0 Stearic	2.35 (0.025) (2.30 - 2.43)	2.46 (0.025) (2.40 - 2.52)	-0.11 (0.028) (-0.16 - -0.097)	-0.18, -0.047	0.006	1.98, 2.95 (1.98 - 2.97)
18:1 Oleic	16.34 (0.078) (16.22 - 16.45)	16.16 (0.078) (15.86 - 16.44)	0.18 (0.11) (-0.14 - 0.53)	-0.083, 0.45	0.143	11.38, 20.64 (13.71 - 18.39)
18:2 Linoleic	55.42 (0.21) (54.97 - 55.95)	55.58 (0.21) (55.18 - 56.13)	-0.15 (0.29) (-1.16 - 0.77)	-0.87, 0.56	0.616	47.49, 63.18 (49.78 - 59.61)
18:3 Linolenic	0.17 (0.0056) (0.17 - 0.18)	0.17 (0.0056) (0.16 - 0.17)	0.0057 (0.0079) (0.00091 - 0.011)	-0.014, 0.025	0.497	0.060, 0.24 (0.10 - 0.29)
20:0 Arachidic	0.28 (0.0055) (0.27 - 0.28)	0.28 (0.0055) (0.28 - 0.29)	-0.0041 (0.0078) (-0.0061 - -0.0022)	-0.023, 0.015	0.618	0.17, 0.38 (0.20 - 0.36)

Table H-17. Statistical Summary of Site TXPL Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
22:0 Behenic	0.16 (0.0015) (0.16 - 0.16)	0.16 (0.0015) (0.16 - 0.16)	0.0020 (0.0020) (-0.00054 - 0.0042)	-0.0029, 0.0068	0.362	0.070, 0.21 (0.051 - 0.19)
Mineral						
Calcium (% dw)	0.16 (0.0016) (0.16 - 0.16)	0.14 (0.0016) (0.13 - 0.14)	0.021 (0.0023) (0.018 - 0.023)	0.015, 0.027	<0.001	0.058, 0.21 (0.081 - 0.18)
Copper (mg/kg dw)	10.49 (0.15) (10.09 - 10.87)	9.98 (0.15) (9.58 - 10.41)	0.51 (0.22) (0.12 - 1.29)	-0.023, 1.04	0.057	2.97, 12.86 (4.46 - 11.62)
Iron (mg/kg dw)	60.47 (5.67) (56.94 - 66.50)	79.02 (5.67) (67.45 - 95.10)	-18.55 (7.06) (-38.15 - -0.95)	-35.82, -1.28	0.039	47.30, 97.12 (39.49 - 114.34)
Magnesium (% dw)	0.35 (0.0045) (0.35 - 0.37)	0.34 (0.0045) (0.33 - 0.34)	0.019 (0.0040) (0.0082 - 0.024)	0.0087, 0.028	0.003	0.28, 0.47 (0.31 - 0.46)
Manganese (mg/kg dw)	10.91 (0.34) (10.18 - 11.37)	9.04 (0.34) (8.83 - 9.54)	1.86 (0.48) (0.64 - 2.54)	0.70, 3.03	0.007	9.07, 17.33 (9.07 - 17.14)

Table H-17. Statistical Summary of Site TXPL Cottonseed Nutrients for MON 88701 vs. Conventional Control (continued)

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (% dw)	0.58 (0.0099) (0.56 - 0.61)	0.57 (0.0099) (0.54 - 0.60)	0.0036 (0.011) (-0.0098 - 0.018)	-0.022, 0.030	0.742	0.49, 0.87 (0.48 - 0.87)
Potassium (% dw)	1.01 (0.023) (0.98 - 1.06)	0.87 (0.023) (0.79 - 0.93)	0.14 (0.033) (0.073 - 0.27)	0.062, 0.22	0.004	0.92, 1.21 (0.90 - 1.26)
Sodium (% dw)	0.024 (0.010) (0.019 - 0.027)	0.047 (0.010) (0.019 - 0.090)	-0.023 (0.014) (-0.065 - 0.0062)	-0.058, 0.012	0.161	0, 0.066 (0.0054 - 0.077)
Zinc (mg/kg dw)	34.10 (0.45) (33.36 - 35.30)	34.96 (0.45) (33.70 - 35.89)	-0.86 (0.64) (-2.31 - 1.61)	-2.44, 0.71	0.227	27.27, 44.95 (25.07 - 48.49)
Vitamin (mg/kg dw)						
Vitamin E	114.39 (2.75) (107.81 - 118.39)	103.66 (2.75) (93.92 - 109.90)	10.73 (3.90) (6.69 - 14.40)	1.20, 20.26	0.033	41.91, 205.89 (84.07 - 162.76)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

Table H-18. Statistical Summary of Site TXPL Cottonseed Anti-nutrients for MON 88701 vs. Conventional Control

Analytical Component (Units)[1]	MON 88701[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88701 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Cyclopropenoid Fatty Acid (% Total FA)						
Dihydrosterculic Acid	0.16 (0.0047) (0.14 - 0.17)	0.16 (0.0047) (0.15 - 0.17)	-0.0044 (0.0066) (-0.026 - 0.021)	-0.021, 0.012	0.533	0.078, 0.25 (0.038 - 0.23)
Malvalic Acid	0.41 (0.020) (0.33 - 0.47)	0.47 (0.020) (0.44 - 0.49)	-0.054 (0.029) (-0.16 - 0.0068)	-0.12, 0.017	0.112	0.23, 0.54 (0.11 - 0.59)
Sterculic Acid	0.23 (0.013) (0.18 - 0.27)	0.26 (0.013) (0.25 - 0.27)	-0.026 (0.018) (-0.085 - 0.024)	-0.070, 0.017	0.189	0.17, 0.27 (0.061 - 0.34)
Gossypol (% dw)						
Free Gossypol	0.98 (0.024) (0.91 - 1.06)	0.93 (0.024) (0.91 - 0.95)	0.054 (0.033) (-0.025 - 0.13)	-0.028, 0.14	0.157	0.099, 1.57 (0.50 - 1.41)
Total Gossypol	1.06 (0.024) (1.03 - 1.11)	1.01 (0.024) (0.97 - 1.05)	0.053 (0.022) (-0.021 - 0.092)	-0.00013, 0.11	0.050	0.064, 1.76 (0.56 - 1.61)

