Food and Feed Safety and Nutritional Assessment of Dicamba-Tolerant Soybean MON 87708 (OECD Unique Identifier MON-877Ø8-9)

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

November 9, 2010

FDA BNF 125

Monsanto 10-SY-216F

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CERTIFICATION

Monsanto Company is submitting this food and feed safety and nutritional assessment in compliance with the FDA's 1992 policy statement regarding foods derived from new plant varieties (57 FR 22984). At the agency's request, and where appropriate, this submission also complies with the recommendations contained in the proposed rule for Premarket Biotechnology Notice (PBN) Concerning Bioengineered Foods (66 FR 4706). Additionally, this submission complies with the Codex Plant Guidelines (CAC/GL 45-2003) 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) soybean MON 87708 is as safe and nutritious as other commercially-available soybean; and (b) the intended uses of the food and feed derived from MON 87708 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 thereafter 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 considerations that may be associated with MON 87708.

Signature: Date:

[redacted] November 9, 2010

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RELEASE OF INFORMATION

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

ABBREVIATIONS AND DEFINTITIONS[1]

~	Approximately
AD_2010	Allergen and gliadin protein sequence database (Release date January 22, 2010)
APHIS	Animal and Plant Health Inspection Service of the United States Department of Agriculture
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
CFR	Code of Federal Regulations
CHT	ceramic hydroxyapatite column
CV	column volume
Da	Dalton
DAP	days after planting
DCSA	3,6-dichlorosalicylic acid also known as 3,6-dichloro-2-hydroxybenzoic acid
DDI	daily dietary intake
DEEM	dietary exposure evaluation model
dicamba	3,6-dichloro-2-methoxybenzoic acid
dmo	Coding sequence of the dicamba mono-oxygenase gene from *Stenotrophomonas maltophilia* encoding DMO
DTT	dithiothreitol
DWCF	dry weight conversion factor
dwt	dry weight of tissue
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FARRP	Food Allergy Research and Resource Program Database
FASTA	algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FFDCA	Federal Food, Drug and Cosmetic Act
fwt	fresh weight of tissue
GenBank	A public genetic database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
GI	Gene Identification number
HRP	horseradish peroxidase
IAA	indole acetic acid
IgE	Immunoglobulin E
ILSI	International Life Sciences Institute
kDa	kiloDalton

[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.

LB	loading buffer
LOD	limit of detection
LOQ	limit of quantitation
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MOE	margin of exposure
MON 87708 DMO	The active form of DMO in MON 87708, a trimer comprised of three monomers. The DMO trimer can be comprised of MON 87708 DMO protein, MON 87708 DMO+27 protein, or a combination of both proteins.
MON 87708 DMO precursor protein	The produced precursor protein from the coding sequence of *dmo* expression cassette, that consists of the MON 87708 DMO protein, a chloroplast transit peptide (CTP), containing the first 24 amino acids from the N-terminus of the mature Rubisco small subunit, and an intervening sequence (IS).
MON 87708 DMO protein	full-length dicamba mono-oxygenase protein
MON 87708 DMO+27 protein	full-length dicamba mono-oxygenase protein with an additional 24 amino acids from the Rubisco small subunit and 3 amino acids from an intervening sequence
MW	molecular weight
MWCO	molecular weight cut off
N/A	not applicable
NADH	nicotinamide adenine dinucleotide
NCBI	National Center of Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
NFDM	non-fat dried milk
NOAEL	no observable adverse effect level
OSL	over-season leaf
PBS	phosphate buffered saline solution
PBST	phosphate buffered saline solution containing 0.05% (v/v) Tween-20
pea	*Pisum sativum*
PMSF	phenylmethylsulfonyl fluoride
PRT_2010	GenBank protein database, 175.0 (Release date January 22, 2010)
PVDF	polyvinylidene difluoride
RT	room temperature
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
S. maltophilia	*Stenotrophomonas maltophilia*
soybean	Glycine max (L.) Merr.
T-DNA	transfer(ed) DNA
TBS	tris buffered saline

TFA	trifluoroacetic acid
Tm	melting temperature
TMB	3,3',5,5' tetramethyl-benzidine
TOX_2010	Toxin protein sequence database (Release date January 22, 2010)
TSSP	tissue-specific pool
v/v	volume per volume
w/v	weight per volume

NAME AND ADDRESS OF SUBMITTER

The submitter of this safety and nutritional assessment summary for dicamba-tolerant soybean MON 87708 is:

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Communications with regard to this submission should be directed to [redacted] Regulatory Affairs Manager, at the Monsanto address listed above, or by telephone at [redacted], or by FAX at [redacted].

STATUS OF SUBMISSION TO USDA-APHIS

Monsanto requested a Determination of Nonregulated Status for MON 87708, including all progenies derived from crosses between MON 87708 and other soybean, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) in July 2010. Under regulations administered by USDA-APHIS (7 CFR 340), MON 87708 is currently considered a "regulated article." Monsanto will continue to conduct all field tests for MON 87708 in strict compliance with USDA field trial regulations until a Determination of Nonregulated Status is granted for MON 87708. Once MON 87708 is deregulated, authorization for import, interstate movement or environmental release of MON 87708 will no longer be required.

STATUS OF SUBMISSIONS TO OTHER GOVERNMENT AGENCIES

Regulatory submissions will be made to countries that import significant soybean or food and feed products derived from U.S. soybean and have functional regulatory review processes in place. This will result in submissions to a number of additional governmental regulatory agencies including, but not limited to Ministry of Agriculture, People's Republic of China; 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 European Food Safety Authority as well as to regulatory authorities in other soybean importing countries with functioning regulatory systems. As appropriate, notifications will be made to countries that import significant quantities of U.S. soybean and soybean products and do not have a formal regulatory review process for biotechnology-derived crops.

Furthermore, a regulatory submission has been made to the EPA requesting the establishment of the new, expanded use of dicamba on MON 87708. Dicamba residues on soybean seed (less than 0.07 ppm average residue and less than 0.5 ppm maximum residue) resulting from its application on MON 87708 at the maximum labeled use rate are well below the established 10 ppm soybean seed pesticide residue tolerance. Therefore, a change to the current soybean seed tolerance is not needed to support the use of dicamba on MON 87708. However Monsanto has requested the establishment of new tolerances for soybean forage and hay, which will allow for the feeding of forage and hay

to livestock. No other revisions to dicamba pesticide residue tolerances are needed including animal products such as meat or milk.

EXECUTIVE SUMMARY

Food and Feed Safety Assessment of MON 87708

Monsanto Company has developed biotechnology-derived soybean MON 87708 that is tolerant to dicamba herbicide. Tolerance of MON 87708 to dicamba will facilitate both preemergence and postemergence in-crop dicamba applications through the early reproductive (R1) growth stage. Dicamba provides effective control of over 95 annual and biennial weed species, and suppression of over 100 perennial broadleaf and woody plant species. Additionally, dicamba provides effective control of broadleaf weeds that are resistant to glyphosate and other commonly used herbicides, such as those in the sulfonylurea and triazine herbicide families. MON 87708 will be combined with glyphosate-tolerant soybean MON 89788 (Roundup Ready 2 Yield® soybean) utilizing traditional breeding techniques. The potential in-crop use of dicamba herbicide, in addition to glyphosate herbicide, enables an improved integrated weed management program to control a broad spectrum of grass and broadleaf weed species and convenient control of weeds resistant to several herbicide families.

MON 87708 soybean contains a gene derived from *Stenotrophomonas maltophilia* that expresses a mono-oxygenase that rapidly demethylates dicamba to an inactive metabolite 3,6-dichlorosalicylic acid (DCSA), a well known metabolite of dicamba in soybean and livestock. The safety of dicamba use on many crops, including soybean, was reviewed in 2006 by the Environmental Protection Agency (EPA) as part of the food, feed, and environmental safety reassessment. The existing 10 ppm pesticide residue tolerance for soybean seed supporting the current uses of dicamba on soybean (40 CFR § 180.227) is for the combined residues of parent dicamba and its metabolites, DCSA and 5-hydroxy dicamba. Studies have shown that the proposed use of dicamba on MON 87708 soybean results in total residue concentrations of parent dicamba and its metabolites (less than 0.07 ppm average residue, less than 0.5 ppm maximum residue) are well below the current 10 ppm tolerance. Consequently, an approval from EPA has been requested only for the expanded use pattern of dicamba on MON 87708 and the feeding of MON 87708 forage and hay to livestock, which requires the establishment of new forage and hay tolerances.

The data and information presented in this safety summary demonstrate that the food and feed derived from MON 87708 are as safe and nutritious as those derived from commercially-available, conventional soybean 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 Organization for Economic Co-operation and Development (OECD). 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).

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Safety of the Donor Organism

The *dmo* gene is derived from the bacterium *Stenotrophomonas maltophilia*. *S. maltophilia* is ubiquitous in the environment, is 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. *S. maltophilia* can be found in healthy individuals without causing any harm to human health, and infections in humans caused by *S. maltophilia* are extremely uncommon. 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, as 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, the presence in healthy individuals, and the incidental presence in foods without any adverse safety reports establishes the safety of the donor organism.

Molecular Characterization of MON 87708 Verifies the Integrity and Stability of the Inserted DNA

MON 87708 was developed through *Agrobacterium tumefaciens*-mediated transformation of conventional soybean A3525 meristem tissue with the 2T-DNA plasmid vector PV-GMHT4355. PV-GMHT4355 contains two separate T-DNAs that are each delineated by Left and Right Border regions. The first T-DNA, designated as T-DNA I, contains the *dmo* expression cassette regulated by the peanut chlorotic streak caulimovirus (*PC1SV*) promoter and the pea *E9* 3′ non-translated region. The second T-DNA, designated as T-DNA II, contains the *cp4 epsps* expression cassette under the regulation of the figwort mosaic virus (*FMV*) promoter and the pea *E9* 3′ non-translated region. During transformation, both T-DNAs were inserted into the soybean genome, where T-DNA II, containing the *cp4 epsps* expression cassette, functioned as a marker gene for the selection of transformed plantlets. Subsequently, self-pollination and segregation were used to isolate a plant containing the *dmo* expression cassette but not containing the *cp4 epsps* expression cassette, resulting in the production of marker-free, dicamba-tolerant soybean MON 87708.

Molecular characterization by Southern blot analyses determined that MON 87708 contains one copy of the T-DNA I at a single integration locus and all elements are present. These data also demonstrated that MON 87708 does not contain detectable backbone sequences from the plasmid vector or T-DNA II sequences. The complete DNA sequence of the insert and adjacent genomic DNA sequence in MON 87708 confirmed the integrity of the inserted *dmo* expression cassette within the inserted sequences and identified the 5and 3′ insert -to-genomic DNA junctions. Furthermore, Southern blot analysis demonstrated that the insert in MON 87708 has been maintained through at least five generations of breeding, thereby confirming the stability of the insert over multiple generations.

Data Confirm the Safety of Expression Products in MON 87708

MON 87708 contains a *dmo* expression cassette that produces a single MON 87708 DMO precursor protein that is post-translationally processed into two forms of the dicamba mono-oxygenase (DMO) protein; referred to as MON 87708 DMO protein and MON 87708 DMO+27 protein (Section VI.A.). The active form of these proteins, necessary to confer dicamba tolerance, is a trimer comprised of three DMO monomers. In MON 87708, the trimer can be comprised of MON 87708 DMO protein, MON 87708 DMO+27 protein, or a combination of both. Unless specified otherwise in this document, MON 87708 DMO will refer to both proteins and all forms of the trimer, collectively.

A multistep approach, conducted according to guidelines established by the CODEX Alimentarius Commission and the Organization for Economic Co-operation and Development (OECD) and which embody the principles and guidance of the U.S. Food and Drug Administration's (FDA) 1992 policy on foods from new plant varieties, was used to characterize MON 87708 DMO. This detailed characterization and assessment confirmed that MON 87708 DMO is safe for human and animal consumption. The assessment involved: 1) characterization of the physicochemical and functional properties of MON 87708 DMO; 2) quantification of MON 87708 DMO expression in MON 87708 plant tissues; 3) examination of the similarity of MON 87708 DMO to known allergens, toxins, or other biologically active proteins known to have adverse effects on mammals; 4) evaluation of the digestibility of MON 87708 DMO in simulated gastrointestinal fluids; 5) documentation of the history of safe consumption of MON 87708 DMO or its structural and functional homology to proteins that lack adverse effects on human or animal health; 6) evaluation of the stability of MON 87708 DMO after heat treatment; 7) investigation of potential mammalian toxicity in an acute mouse gavage and calculation of margins of exposure; and 8) assessment of the potential for allergenicity, toxicity, and adverse biological activity of putative polypeptides encoded by the insert and flanking sequences.

MON 87708 DMO was fully characterized and the enzymatic activity was found to be specific for dicamba when tested using structurally similar soybean endogenous substrates. MON 87708 DMO was expressed in all tissues of MON 87708 at varying levels. MON 87708 DMO has no relevant amino acid sequence similarities with known allergens, gliadins, glutenins, or toxins that may have adverse effects on mammals. MON 87708 DMO was rapidly digested in simulated gastric and intestinal fluids. MON 87708 DMO was completely deactivated after heating at temperatures above 55°C. MON 87708 DMO was not acutely toxic and did not cause any observable adverse effects when tested in a mouse acute oral toxicity analysis. Large margins of exposure were demonstrated for human and animal exposure to MON 87708 DMO from the consumption of MON 87708 and MON 87708 derived products. An open reading frame bioinformatic analyses of the junction site between the soybean genomic DNA and the insert confirm no relevant similarities exist between any putative polypeptides and known toxins or allergens. In addition, results from an IgE binding study using sera from soybean allergic individuals demonstrate MON 87708 does not pose an increased endogenous soybean allergenic risk compared to commercially available conventional

reference soybean varieties. The safety assessment supports the conclusion that exposure to MON 87708 DMO poses no meaningful risk to human and animal health.

Food and Feed Safety Assessments of MON 87708 Demonstrate Compositional Equivalence to Conventional Crop

Detailed compositional analyses, in accordance with OECD guidelines, were conducted to assess whether levels of key nutrients and anti-nutrients in MON 87708 were comparable to levels present in the near isogenic conventional soybean control A3525 and several commercial reference soybean varieties. Seed and forage were harvested from five individual sites in which MON 87708 (treated with dicamba herbicide), 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 for the key nutrients and anti-nutrients in commercial soybean varieties that have a history of safe consumption. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), fiber, amino acids (18 components), fatty acids (FA, C8-C22), and vitamin E (α-tocopherol) in seed; and proximates (ash, carbohydrates by calculation, moisture, protein, and fat) and fiber in forage. The anti-nutrients assessed in seed included lectin, phytic acid, raffinose, stachyose, trypsin inhibitors, and isoflavones (daidzein, genistein, and glycitein).

A combined-site analysis was conducted to determine statistically significant differences (5% level of significance) between MON 87708 and the conventional control. The results from the combined-site data were reviewed using considerations relevant to food and feed safety and nutritional quality. These considerations included assessments of: 1) the relative magnitudes of the difference in the mean values of nutrient and anti-nutrient components of MON 87708 and the conventional control, 2) whether the MON 87708 component mean value was 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 field trial, 3) analysis of the reproducibility of the statistically significant combined-site component differences at individual sites, and 4) assessing the differences within the context of natural variability of commercial soybean composition published in the scientific literature and in the International Life Sciences Institute (ILSI) Crop Composition Database.

Based on assessments of the levels of the components analyzed in MON 87708, it was determined that MON 87708 is compositionally equivalent to the conventional control and within the range of variability of the commercial reference varieties that were grown concurrently in the same field trial. There were either no statistically significant differences between MON 87708 and the conventional control or the differences observed in the combined-site analysis were deemed to not be meaningful to food and feed safety or the nutritional quality of MON 87708. These results support the overall food and feed safety of MON 87708.

Conclusion

The data and information presented in this safety assessment demonstrate that the food and feed derived from MON 87708 are as safe and nutritious as conventional soybean, a familiar crop with a long history of safe consumption. The food and feed safety of MON 87708 was confirmed through multiple, well established lines of evidence:

1. The safety of the donor organism, *S. maltophilia*, based on its ubiquitous presence in the environment, presence in healthy individuals, and the incidental presence in foods without any adverse safety reports.

2. A detailed molecular characterization of the inserted DNA demonstrated a single, intact copy of the T-DNA stably inserted in a single locus within the soybean genome.

3. A history of safe use has been established for MON 87708 DMO. Data confirmed that MON 87708 DMO is unlikely to be a toxin or allergen based on extensive information collected. MON 87708 DMO was readily digestible in simulated gastric and simulated intestinal fluids, inactivated when exposed to heat, and showed no oral toxicity or cause any adverse effect in mice. Large margins of exposures (MOE) have been demonstrated for human and animal consumption of MON 87708 DMO derived from MON 87708.

4. A compositional assessment of seed and forage confirmed that MON 87708 is compositionally equivalent to conventional soybean.

All data strongly support the conclusion that food and feed derived from MON 87708 will be as safe and nutritious as food and feed derived from conventional soybean. Therefore, the consumption of MON 87708 and the food and feed derived from it will be fully consistent with FDA's Policy (U.S. FDA, 1992) and such use will be in compliance with all applicable requirements of the Federal Food, Drug, and Cosmetic Act.

I. DESCRIPTION OF MON 87708

This section provides a description of MON 87708 being presented for food and feed safety and nutritional assessment. The description identifies the crop, the transformation event 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 87708. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraph 22.

I.A. MON 87708 Summary

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants" MON 87708 has been assigned the unique identifier MON-877Ø8-9.

Monsanto Company has developed biotechnology-derived soybean MON 87708 that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide. MON 87708 offers growers an expanded use of dicamba in soybean production from the current preplant and preharvest labeled uses. The tolerance of MON 87708 to dicamba facilitates a wider window of application on soybean, allowing preemergence application of the herbicide up to the day of crop emergence and postemergence in-crop applications through the early reproductive (R1) growth stage. Dicamba provides effective control of over 95 annual and biennial weed species, and suppression of over 100 perennial broadleaf and woody plant species. Dicamba is efficacious on broadleaf weeds that are hard-to-control with glyphosate, such as common lambsquarters, hemp sesbania, morning glory species, nightshade, Pennsylvania smartweed, prickly sida, and wild buckwheat. Additionally, dicamba provides effective control of herbicide-resistant broadleaf weeds, including glyphosate-resistant weeds such as marestail, common ragweed, giant ragweed, palmer pigweed, and waterhemp.

MON 87708 will be combined with glyphosate-tolerant soybean MON 89788 (Roundup Ready 2 Yield® soybean) utilizing traditional breeding techniques. The potential use of dicamba or glyphosate herbicides, in addition to the other herbicide options currently labeled for use on soybean, enables an integrated weed management program to control a broad spectrum of grass and broadleaf weed species. Successful integration of MON 87708 into the Roundup Ready® soybean system will 1) provide growers with an opportunity for an efficient, effective weed management system for control of glyphosate's hard-to-control and resistant broadleaf weeds; 2) provide an easy system for an additional in-crop herbicide mode-of-action in current soybean production practices as recommended by weed science experts to manage future weed resistance development; and 3) continue to provide soybean growers with effective weed control systems necessary for yields to meet the growing needs of the food, feed, and industrial markets.

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MON 87708 contains a gene derived from *Stenotrophomonas maltophilia* that expresses a mono-oxygenase that rapidly demethylates dicamba rendering it inactive, thereby conferring tolerance to dicamba. The demethylation of dicamba produces 3,6-dichlorosalicylic acid (DCSA). DCSA is a known metabolite of dicamba in soybean, soil, and livestock whose safety has been evaluated by the Environmental Protection Agency (EPA) as part of the food, feed, and environmental safety assessment. DCSA, in addition to dicamba, is included in the current 10 ppm pesticide residue tolerance for soybean seed that supports the existing uses of dicamba on soybean (40 CFR § 180.227). With the proposed expanded use of dicamba on MON 87708, compared to current uses on soybean, the rapid metabolism of dicamba results in residues in dicamba-treated MON 87708 seed, including the DCSA metabolite, that are well below the established 10 ppm tolerance established for current uses in soybean. Consequently, only an approval for the expanded use pattern of dicamba on MON 87708 and the feeding of MON 87708 forage and hay to livestock, which requires the establishment of new forage and hay tolerances, has been requested of EPA.

EPA has reviewed the safety of dicamba and DCSA during the reregistration of dicamba in 2006. EPA concluded in the 2006 dicamba Reregistration Eligibility Decision (RED) document that risks to human health and the environment associated with exposure to dicamba and its metabolites, including DCSA, were below the Agency's level of concern for all registered uses of dicamba including conventional soybean (U.S. EPA, 2009). Dicamba residues on soybean seed (less than 0.07 ppm average residue and less than 0.5 ppm maximum residue) resulting from dicamba applications on MON 87708 at the maximum labeled use rate are well below the established 10 ppm soybean seed pesticide residue tolerance. Therefore, a change to the current soybean seed tolerance is not necessary to support the use of dicamba on MON 87708. However, Monsanto has requested the establishment of new tolerances for soybean forage and hay that will allow for the feeding of forage and hay to livestock. No other revisions to the dicamba pesticide residue tolerances are necessary including animal products such as meat or milk. Furthermore, the use of dicamba on MON 87708 does not present any new environmental exposure scenarios not previously evaluated and deemed acceptable by EPA, including estimates of drinking water exposure.

The data and information presented in this safety summary demonstrate that the food and feed derived from MON 87708 are as safe and nutritious as those derived from commercially-available, conventional soybean 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 Organization for Economic Co-operation and Development (OECD). 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 87708 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 87708 is Not Suitable

Monsanto Company is aware of no food or feed uses of conventional soybean that are not applicable to MON 87708.

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 Soybean

The OECD Consensus Document (OECD, 2000) on the biology of soybean provides key information on:

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

The taxonomic information for soybean is available in the USDA's PLANTS Profile (USDA-NRCS, 2010).

II.A.1. Soybean as a Crop

Soybean is the most widely grown oilseed in the world. Approximately 211 million metric tons of harvested seed was produced in 2009, representing 56% of world oilseed production (Soyatech, 2010). Soybean is grown as a commercial crop in over 35 countries. The major producers are the U.S., Brazil, Argentina, China, India, and Paraguay, accounting for approximately 94% of the global soybean production in 2009 (Soyatech, 2010). Approximately one third of the 2009 world soybean production was in the U.S. (Soyatech, 2010). The U.S. was also the largest soybean exporting country in 2009 (ASA, 2010b).

Soybean has a long history of planting and production in North America. Soybean was originally introduced into North America from China in 1765 and has been reintroduced several times by scientists, seed dealers, merchants, military expeditions, and various individuals (Singh and Hymowitz, 1999).

II.B. The Recipient Plant

II.B.1. Characteristics of the Recipient Plant

The soybean variety used as the recipient for the DNA insertion to create MON 87708 was A3525, a non-transgenic conventional variety developed by Asgrow Seed Company. A3525 is a mid maturity group III soybean variety with very high yield potential. A3525 has superior yields relative to varieties of similar maturity and has excellent agronomic characteristics (Monsanto Technology, 2004).

Soybean variety A3525 is the near isogenic line to MON 87708 and was used as the conventional soybean comparator (hereafter referred to as the conventional control) in the safety assessment of MON 87708. MON 87708 and the conventional control have similar genetic backgrounds with the exception of the *dmo* expression cassette, thus, the effect of the *dmo* expression cassette and the expressed MON 87708 DMO could be assessed in an objective manner. In addition, commercial soybean varieties included conventional and Roundup Ready® soybean varieties (hereafter both are referred to as commercial reference varieties), were used as reference materials to establish ranges of natural variability representative of commercial soybean 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.2. Known Toxicity or Allergenicity of Recipient Plant

Soybean has had a long history of domestication and consumption by humans, and foods containing soybean-derived products are consumed by a large proportion of the global population (Liu, 2004a). Soybean seed contains several well-described anti-nutritional factors, including trypsin inhibitors, lectins, isoflavones (daidzein, genistein, and glycitein), stachyose, raffinose, and phytic acid (OECD, 2001). The effects of these anti-nutrients to human or animal health are reduced through proper processing (OECD, 2001).

Soybean is one of eight allergenic foods that are responsible for approximately 90% of all food allergies (Cordle, 2004). Soybean is less allergenic than other foods in this group and is rarely responsible for severe, life-threatening reactions (Cordle, 2004). Allergy to soybean is more prevalent in children than adults and is considered a transient allergy of infancy/childhood (Sicherer, et al., 2000).

II.C. Soybean as a Food Source

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

1. Soybean oil: Soybean oil constitutes approximately 69% of consumption of edible fats and oil in the U.S. (ASA, 2010c), and is the second largest source of vegetable oil worldwide (Soyatech, 2010). Refined, bleached, and deodorized soybean oil can be further processed to produce cooking oils, shortening, margarine, mayonnaise, salad dressings, and a wide variety of products that are either based entirely on fats and oils or contain fat or oil as a principal ingredient.

2. Traditional soyfoods: Traditional soyfoods are primarily made from whole soybean. The nonfermented traditional soyfoods include soymilk, tofu, soybean sprouts, soymilk film (yuba), soynuts, and green vegetable soybean (*e.g.*, edamame), whereas the fermented soyfoods include soybean paste (miso), soybean sauce, natto, and tempeh.