[1]dw = dry weight; FA = fatty acid.
[2]MON 88701 plants were sprayed with dicamba and glufosinate.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the non-biotechnology derived, conventional control (Coker 130).
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

References for Appendix H

AOAC. 2011a. AOAC official method 988.15: Tryptophan in foods and food and feed ingredients. Official Methods of Analysis of AOAC International, Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011b. AOAC official method 923.03: Ash of flour. Official Methods of Analysis of AOAC International, Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011c. AOAC official method 962.09: Fiber (crude) in animal feed and pet food. Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011d. AOAC official method 948.22: Fat (crude) in nuts and nut products. Official Methods of Analysis of AOAC International, Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011e. AOAC official method 960.39: Fat (crude) or ether extract in meat. Official Methods of Analysis of AOAC International, Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011f. AOAC official method 984.27: Calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc in infant formula. Official Methods of Analysis of AOAC International, Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011g. AOAC official method 985.01: Metals and other elements in plants and pet foods. Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011h. AOAC official method 925.09: Solids (total) and loss on drying (moisture) in flour. Official Methods of Analysis of AOAC International, Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011i. AOAC official method 926.08: Loss on drying (moisture) in cheese. Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011j. AOAC official method 955.04: Nitrogen (total) in fertilizers Kjeldahl method. Official Methods of Analysis of AOAC International, Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011k. AOAC official method 979.09: Protein in grains. Official Methods of Analysis of AOAC International, Association of Analytical Communities International, Gaithersburg, Maryland.

AOAC. 2011l. AOAC official method 985.29: Total dietary fiber in foods. Association of Analytical Communities International, Gaithersburg, Maryland.

AOCS. 1997. Preparation of methyl esters of fatty acids. Method Ce 2-66, American Oil Chemists' Society, Champaign, Illinois.

AOCS. 2001. Determination of fatty acids in edible oils and fats by capillary GLC. Method Ce 1e-91, American Oil Chemists' Society, Champaign, Illinois.

AOCS. 2009a. Official Methods and Recommended Practices of the American Oil Chemists' Society. Method Ce 1i-07, American Oil Chemists' Society, Champaign, Illinois.

AOCS. 2009b. Nitrogen-ammonia-protein modified Kjeldahl method titanium dioxide + copper sulfate catalyst. Official Methods and Recommended Practices of the American Oil Chemists' Society, 5th Edition. Method Ac 4-91, American Oil Chemists' Society, Champaign, Illinois.

AOCS. 2009c. Direct methylation of lipids in foods for the determination of total fat, saturated, *cis*-monounsaturated, *cis*-polyunsaturated, and *trans* fatty acids by gas chromatography. Method Ce 1k-09, American Oil Chemists' Society, Champaign, Illinois.

AOCS. 2011a. Total gossypol. Method Ba 8-78, American Oil Chemists' Society, Champaign, Illinois.

AOCS. 2011b. Free gossypol. Method Ba 7-58, American Oil Chemists' Society, Champaign, Illinois.

Barkholt, V. and A.L. Jensen. 1989. Amino acid analysis determination of cysteine plus half-cystine in proteins after hydrochloric acid hydrolysis with a disulfide compound as additive. Analytical Biochemistry 177:318-322.

Cort, W., T.S. Vicente, E.H. Waysek and B.D. Williams. 1983. Vitamin E content of feedstuffs determined by high-performance liquid chromatographic fluorescence. Journal of Agriculture and Food Chemistry 31:1330-1333.

[redacted] and [redacted]. 2010. Improved amino acid methods using Agilent ZORBAX Eclipse Plus C18 columns for a variety of Agilent LC instrumentation and separation goals. Agilent Technologies, Inc., Wilmington, Delaware.

[redacted] and [redacted]. 2000. Rapid, accurate, sensitive, and reproducible HPLC analysis of amino acids. Agilent Technologies, Inc., Wilmington, Delaware.

Komarek, A.R., J.B. Robertson and P.J. Van Soest. 1993. A comparison of methods for determining ADF using the Filter Bag Technique versus conventional filtration. Journal of Animal Science 72: 114.

McMurray, C.H., W.J. Blanchflower and D.A. Rice. 1980. Influence of extraction techniques on determination of α–Tocopherol in animal feedstuffs. Journal of the Association of Official Analytical Chemists 63:1258-1261.

Schuster, R. 1988. Determination of amino acids in biological pharmaceutical plant and food samples by automated precolumn derivatization and high-performance liquid chromatography. Journal of Chromatography 431:271-284.

Speek, A.J., J. de Schrijver and W.H.P. Schreurs. 1985. Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorometric detection. Journal of Food Science 50:121-124.

USDA. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). Agricultural Handbook No. 379. U.S. Department of Agriculture, Washington, DC.

USDA. 1973. Energy value of foods: Basis and derivation. Agricultural Handbook No. 74. US Department of Agriculture, Agricultural Research Service, Washington, D.C.

Wood, R. 1986. High-performance liquid chromatography analysis of cyclopropene fatty-acids. Biochemical Archives 2:63-72.