3. Soybean protein products: Soybean protein products are mostly made from defatted soybean flakes, and include soybean flour, soybean protein concentrate, and soybean protein isolate. Soybean flour has a protein content of approximately 50% and is used mainly as an ingredient in the baking industry. Soybean protein concentrate has a protein content of approximately 70% and is used widely in the meat industry to bind water, emulsify fat, or as a key ingredient of meat alternative products. Soybean protein isolate has a protein content of 90%, and possesses many functional properties such as gelation and emulsification. As a result, it can be used in a wide range of food applications, including processed meats, meat analogs, soup and sauce bases, energy bars, nutritional beverages, infant formula, and dairy replacements. Soybean protein may also be texturized through various mechanisms to achieve a particular structure similar to fiber that is used as a meat analog.

4. Modern soyfoods: Modern soyfoods resulted from modification of traditional soyfoods to suit local tastes in the West. Blending of soybean products and processing techniques have resulted in two major subgroups including meat and dairy alternatives. Examples of modern soyfoods include soy ice cream, soy yogurt, soy cheese, soy burgers, and meatless meatballs.

5. Soybean-enriched foods: Soybean-enriched foods are similar to modern soyfoods, with the exception that soybean is not the main ingredient in soybean-enriched foods. Soybean protein is an ideal choice for increasing the protein content of many common foods. A wide variety of food can be enriched with soybean, including soy bread, soy pastes, and soy cereals.

6. Functional soybean ingredients/dietary supplements: Soybean is a rich source of certain phytochemicals, including lecithin and isoflavones. Often the result of modern processing, these phytochemicals can be recovered and used as a food ingredient or dietary supplements.

II.D. Soybean as a Feed Source

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

The majority of soybean meal is used by the animal feed industry as a cost-effective protein and amino acid source to animal diets. Soybean meal can serve as a excellent protein source that complements the limiting amino acid profile of feeds derived from corn (Kerley and Allee, 2003). Poultry and swine account for most of the soybean meal used in the U.S., with poultry consuming 48%, swine 26%, beef 12%, dairy 9%, pet food 2%, and other 3% (ASA, 2010a). Globally, soybean meal accounts for approximately two-thirds of the protein meal consumed, with the remainder divided between rapeseed, cottonseed, sunflower, peanut, and other crops (ASA, 2010d).

Dairy and livestock producers use soybean forage as feed. Soybean forage is an inexpensive, readily available, on-farm source of high-quality, high-protein forage adapted to growth during the summer months when other forage legume species typically are restricted in growth (USDA-ARS, 2006). Soybean forage can be used as hay or to produce silage (MAFRI, 2004). An additional use of soybean for feed can be full-fat (whole) soybean for dairy cattle and swine, but for swine it is limited due to the high oil content to a maximum of 20% of the total diet (Yacentiuk, 2008).

III. DESCRIPTION OF THE DONOR ORGANISMS

This section describes the donor organism for the genetic material encoding for the introduced protein. It contains information describing if the donor organism exhibits 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.

III.A. Donor Organism

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, chicory, 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 cotton seed, 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 dishwashers, sponges, toothbrushes, flowers, plants, fruits, vegetables, frozen fish, milk, and poultry (Ryan et al., 2009). 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 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.B. Identity and Sources of the Genetic Material Introduced into MON 87708

MON 87708 was developed through *Agrobacterium tumefaciens*-mediated transformation using the binary plasmid vector PV-GMHT4355. The insert present in MON 87708 contains the *dmo* coding sequence under regulation of the peanut chlorotic streak caulimovirus (*PC1SV*) promoter, Tobacco Etch virus (*TEV*) leader, Rubisco targeting sequence, and the pea (*Pisum sativum*) *E9* 3′ non-translated region. As described in Tables IV-1 and V-2, the *PC1SV* promoter is the promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus, the *TEV* leader is the 5′ non-translated region from the Tobacco Etch virus, the *RbcS* targeting sequence is the sequence encoding the chloroplast transit peptide and the first 24 amino acids of the mature protein of the *RbcS* gene from pea, and the *E9* 3′ non-translated region is the 3′ non-translated region from the *RbcS2* gene of pea encoding the Rubisco small subunit.

There is no evidence of any safety issues related to the use of MON 87708 and there is no evidence of human or animal pathogenicity for any of the donor organisms of the coding and non-coding DNA sequences present in MON 87708. DNA has always been present in food and, upon consumption, is quickly degraded by restriction nucleases present in the gastrointestinal tract of humans and animals to nucleic acids. According to the U.S. 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 non-recombinant DNA; and 3) there is no evidence that DNA from dietary sources has ever been incorporated into the mammalian genome. Additionally, the European Food Safety Authority (EFSA) has reported that a large number of experimental studies have shown that recombinant DNA consumed by livestock has not been subsequently detected in tissues, fluids, or edible products of these farm animals (EFSA, 2007).

IV. DESCRIPTION OF THE GENETIC MODIFICATION

This section provides a description of the transformation process and plasmid vector used in the development of MON 87708. 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.

MON 87708 was developed through *Agrobacterium tumefaciens*-mediated transformation of conventional soybean A3525 meristem tissue utilizing transformation plasmid vector PV-GMHT4355. This section describes the plasmid vector, the donor genes, and the regulatory elements used in the development of MON 87708 and the deduced amino acid sequence of the MON 87708 DMO. 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. The Plasmid Vector PV-GMHT4355

PV-GMHT4355 was used for the transformation of conventional soybean to produce MON 87708 and is shown in Figure IV-1; PV-GMHT4355 is approximately 11.4 kb and contains two T-DNAs, each delineated by Left and Right Border regions to facilitate transformation. The first T-DNA, designated as T-DNA I, contains the *dmo* coding sequence under regulation of the peanut chlorotic streak caulimovirus (*PC1SV*) promoter and the pea *E9* 3′ non-translated region. The second T-DNA, designated as T-DNA II, contains the *cp4 epsps* coding sequence under the regulation of the figwort mosaic virus (*FMV*) promoter and the pea *E9* 3′ non-translated region. During transformation, both T-DNAs were inserted into the soybean genome (Section IV.B) where T-DNA II, containing the *cp4 epsps* expression cassette, functioned as a marker gene for the selection of transformed plantlets. Subsequently, conventional self-pollinated breeding methods and segregation, along with a combination of analytical techniques, were used to isolate those plants that contain the *dmo* expression cassette (T-DNA I) and did not contain the *cp4 epsps* expression cassette (T-DNA II).

The backbone region of PV-GMHT4355 that is outside both of the T-DNAs contains two origins of replication for maintenance of the plasmid vector in bacteria (*ori V*, *ori-pBR322*), a bacterial selectable marker gene (*aadA*), and a coding sequence for repressor of primer (*rop*) protein which is necessary for the maintenance of the plasmid vector copy number in *E. coli*. A description of the genetic elements and their prefixes (*e.g.,* P-, L-, I-, TS-, OR-, B-, CS-, and T-) in PV-GMHT4355 is provided in Table IV-1.

IV.B. Description of the Transformation System

The *Agrobacterium*-mediated soybean transformation used to produce MON 87708 was based on the method described by Martinell et al. (Martinell, et al., 2002), which allows for the generation of transformed plants without utilization of callus. Briefly, meristem tissues were excised from the embryos of germinated conventional seed. After co-culturing with the *Agrobacterium* carrying the vector, the meristems were placed on selection medium containing glyphosate, carbenicillin, cefotaxime, and ticarcillin/clavulanate acid mixture, to inhibit the growth of untransformed plant cells and excess *Agrobacterium*. The meristems were then placed in media conducive to shoot and root development. Rooted plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

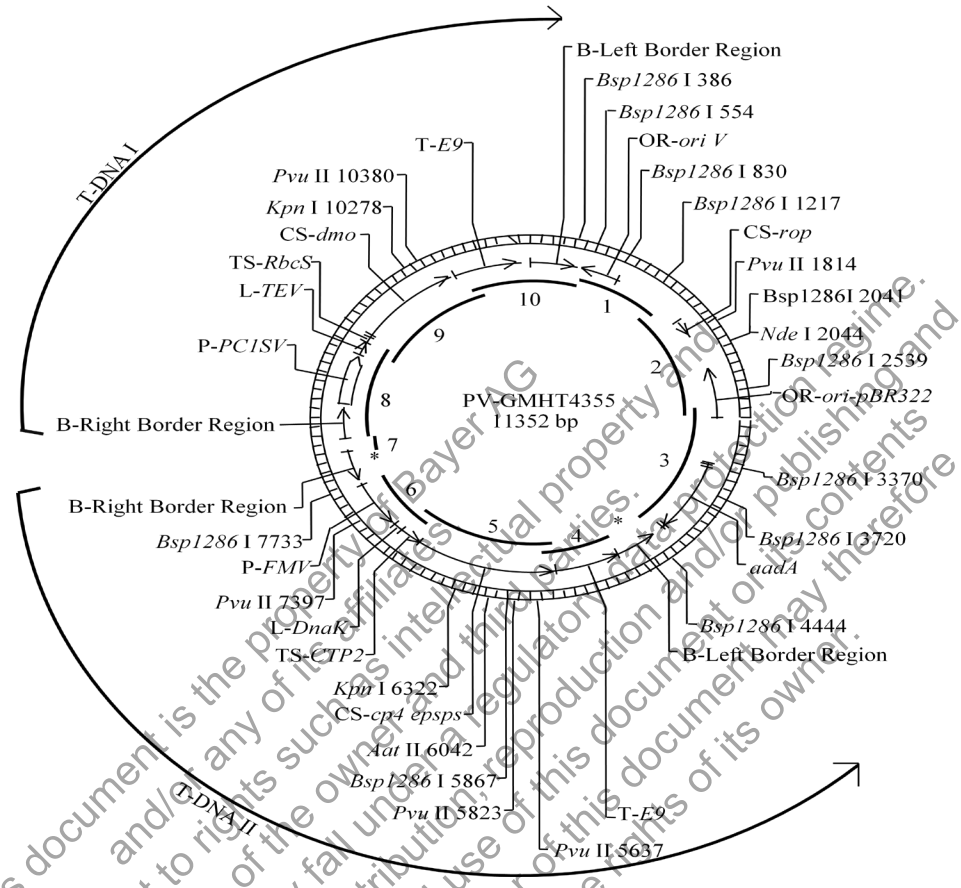
The R_0 plants generated through this transformation were self-pollinated to produce R_1 plants, and the unlinked insertions of T-DNA I and T-DNA II were segregated. A non-lethal dose of glyphosate was applied to R_1 plants and those plants with minor herbicide injury were selected for further analyses, whereas plants showing no injury, indicating that they contained the *cp4 epsps* coding sequence from T-DNA II, were eliminated from further development. Subsequently, plants that were homozygous for T-DNA I were identified by quantitative polymerase chain reaction (PCR) analysis. MON 87708 was selected as the lead event based on superior phenotypic characteristics, dicamba tolerance, and its molecular profile. The major development steps of MON 87708 are depicted in Figure IV-2. The result of this process was the production of marker-free, dicamba-tolerant soybean MON 87708.

IV.C. The *dmo* Coding Sequence and MON 87708 DMO (T-DNA I)

The *dmo* expression cassette (T-DNA I) present in MON 87708 encodes MON 87708 DMO (Figure IV-3). The *dmo* expression cassette contains the coding region for the DMO from *Stenotrophomonas maltophilia* (Herman, et al., 2005; Wang, et al., 1997). The presence of MON 87708 DMO confers tolerance to dicamba (refer to Section V.B. for more details).

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

The *cp4 epsps* expression cassette (T-DNA II), that is not present in MON 87708, encoded a 47.6 kDa CP4 EPSPS protein, consisting of a single polypeptide of 455 amino acids (Padgette, et al., 1996a). The *cp4 epsps* coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry, et al., 1997; Padgette, et al., 1996b). CP4 EPSPS confers tolerance to glyphosate and was used as a selectable marker during the transformation selection process. Through conventional self-pollinated breeding methods and segregation, along with a combination of analytical techniques, plants that did not contain the *cp4 epsps* expression cassette were isolated.



Probe Number	Probe Type	Start Position (bp)	End Position (bp)	Total Length (bp)
1	Backbone Probe	443	1328	886
2	Backbone probe	1250	2754	1505
3	Backbone Probe	2625	4384	1760
4	T-DNA II Probe	4796	5637	842
5	T-DNA II Probe	5575	7021	1447
6	T-DNA II Probe	6937	7761	825
7	Backbone Probe	8119	8289	171
8	T-DNA I Probe	8290	9523	1234
9	T-DNA I Probe	9448	10668	1221
10	T-DNA I Probe	10610	442	1185

Figure IV-1. Circular Map of Plasmid Vector PV-GMHT4355 Showing Probes 1-10
The plasmid vector PV-GMHT4355 containing the T-DNAs used in *Agrobacterium*-mediated transformation to produce MON 87708. Genetic elements and restriction sites for enzymes used in the Southern blot analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern blot analyses (labeled 1-10 on the interior of the map) are detailed in the accompanying table above.

*The Left and Right Border regions of T-DNA II share 100% identity to those of T-DNA I, which were covered by probes 8 and 10 and thus not included in the T-DNA II probes.

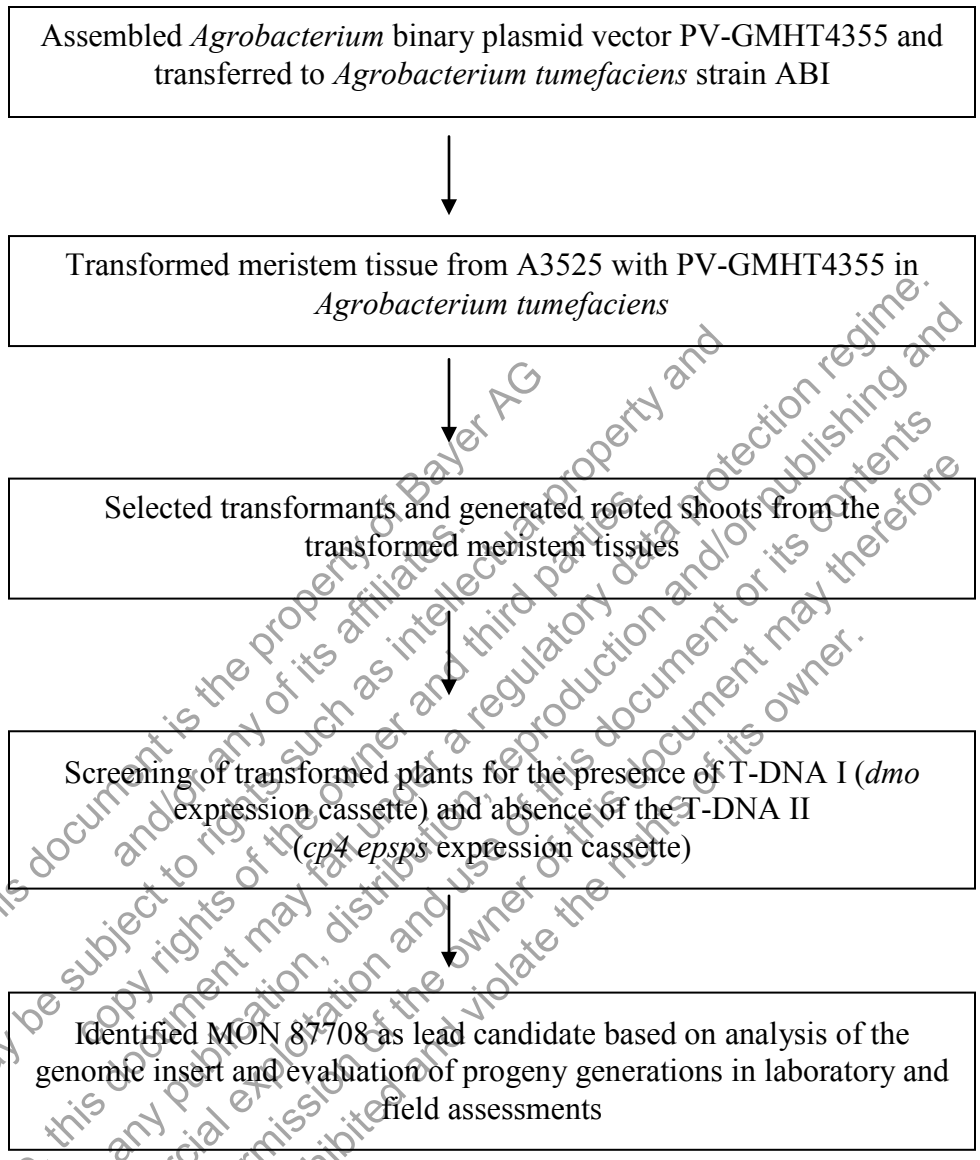


Figure IV-2. Schematic of the Development of MON 87708

IV.E. Regulatory Sequences

The *dmo* coding sequence in T-DNA I is under the regulation of the *PC1SV* promoter, *TEV* leader, the *RbcS* targeting sequence, and the *E9* 3′ non-translated 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′ non-translated region from the Tobacco Etch virus (Niepel and Gallie, 1999) and is involved in regulating gene expression. The *RbcS* targeting sequence is the sequence encoding the chloroplast transit peptide and the first 24 amino acids of the mature protein of the ribulose-1,5-bisphosphate carboxylase oxygenase gene from pea (*Pisum sativum*) (Fluhr, et al., 1986) that directs transport of the DMO precursor protein to the chloroplast. The *E9* 3′ non-translated region is the 3′ non-translated region from the *RbcS2* gene of pea encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi, et al., 1984).

T-DNA II contains the *cp4 epsps* coding sequence under the regulation of the *FMV* promoter, *DnaK* leader, the *CTP2* targeting sequence, and the *E9* 3′ non-translated region. The *FMV* promoter is the promoter for the 35S RNA from figwort mosaic virus (Rogers, 2000) that directs transcription in plant cells. The *DnaK* leader is the 5′ non-translated leader sequence from the *Petunia hybrida Hsp70* gene (Rensing and Maier, 1994) that is involved in regulating gene expression. The *CTP2* targeting sequence is the sequence encoding the chloroplast transit peptide region from the *shkG* gene of *Arabidopsis thaliana* encoding EPSPS (Herrmann, 1995; Klee, et al., 1987) that directs transport of the CP4 EPSPS precursor protein to the chloroplast. The *E9* 3′ non-translated region is the 3′ non-translated region from the *RbcS2* gene of pea encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984).

IV.F. T-DNA Borders

PV-GMHT4355 contains Right and Left Border regions (Figure IV-1 and Table IV-1) that were derived from *Agrobacterium tumefaciens* (Barker, et al., 1983; Depicker, et al., 1982; Zambryski, et al., 1982). The border regions each contain a 24-25 bp nick site that is the site of DNA exchange during transformation. The border regions separate the T-DNA from the backbone region and are involved in their efficient transfer into the soybean genome. Because PV-GMHT4355 is a 2T-DNA vector, it contains two Right Border regions and two Left Border regions, where one set flanks T-DNA I and the other set flanks T-DNA II.

IV.G. Genetic Elements Outside the T-DNA Borders

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV-GMHT4355 in bacteria and are referred to as the plasmid backbone. The *ori V*, derived from the broad host plasmid RK2, is required for the maintenance of the plasmid vector in *Agrobacterium* (Stalker, et al., 1981), whereas the *ori-pBR322*, derived from the plasmid vector pBR322, is required for the maintenance of the plasmid vector in *E. coli* (Sutcliffe, 1979). The *rop* is necessary

for the maintenance of plasmid vector copy number in *E. coli* (Giza and Huang, 1989). The *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 were not expected to be transferred into the soybean genome. The absence of the backbone sequence in MON 87708 was confirmed by Southern blot analyses (Section V.C.).

Table IV-1. Summary of Genetic Elements in the Plasmid Vector PV-GMHT4355

Genetic Element	Location in Plasmid (bp)	Function (Reference)
	T-DNA I (Present in MON 87708)	
B[1]-Right Border Region	8290-8646	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	8647-8691	Sequence used in DNA cloning
P[2]-*PC1SV*	8692-9124	Promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (Maiti and Shepherd, 1998) that directs transcription in plant cells
Intervening sequence	9125-9144	Sequence used in DNA cloning
L[3]-*TEV*	9145-9276	5′ non-translated region from the Tobacco Etch virus genome (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening sequence	9277	Sequence used in DNA cloning
TS[4]-*RbcS*	9278-9520	Sequences encoding the transit peptide and the first 24 amino acids of the mature protein of the *RbcS* gene from *Pisum sativum* (pea) (Fluhr et al., 1986) that directs transport to the DMO precursor protein to the chloroplast
Intervening Sequence	9521-9529	Sequence used in DNA cloning
CS[5]-*dmo*	9530-10552	Coding sequence for the dicamba mono-oxygenase derived from *Stenotrophomonas maltophilia* (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	10553-10620	Sequence used in DNA cloning
T[6]-*E9*	10621-11263	3′ non-translated region from the *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	11264-11352	Sequence used in DNA cloning
B-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)

Table IV-1 (continued). Summary of Genetic Elements in the Plasmid Vector PV-GMHT4355

Genetic Element	Location in Plasmid (bp)	Function (Reference)
Plasmid Vector Backbone (Not present in MON 87708)		
Intervening Sequence	443-528	Sequence used in DNA cloning
OR[7]-*ori V*	529-925	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	926-1662	Sequence used in DNA cloning
CS-*rop*	1663-1854	Coding sequence for repressor of primer protein derived from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	1855-2281	Sequence used in DNA cloning
OR-*ori-pBR322*	2282-2870	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
Intervening Sequence	2871-3400	Sequence used in DNA cloning
aadA	3401-4289	Bacterial promoter, coding and 3′UTR sequences for an aminoglycoside-modifying enzyme, 3′ (9) -O-nucleotidyltransferase from transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	4290-4384	Sequence used in DNA cloning
T-DNA II (Not present in MON 87708)		
B-Left Border Region	4385-4795	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	4796-4809	Sequence used in DNA cloning
T-*E9*	4810-5452	3′ non-translated sequence from *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	5453-5458	Sequence used in DNA cloning
CS-*cp4 epsps*	5459-6826	Codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry et al., 1997; Padgette et al., 1996b)

Table IV-1 (continued). Summary of Genetic Elements in the Plasmid Vector PV-GMHT4355

Genetic Element	Location in Plasmid (bp)	Function (Reference)
TS-*CTP2*	6827-7054	Sequences encoding the chloroplast transit peptide region from the *shkG* gene of *Arabidopsis thaliana* encoding EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS precursor protein to the chloroplast
Intervening Sequence	7055-7063	Sequence used in DNA cloning
L-*DnaK*	7064-7159	5′ non-translated leader sequence from the *Petunia hybrida Hsp70* gene (Rensing and Maier, 1994) that is involved in regulating gene expression
Intervening Sequence	7160-7162	Sequence used in DNA cloning
P-*FMV*	7163-7714	Promoter for the 35S RNA from figwort mosaic virus (Rogers, 2000) that directs transcription in plant cells
Intervening Sequence	7715-7761	Sequence used in DNA cloning
B-Right Border Region	7762-8118	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	8119-8289	Sequence used in DNA cloning

[1]B -border.
[2]P-promoter.
[3]L- leader.
[4]TS- targeting sequence.
[5]CS-coding sequence.
[6]T- 3' non-translated transcriptional termination sequence and polyadenylation signal sequences.
[7]OR-origin of replication.

```
1   MASMISSSAV TTVSRASRGQ SAAMAPFGGL KSMTGFPVRK VNTDITSITS NGGRVKCMQV
61  WPPIGKKKFE TLSYLPPLTR DSRAMATFVR NAWYVAALPE ELSEKPLGRT ILDTPLALYR
121 QPDGVVAALL DICPHRFAPL SDGILVNGHL QCPYHGLEFD GGGQCVHNPH GNGARPASLN
181 VRSFPVVERD ALIWICPGDP ALADPGAIPD FGCRVDPAYR TVGGYGHVDC NYKLLVDNLM
241 DLGHAQYVHR ANAQTDAFDR LEREVIVGDG EIQALMKIPG GTPSVLMAKF LRGANTPVDA
301 WNDIRWNKVS AMLNFIAVAP EGTPKEQSIH SRGTHILTPE TEASCHYFFG SSRNFGIDDP
361 EMDGVLRSWQ AQALVKEDKV VVEAIERRRA YVEANGIRPA MLSCDEAAVR VSREIEKLEQ
421 LEAA
```

Figure IV-3. Deduced Amino Acid Sequence of the MON 87708 DMO Precursor Protein

The chloroplast transit peptide and the first 24 amino acids of the mature protein of the *RbcS* elementare underlined and followed by three amino acids of an intervening sequence. The methionine in position 85 is the N-terminus of the MON 87708 DMO protein deduced from the *dmo* element (see Table IV-1 and Section VI.A, Figure VI-1 for more detail).

V. CHARACTERIZATION OF THE GENETIC MODIFICATION

This section contains a comprehensive molecular characterization of the genetic modification present in MON 87708. It provides information on the DNA insertion into the plant genome of MON 87708, 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.

A multi-faceted approach was taken to characterize the genetic modification that produced MON 87708. The results confirmed that MON 87708 contains a single copy of the *dmo* expression cassette (T-DNA I) that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations (Section V.G.). The results confirmed that no T-DNA II or plasmid vector backbone sequences are detected in MON 87708. These conclusions are based on several lines of evidence: 1) Southern blot analyses to assay the entire soybean genome for the presence of DNA derived from PV-GMHT4355, and to confirm that a single copy of T-DNA I was inserted at a single site and that the insert is stably inherited; 2) DNA sequencing analyses to determine the exact sequence of the inserted DNA and allowed a comparison to the T-DNA I sequence in PV-GMHT4355 to confirm that only the expected sequences were integrated; and 3) a comparison of the DNA flanking T-DNA I to the sequence of the insertion site in conventional soybean 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 I was inserted at a single locus of the genome.

Southern blot analyses were used to determine the number of copies and the insertion sites of T-DNA I as well as the presence or absence of T-DNA II and plasmid vector backbone sequences. The Southern blot strategy was designed to ensure that all potential inserted segments would be identified. The entire soybean genome was assayed with probes that spanned the complete plasmid vector PV-GMHT4355 to detect the presence of T-DNA I as well as confirm the lack of any detectable T-DNA II and plasmid vector backbone sequences. This was accomplished by using probes that were less than 2 kb in length, ensuring 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 restriction enzyme sets were specifically chosen to fully characterize T-DNA I and look for any potential fragments of T-DNA I. This two enzyme set design also maximizes the possibility of detecting an insertion elsewhere in the genome that could be overlooked if that band co-migrated with an expected band. Additionally, the restriction enzyme sets were chosen such that at least one enzyme from each set resides in the known 5′ or 3′ flanking sequence and that together the enzyme sets result in overlapping segments covering the entire insert. Therefore, at least one segment for each flank is of a predictable size and overlaps with another predictable size segment. This overlapping strategy confirms that the entire insert sequence is identified in a predictable hybridization pattern.

To determine the number of copies and the insertion sites of T-DNA I, and the presence or absence of T-DNA II and 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 the detection of small molecular weight DNA. 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.

The DNA sequencing analyses complement the Southern blot analyses. Southern blot results demonstrated that MON 87708 contains a single copy of T-DNA I at a single insertion site. Sequencing of the insert and the flanking DNA confirmed the organization of the elements within the insert, determined the 5′ and 3′ insert-to-plant junctions, as well as the complete DNA sequence of the insert and adjacent DNA. In addition, DNA sequencing analyses confirmed that each genetic element in the insert is intact and the sequence of the insert matches the corresponding sequence in PV-GMHT4355. Furthermore, genomic organization at the insertion site was assessed by comparing the insert and flanking sequence to the insertion site in conventional soybean.

The stability of the T-DNA I present in MON 87708 across multiple generations (R_2-R_6) was demonstrated by Southern blot analysis. Genomic DNA from five generations of MON 87708 was digested with one of the enzyme sets used for the insert and copy number analysis and was hybridized with a probe that detects restriction segments that encompass the entire T-DNA I. This fingerprint strategy consists of two border segments that assess not only the stability of T-DNA I, but also the stability of genomic DNA directly adjacent to T-DNA I.

The results of these analyses for MON 87708 demonstrated that a single copy of the T-DNA I was inserted at a single locus of the genome. Generational stability analysis demonstrated that an expected Southern blot fingerprint of MON 87708 was maintained through five generations of the breeding history, thereby confirming the stability of T-DNA I in MON 87708. Results from segregation analyses showed heritability and stability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA I at a single chromosomal locus.

The Southern blot analysis confirmed that T-DNA I reported in Figure V-1 represents the only detectable insert in MON 87708. Figure V-1 is a linear map depicting restriction sites within the insert as well as within the known soybean genomic DNA immediately flanking the insert in MON 87708. The circular map of PV-GMHT4355 annotated with the probes used in the Southern blot analysis is presented in Figure IV-1. Based on the linear map of the insert and the plasmid map, a table summarizing the expected DNA segments for Southern analyses is presented in Table V-1. The genetic elements integrated in MON 87708 are summarized in Table V-2. The generations used are

depicted in the breeding history shown in Figure V-11. Materials and methods used for characterization of T-DNA I in MON 87708 are found in Appendix A.

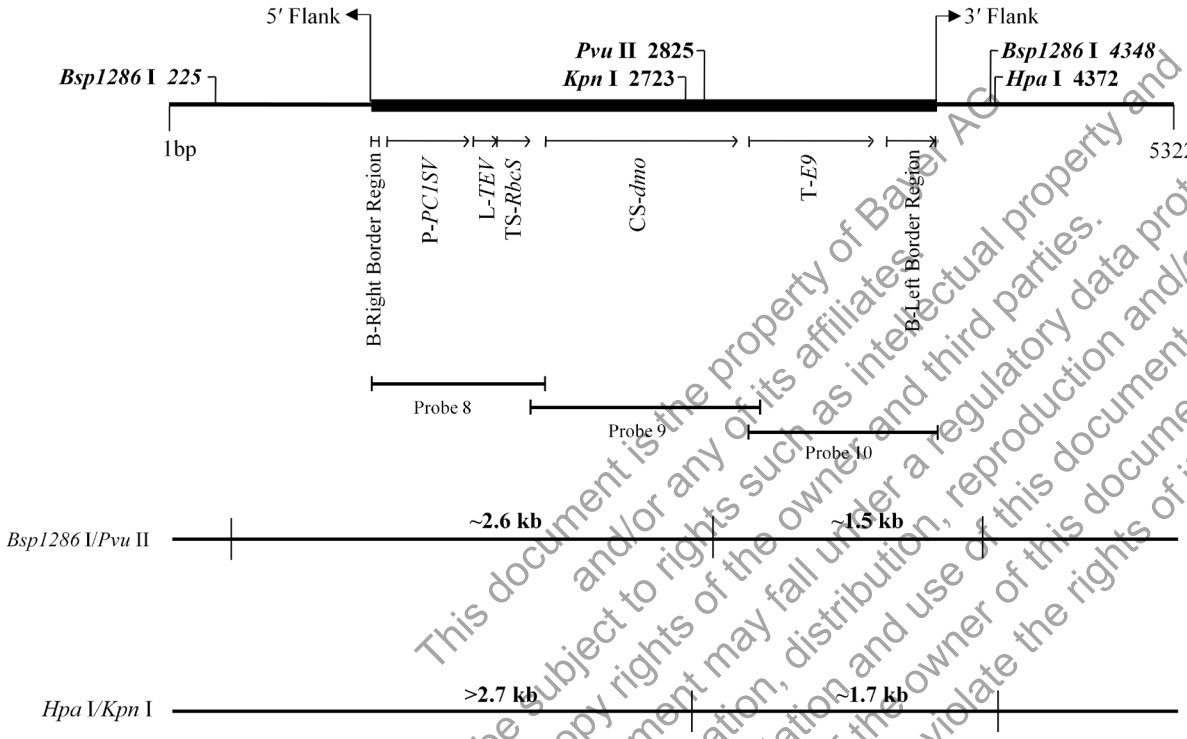


Figure V-1. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87708

A linear map of the insert and genomic DNA flanking the insert in MON 87708 is shown. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the linear map for enzymes used in the Southern analyses. The relative sizes and locations of the T-DNA I probes, which are described in Figure IV-1, are shown on the middle portion. Shown on the lower portion of the map are the expected sizes of the DNA segments after digestion with respective restriction enzymes. Arrowheads (⟶) indicate the end of the insert and the beginning of the genomic DNA sequence flanking the 5′ and 3′ end of the insert. The arrows (→) indicate the sequence direction of the elements in MON 87708.

Table V-1. Summary Chart of the Expected DNA Segments Based on Hybridizing Probes and Restriction Enzymes Used in MON 87708 Analysis

Southern Blot Figure		V-2	V-3	V-4	V-5	V-6	V-7	V-8	V-12
Probe Used		8	9	10	4	5	6	1, 2, 3, and 7	9
Probing Target	Digestion Enzyme	Expected Band Sizes (kb) on Each Southern Blot							
Plasmid Vector PV-GMHT4355	*Aat* II/*Nde* I	~7.4	~7.4	~4.0 ~7.4	~4.0 ~7.4	~4.0 ~7.4	~7.4	~4.0 ~7.4	~7.4
Probe Templates[1]	N/A	~~[2]	~~[2]	~~[2]	~~[2]	~~[2]	~~[2]	~0.2 ~0.9 ~1.5 ~1.8	~~[2]
Conventional Control A3525	*Bsp1286* I/*Pvu* II	None	None	None	None	None	None	None	None
	Hpa I/*Kpn* I	None	None	None	None	None	None	None	None
MON 87708	*Bsp1286* I/*Pvu* II	~2.6	~2.6 ~1.5	~1.5	~1.5	None	None	None	~2.6 ~1.5
	Hpa I/*Kpn* I	>2.7*	>2.7* ~1.7	~1.7	~1.7	None	None	None	--[3]

[1] Probe templates were spiked when multiple probes are used in Southern blot analysis.
[2] '~~' indicates that only plasmid template was used since the Southern blot was hybridized with one probe.
[3] '--' indicates that the particular restriction enzyme or the combination of the enzymes was not used in the analysis.
* Southern analysis indicates this segment to be ~5.6 kb.

Table V-2. Summary of Genetic Elements in MON 87708

Genetic Element	Location (bp)	Function (Reference)
5′ Flanking Sequences	1-1048	DNA sequence adjacent to the 5′ end of the insertion site
B[1]-Right Border* Region	1049-1091	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	1092-1136	Sequence used in DNA cloning
P[2]-*PC1SV*	1137-1569	Promoter for the Full-Length Transcript (FLt) of peanut chlorotic streak caulimovirus (Maiti and Shepherd, 1998) that directs transcription in plant cells
Intervening sequence	1570-1589	Sequence used in DNA cloning
L[3]-*TEV*	1590-1721	5′ non-translated region from the Tobacco Etch virus genome (Niepel and Gallie, 1999) that is involved in regulating gene expression
Intervening sequence	1722-1722	Sequence used in DNA cloning
TS[4]-*RbcS*	1723-1965	Sequences encoding the transit peptide and the first 24 amino acids of the mature protein of the *RbcS* gene from *Pisum sativum* (pea) (Fluhr et al., 1986) that directs transport of the DMO precursor protein to the chloroplast
Intervening Sequence	1966-1974	Sequence used in DNA cloning
CS[5]-*dmo*	1975-2997	Coding sequence for the dicamba mono-oxygenase derived from *Stenotrophomonas maltophilia* (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	2998-3065	Sequence used in DNA cloning
T[6]-*E9*	3066-3708	3′ non-translated region from the *RbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi et al., 1984)
Intervening Sequence	3709-3797	Sequence used in DNA cloning
B-Left Border* Region	3798-4051	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
3′ Flanking Sequences	4052-5322	DNA sequence adjacent to the 3′ end of the insertion site

[1]B-border.
[2]P-promoter.
[3]L-leader.
[4]TS- targeting sequence.
[5]S- coding sequence.
[6]T-3' non-translated transcriptional termination sequence and polyadenylation signal sequences.
*These borders are truncated.

V.A. Insert and Copy Number of T-DNA I in MON 87708

The copy number and insertion site of T-DNA I was assessed by digesting MON 87708 genomic DNA with the restriction enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I and hybridizing Southern blots with probes that span T-DNA I (Figure IV-1). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table V-1). Since each detected segment contains flanking genomic DNA, any additional integrated sites would produce a different banding pattern with additional bands.

The restriction enzyme combination *Bsp1286* I/*Pvu* II cuts once within T-DNA I and once within each of the known genomic DNA sequences flanking the 5′ and 3′ ends of T-DNA I (Figure V-1). Therefore, if T-DNA I sequences are present at a single integration site in MON 87708, the digestion with *Bsp1286* I/*Pvu* II was expected to generate two border segments with expected sizes of ~2.6 kb and ~1.5 kb (Figure V-1, and Table V-1). The ~2.6 kb restriction segment contained genomic DNA flanking the 5′ end of T-DNA I, the Right Border, the *PC1SV* promoter, the *TEV* leader, the *RbcS* targeting sequence, and a portion of the *dmo* coding sequence. The ~1.5 kb restriction segment contained a portion of the *dmo* coding sequence, the *E9 3′* non-translated sequence, the Left Border, and genomic DNA flanking the 3′ end of T-DNA I.

The restriction enzyme combination *Hpa* I/*Kpn* I cuts once within T-DNA I and once within the known genomic DNA flanking the 3′ end of T-DNA I (Figure V-1). Therefore, if T-DNA I sequences are present at a single integration site in MON 87708, the digestion with *Hpa* I/*Kpn* I was expected to generate two border segments with expected sizes of ~1.7 kb and greater than 2.7 kb (Figure V-1, and Table V-1). Since the *Hpa* I/*Kpn* I restriction site in the genomic DNA flanking the 5′ end of the insert lies outside of the known sequence, it was not possible to predict a precise segment size. However, the segment size was determined by Southern blot analyses to be ~5.6 kb (Figures V-2 and V-3). The ~5.6 kb restriction segment contained genomic DNA flanking the 5′ end of T-DNA I, the Right Border, the *PC1SV* promoter, the *TEV* leader, the *RbcS* targeting sequence, and a portion of *dmo* coding sequence. The ~1.7 kb restriction segment contained a portion of the *dmo* coding sequence, the *E9* 3′ non-translated sequence, the Left Border, and genomic DNA flanking the 3′ end of T-DNA I.

In the Southern blot analyses performed, each Southern blot contained a negative and a positive control. Conventional control genomic DNA digested with either the restriction enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I was used as a negative control to determine if the probes hybridized to any endogenous soybean sequences. As a positive control on the Southern blots, PV-GMHT4355 digested with the restriction enzyme combination *Aat* II/*Nde* I was mixed with predigested conventional control DNA. The positive hybridization control was spiked at 0.1 and 1 genome equivalent to demonstrate sufficient sensitivity of the Southern blot. Individual Southern blots were hybridized with the following probes: probes 8, 9, and 10 (refer to Figure IV-1 and Table V-1). The results of this analysis are shown in Figures V-2 through V-4.

V.A.1. Probe 8

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-2, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure V-2, lanes 3 and 7) and hybridized with probe 8 (Figure IV-1) produced no detectable hybridization bands as expected for the negative control. PV-GMHT4355, digested with the restriction enzyme combination *Aat* II/*Nde* I and mixed with conventional control DNA predigested with the restriction enzyme combination *Hpa* I/*Kpn* I (Figure V-2, lanes 10 and 11), produced the expected size band at ~7.4 kb (refer to Figure V-1 and Table V-1). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 8 (Figure IV-1) produced one unique band at ~2.6 kb (Figure V-2, lanes 2 and 6). The ~2.6 kb band is the expected size for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure V-1).

MON 87708 DNA digested with the restriction enzyme combination *Hpa* I/*Kpn* I and hybridized with probe 8 (Figure IV-1) produced one unique band at ~5.6 kb (Figure V-2, lanes 4 and 8). The ~5.6 kb band is consistent with the expected band being greater than 2.7 kb for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure V-1).

No additional bands were detected using probe 8. Based on the results presented in Figure V-2, it was concluded that T-DNA I sequences covered by probe 8 reside at a single integration locus as one copy in MON 87708.

V.A.2. Probe 9

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-3, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure V-3, lanes 3 and 7) and hybridized with probe 9 (Figure IV-1) produced no detectable hybridization bands as expected for the negative control. PV-GMHT4355, digested with the restriction enzyme combination *Aat* II/*Nde* I and mixed with conventional control DNA predigested with the restriction enzyme combination *Hpa* I/*Kpn* I (Figure V-3, lanes 10 and 11), produced the expected size band at ~7.4 kb (refer to Figure V-1 and Table V-1). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 9 (Figure IV-1) produced two unique bands at ~1.5 kb and ~2.6 kb (Figure V-3, lanes 2 and 6). The ~1.5 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure V-1). The ~2.6 kb band is the expected size for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure V-1).

MON 87708 DNA digested with the restriction enzyme combination *Hpa* I/*Kpn* I and hybridized with probe 9 (Figure IV-1) produced two unique bands at ~1.7 kb and ~5.6 kb

(Figure V-3, lanes 4 and 8). The ~1.7 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure V-1). The ~5.6 kb band is consistent with the expected band being greater than 2.7 kb for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure V-1).

No additional bands were detected using probe 9. Based on the results presented in Figure V-3, it was concluded that T-DNA I sequences covered by probe 9 reside at a single integration locus as one copy in MON 87708.

V.A.3. Probe 10

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-4, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure V-4, lanes 3 and 7) and hybridized with probe 10 (Figure IV-1) produced no detectable hybridization bands as expected for the negative control. PV-GMHT4355, digested with the restriction enzyme combination *Aat* II/*Nde* I and mixed with conventional control DNA predigested with the restriction enzyme combination *Hpa* I/*Kpn* I (Figure V-4, lanes 10 and 11), produced two bands at ~4.0 kb and ~7.4 kb. Both bands were expected because probe 10 contains *E9* and Left Border regions that hybridized to both the ~4.0 kb and the ~7.4 kb fragments from the digested plasmid (refer to Figure V-1 and Table V-1). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 10 (Figure IV-1) produced a unique band at ~1.5 kb (Figure V-4, lanes 2 and 6). The ~1.5 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure V-1).

MON 87708 DNA digested with the restriction enzyme combination *Hpa* I/*Kpn* I and hybridized with probe 10 (Figure IV-1) produced a unique band at ~1.7 kb (Figure V-4, lanes 4 and 8). The ~1.7 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure V-1).

No additional bands were detected using probe 10. Based on the results presented in Figure V-4, it was concluded that T-DNA sequences covered by probe 10 reside at a single integration locus as one copy in MON 87708.

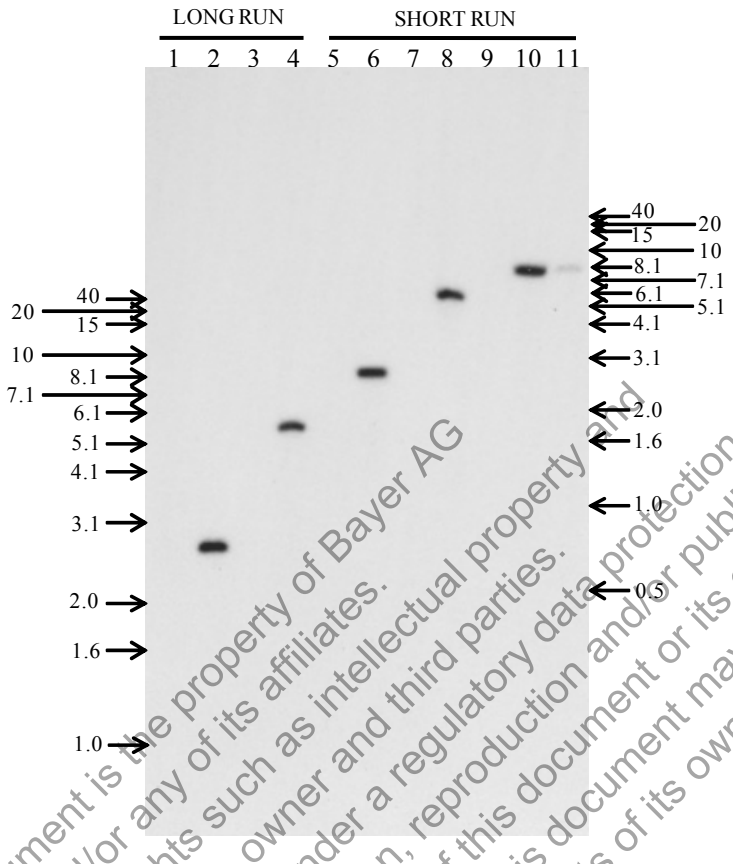


Figure V-2. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 8

The blot was hybridized with a ^{32}P labeled T-DNA I probe that spans a portion of the T-DNA I sequence (Probe 8, Figure IV-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

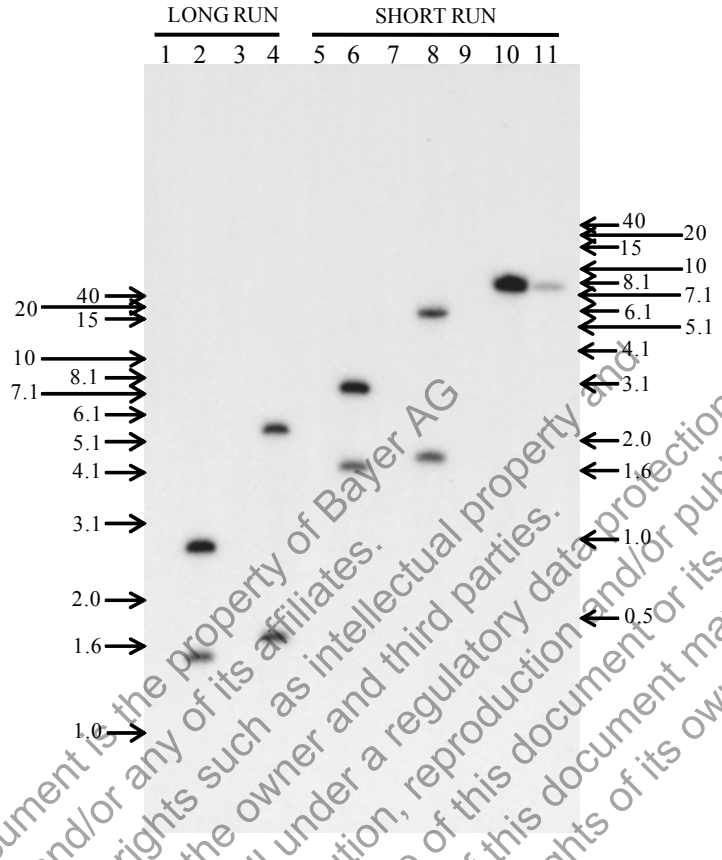


Figure V-3. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 9

The blot was hybridized with a ^{32}P labeled T-DNA I probe that spans a portion of the T-DNA I sequence (Probe 9, Figure IV-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

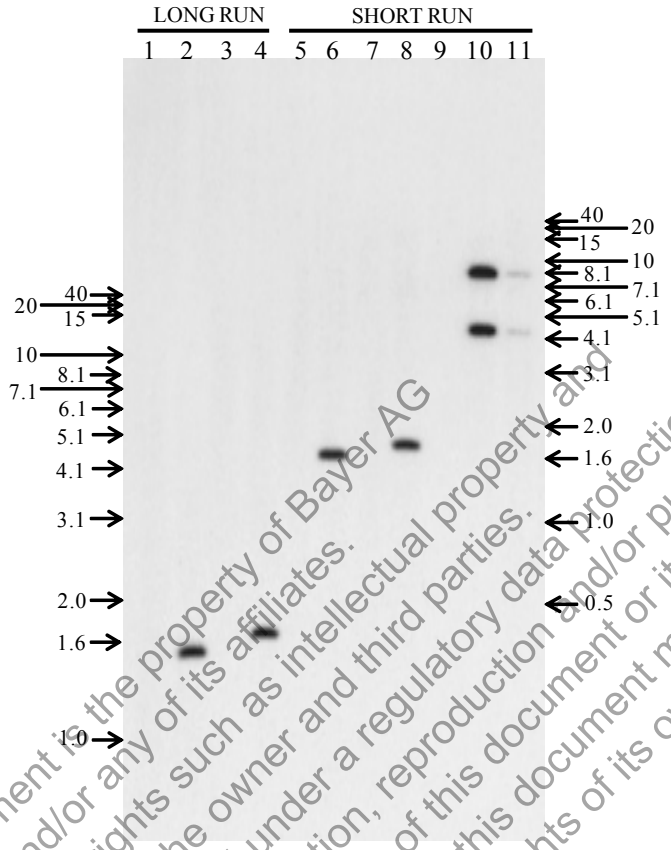


Figure V-4. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA I in MON 87708: Probe 10

The blot was hybridized with a ^{32}P labeled T-DNA I probe that spans a portion of the T-DNA I sequence (Probe 10, Figure IV-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

V.B. Southern Blot Analysis to Determine the Presence or Absence of T-DNA II Sequences in MON 87708

To determine the presence or absence of T-DNA II sequences, MON 87708 and conventional control genomic DNA were digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I and Southern blots were hybridized with probes that span the T-DNA II sequence (Figure IV-1). As a positive control on the Southern blots, PV-GMHT4355 digested with the restriction enzyme combination *Aat* II/*Nde* I was mixed with predigested conventional control DNA. The positive hybridization control was spiked at 0.1 and 1 genome equivalent to demonstrate sufficient sensitivity of the Southern blot. Each blot was hybridized with one of three overlapping probes spanning the T-DNA II sequence other than the two border regions that share the same sequences as present in T-DNA I (Probes 4, 5 and 6, Figure IV-1). If T-DNA II sequences were present in MON 87708, then probing with the T-DNA II sequences should result in unique hybridizing bands. The results of this analysis are shown in Figures V-5 through V-7.

V.B.1. Probe 4

Conventional control DNA digested with *Bsp1286* I/*Pvu* II (Figure V-5, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure V-5, lanes 3 and 7) and hybridized with probe 4 showed no detectable hybridization bands, as expected for the negative control. PV-GMHT4355, previously digested with *Aat* II/*Nde* I and mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure V-5, lanes 10 and 11), produced two bands at ~4.0 kb and ~7.4 kb. Both bands were expected because probe 4 contains *E9* sequence that hybridized to both the ~4.0 kb and the ~7.4 kb fragments from the digested plasmid (refer to Figure V-1 and Table V-1). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 4 (Figure IV-1) produced one unique band at ~1.5 kb (Figure V-5, lanes 2 and 6). MON 87708 DNA digested with *Hpa* I/*Kpn* I and hybridized with probe 4 (Figure IV-1) produced one unique band at ~1.7 kb (Figure V-5, lanes 4 and 8). Probe 4 contains the *E9* 3' non-translated region sequence that is also contained in T-DNA I (Figure IV-1). Therefore, probe 4 was expected to hybridize to the ~1.5 kb and ~1.7 kb fragments (Figure V-1) derived from the T-DNA I insert. These bands were also detected by probe 10 (Figure V-4, lanes 2 and 6, and lanes 4 and 8). Any T-DNA II sequences other than those associated with T-DNA I would be detected as novel bands. No unexpected bands were detected indicating that MON 87708 contains no detectable T-DNA II elements covered by probe 4.

V.B.2. Probe 5

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-6, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure V-6, lanes 3 and 7) and hybridized with probe 5 (Figure IV-1) showed no detectable hybridization bands, as expected for the negative control. PV-GMHT4355, previously digested with *Aat* II/*Nde* I

and mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure V-6, lanes 10 and 11), produced two expected size bands at ~4.0 kb and ~7.4 kb (refer to Figure V-1 and Table V-1). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-6, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure V-6, lanes 4 and 8) and hybridized with probe 5, produced no detectable hybridization bands. These results indicate that MON 87708 contains no detectable T-DNA II elements covered by probe 5.

V.B.3. Probe 6

Conventional control DNA digested with *Bsp1286* I/*Pvu* II (Figure V-7, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure V-7, lanes 3 and 7) and hybridized with probe 6 (Figure IV-1) showed no detectable hybridization bands, as expected for the negative control. PV-GMHT4355 previously digested with *Aat* II/*Nde* I and mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure V-7, lanes 10 and 11) produced one expected size band at ~7.4 kb (refer to Figure V-1, and Table V-1). These results indicate that the probe is hybridizing to its target sequence.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-7, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure V-7, lanes 4 and 8) and hybridized with probe 6 produced no detectable hybridization bands. These results indicated that MON 87708 contains no detectable T-DNA II elements covered by probe 6.

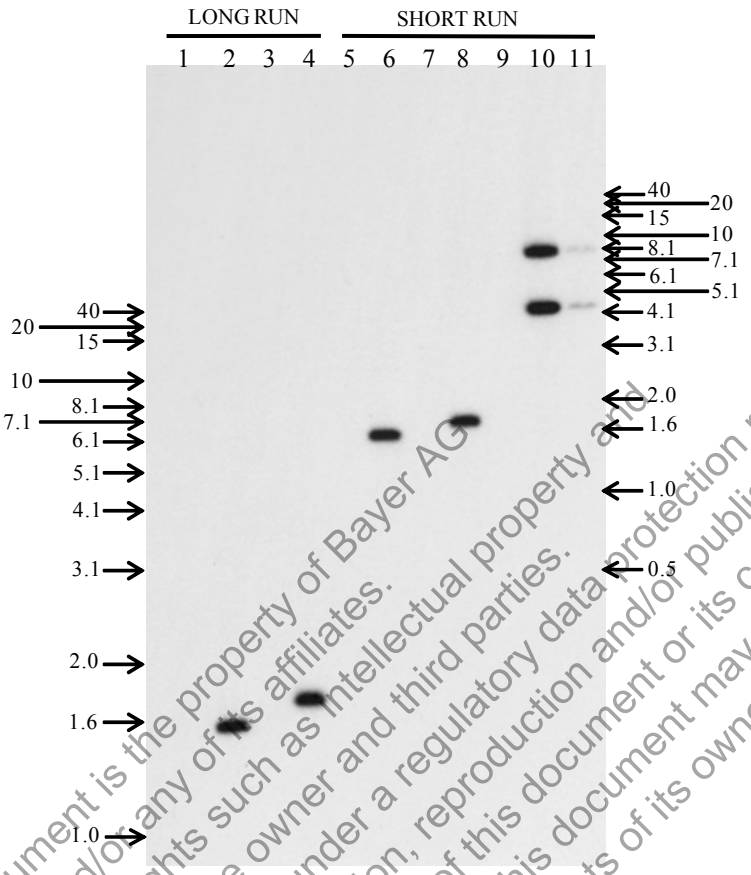


Figure V-5. Sothern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 4

The blot was hybridized with a ^{32}P labeled T-DNA II probe that spans a portion of the T-DNA II sequence (Probe 4, Figure IV-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

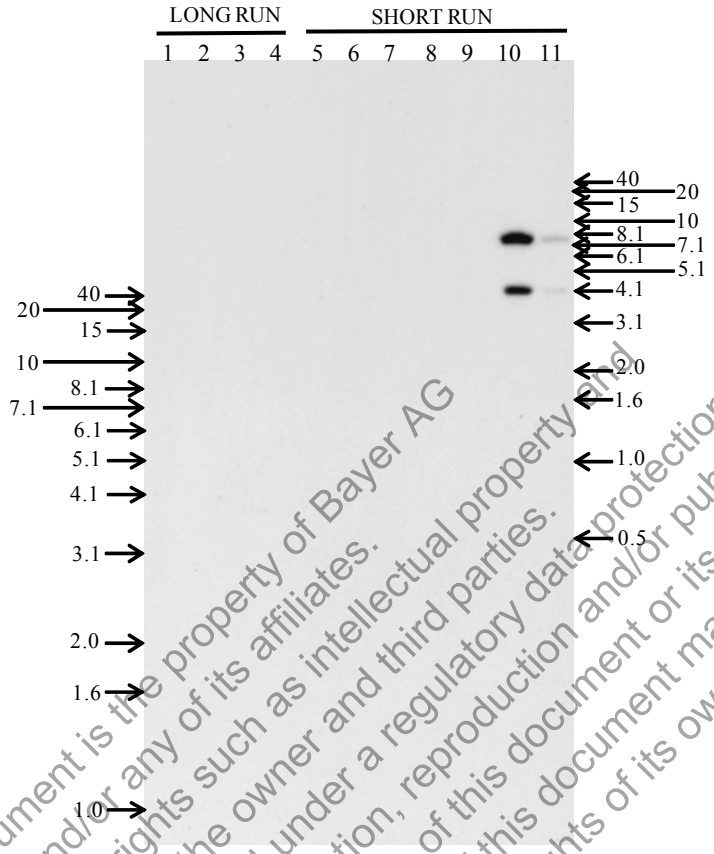


Figure V-6. Sothern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 5

The blot was hybridized with a ^{32}P labeled T-DNA II probe that spans the coding region of the T-DNA II sequence (Probe 5, Figure IV-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

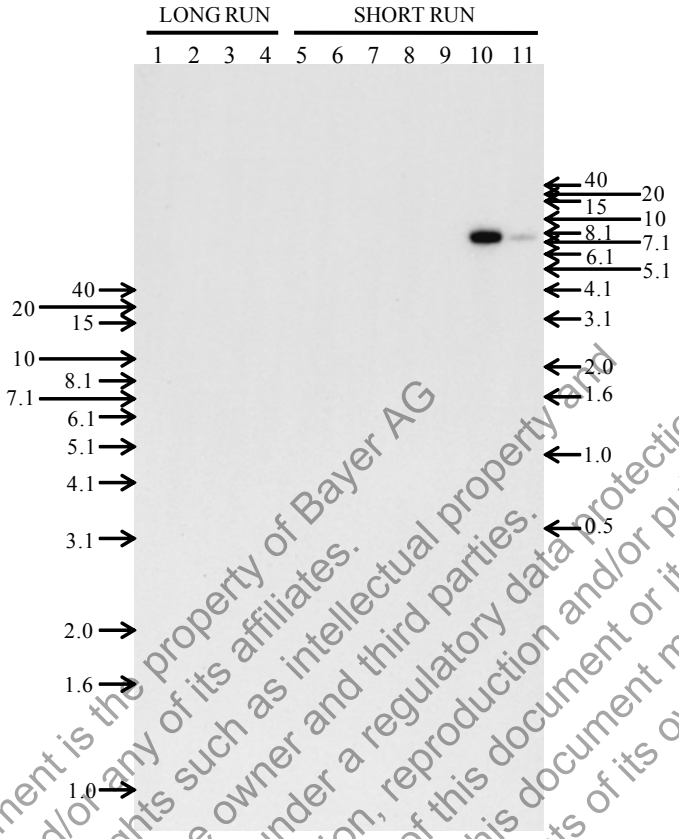


Figure V-7. Sothern Blot Analysis to Detect the Presence or Absence of T-DNA II Sequences in MON 87708: Probe 6

The blots were hybridized with a ^{32}P labeled T-DNA II probe that spans a portion of the T-DNA II sequence (Probe 6, Figure IV-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/ *Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

V.C. Southern Blot Analysis to Determine the Presence or Absence of Plasmid Vector PV-GMHT4355 Backbone Sequences in MON 87708

To determine the presence or absence of PV-GMHT4355 backbone sequences, MON 87708 and conventional control genomic DNA were digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I and Southern blots were hybridized with probes that span the plasmid vector backbone sequence (Figure IV-1). As a positive control on the Southern blots, digested PV-GMHT4355 and probe templates generated from PV-GMHT4355 were used. Approximately 1 genome equivalent of PV-GMHT4355 digested with the restriction enzyme combination *Aat* II/*Nde* I was mixed with predigested conventional control DNA. As an additional positive control, approximately 0.1 and 1 genome equivalent of probe templates (Figure IV-1, probes 1, 2, 3, and 7) generated from PV-GMHT4355 were mixed with predigested conventional control DNA. The blot was hybridized with probes 1, 2, 3, and 7 (Figure IV-1). If backbone sequences are present in MON 87708, then probing with backbone probes should result in hybridizing bands. The results of this analysis are shown in Figure V-8.

V.C.1. Plasmid Vector Backbone Probes 1, 2, 3, and 7

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-8, lanes 1 and 5) or *Hpa* I/*Kpn* I (Figure V-8, lanes 3 and 7) and hybridized simultaneously with the probes 1, 2, 3, and 7 (Figure IV-1) spanning the entire backbone sequence of PV-GMHT4355 showed no detectable hybridization bands, as expected for the negative control. PV-GMHT4355, previously digested with *Aat* II/*Nde* I and mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure V-8, lane 10), produced two expected size bands at ~4.0 kb and ~7.4 kb (refer to Figure V-1 and Table V-1). In addition, there are two faint hybridization bands at ~4.5 kb and ~11 kb (Figure V-8, lane 10). The ~4.5 kb band was likely due to an artifact that occurred during the electrophoresis, and the ~11 kb band was likely due to undigested plasmid DNA or an artifact that occurred during the electrophoresis. Since these faint bands appeared only in the plasmid spike and the expected bands were observed, they have no negative impact on the conclusions made from this blot. Probe template spikes of probes 1, 2, 3, and 7 (Figure IV-1) generated from PV-GMHT4355 mixed with conventional control DNA predigested with *Hpa* I/*Kpn* I (Figure V-8, lanes 11 and 12) produced the expected size bands at ~0.2 kb, ~0.9 kb, ~1.5 kb, and ~1.8 kb, respectively. The 0.1 genome equivalent copy of the expected ~0.2 kb band was not observed on the exposure of the Southern blot that is reported in Figure V-8, lane 12; however, the band was observed on the same blot with a longer exposure. These results indicate that the probes are hybridizing to their target sequences.

MON 87708 DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-8, lanes 2 and 6) or *Hpa* I/*Kpn* I (Figure V-8, lanes 4 and 8) and hybridized simultaneously with probes 1, 2, 3, and 7 produced no detectable bands. The data indicate MON 87708 contains no detectable backbone sequences from PV-GMHT4355.

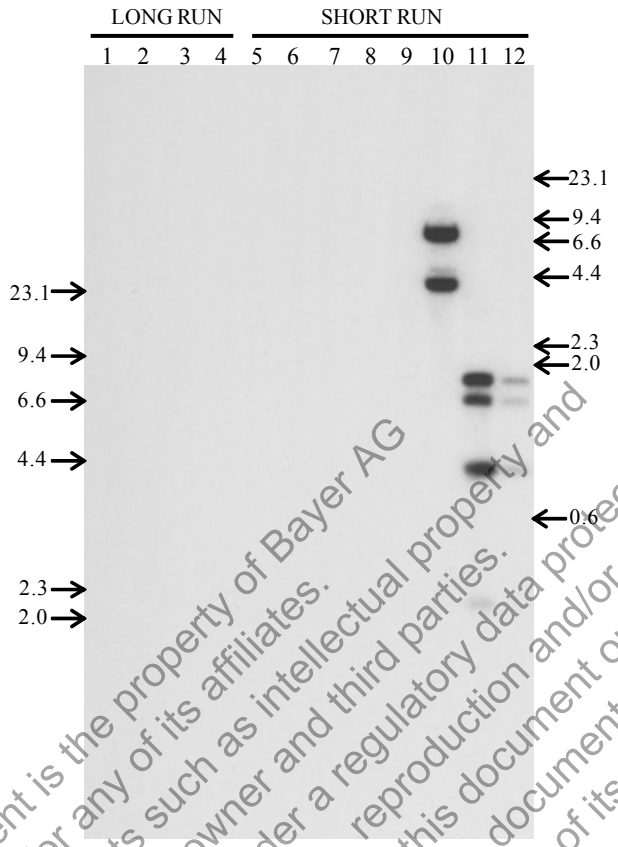


Figure V-8. Southern Blot Analysis to Determine the Presence or Absence of PV-GMHT4355 Backbone Sequences in MON 87708: Probes 1, 2, 3, and 7

The blot was hybridized simultaneously with four ^{32}P labeled backbone probes (Probes 1, 2, 3, and 7, Figure IV-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	MON 87708 (*Bsp1286* I/*Pvu* II)
3.	Conventional control (*Hpa* I/*Kpn* I)
4.	MON 87708 (*Hpa* I/*Kpn* I)
5.	Conventional control (*Bsp1286* I/*Pvu* II)
6.	MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Conventional control (*Hpa* I/*Kpn* I)
8.	MON 87708 (*Hpa* I/*Kpn* I)
9.	Blank
10.	Conventional control (*Hpa* I/*Kpn* I) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
11.	Conventional control (*Hpa* I/*Kpn* I) spiked with probe templates [~1 genome equivalent]
12.	Conventional control (*Hpa* I/*Kpn* I) spiked with probe templates [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

V.D. Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 87708

The organization of the elements within the T-DNA I was confirmed by DNA sequence analyses. PCR primers were designed with the intent to amplify two overlapping regions of the DNA that span the entire length of T-DNA I (Figure V-9). The amplified DNA segments were subjected to DNA sequencing analyses. The T-DNA I in MON 87708 is 3003 bp and matches the sequence of plasmid vector PV-GMHT4355, as described in Tables IV-1 and V-2

V.E. PCR and DNA Sequence Analyses to Examine the MON 87708 Insertion Site

PCR and sequence analyses were performed on genomic DNA extracted from MON 87708 and conventional control to examine the insertion sites. The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5′ end of T-DNA I paired with a second primer specific to the genomic DNA sequence flanking the 3′ end of T-DNA I (Figure V-10). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5′ and 3′ flanking sequences of T-DNA I in MON 87708 indicates there was an 899 bp deletion and a 128 bp insertion just 5′ of T-DNA I, and a 35 bp insertion just 3′ of T-DNA I. These molecular rearrangements presumably resulted from double stranded break repair mechanisms in the plant during the *Agrobacterium* mediated transformation process (Salomon and Puchta, 1998).

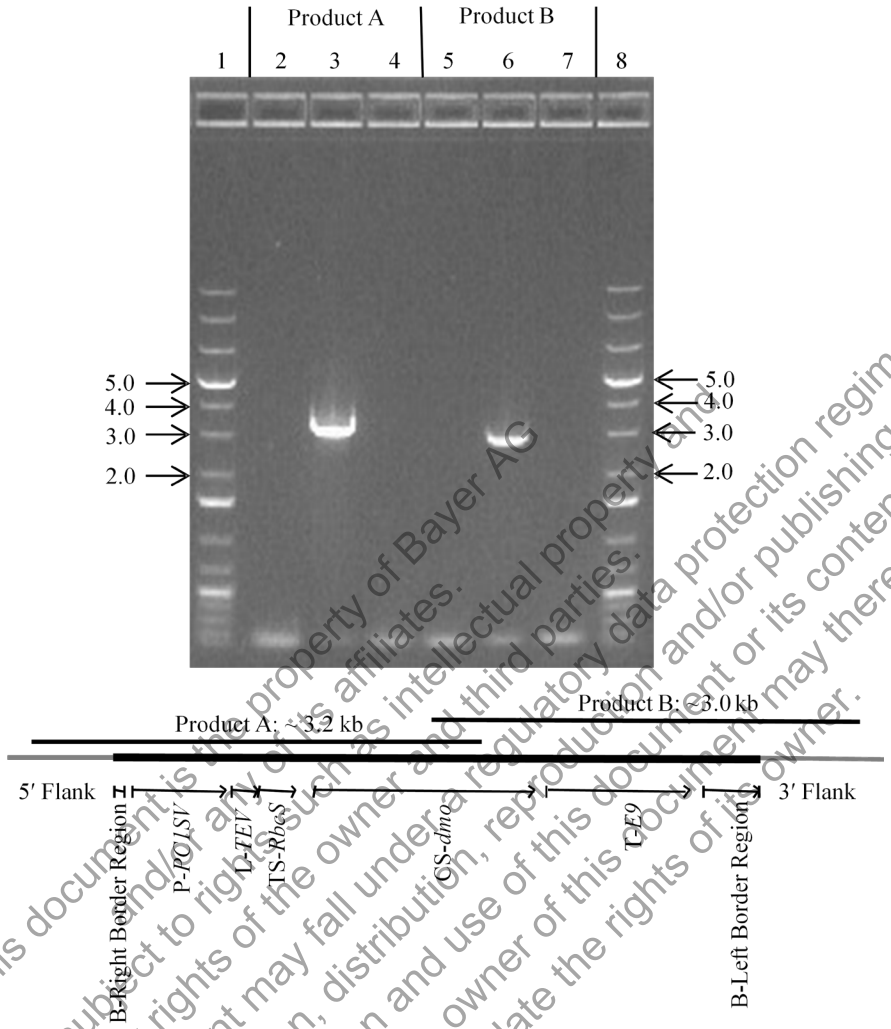


Figure V-9. Overlapping PCR Analysis Across the Insert in MON 87708

PCR analyses were performed on MON 87708 genomic DNA extracted from leaf (Lanes 3 and 6). Lanes 2 and 5 contain reactions with conventional control DNA while lanes 4 and 7 are reactions containing no template DNA. Lanes 1 and 8 contain Fermentas GeneRuler™ 1 kb Plus DNA Ladder. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87708 that appears at the bottom of the figure. Five microliters of each of the PCR products was loaded on the gel. This figure is representative of the data generated; however, the specific bands from this gel were not excised and sequenced.

Lane:

1. GeneRuler™ 1 kb Plus DNA Ladder
2. Conventional control DNA
3. MON 87708 genomic DNA
4. No template DNA control
5. Conventional control DNA
6. MON 87708 genomic DNA
7. No template DNA control
8. GeneRuler™ 1 kb Plus DNA Ladder

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel

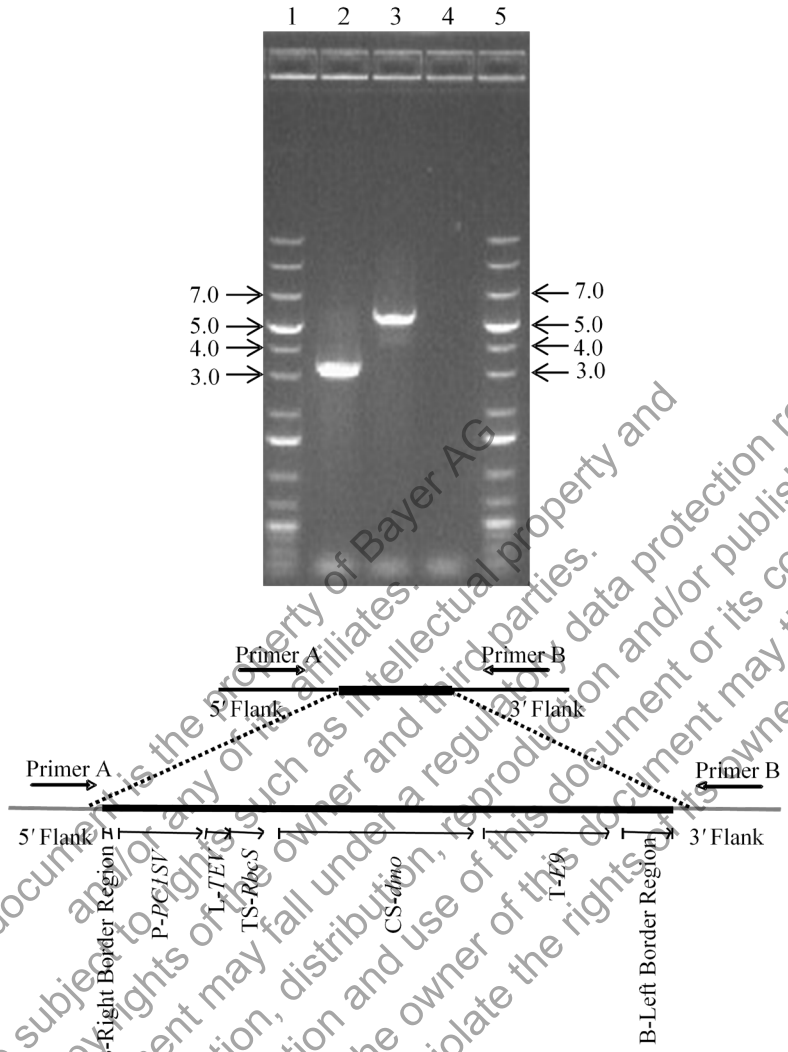


Figure V-10. PCR Amplification of the MON 87708 Insertion Site

Depiction of the MON 87708 insertion locus in conventional control and MON 87708. PCR amplification was performed using Primer A in the 5′ flanking sequence and Primer B in the 3′ flanking sequence of the insert in MON 87708 to examine the insertion site in conventional soybean and MON 87708.

Lane	Description
1	GeneRuler™ 1 kb Plus DNA Ladder
2	Conventional control DNA
3	MON 87708 genomic DNA
4	No template DNA control
5	GeneRuler™ 1 kb Plus DNA Ladder

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

V.F. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87708

In order to demonstrate the stability of the T-DNA I insert present in MON 87708 through multiple generations, Southern blot analysis was performed using DNA obtained from five breeding generations of MON 87708. For reference, the breeding history of MON 87708 is presented in Figure V-11. The specific generations tested are indicated in the legend of Figure V-11. The R_3 generation was used for the molecular characterization analyses shown in Figures V-2 through V-8. To analyze stability, four additional generations were evaluated by Southern blot analysis and compared to the fully characterized R_3 generation. Genomic DNA, isolated from each of the selected generations of MON 87708 and the conventional control, was digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-1) and hybridized with probe 9 (Figure IV-1). Probe 9 will detect both border fragments generated by the *Bsp1286* I/*Pvu* II digestion. Any instability associated with the T-DNA I insert would be detected as novel bands within the fingerprint on the Southern blot. The Southern blot has the same positive hybridization controls as described in Section V.A. The results are shown in Figure V-12.

V.F.1. Probe 9

Conventional control DNA digested with the restriction enzyme combination *Bsp1286* I/*Pvu* II produced no hybridization signals (Figure V-12, lane 1) as expected for the negative control. PV-GMHT4355, digested with the restriction enzyme combination *Aat* II/*Nde* I and mixed with conventional control DNA predigested with the restriction enzyme combination *Bsp1286* I/*Pvu* II (Figure V-12, lanes 8 and 9), produced the expected size band at ~7.4 kb (refer to Figure V-1 and Table V-1). Additionally, there were two very faint hybridization bands in the ~1 genome equivalent plasmid vector PV-GMHT4355 spike at ~4.3 kb and ~6.5 kb observed in a longer exposure of the Southern blot (data not shown). These bands were likely due to an artifact that occurred during the electrophoresis. Since these faint bands appeared only in the plasmid vector spike and the expected ~7.4 kb band was observed, they do not have any negative impact on the conclusions from this Southern blot analysis. These results indicate that the probe is hybridizing to its target sequence.

Digestion of MON 87708 genomic DNA from multiple generations with the restriction enzyme combination *Bsp1286* I/*Pvu* II and hybridized with probe 9 (Figure IV-1) produced two bands at ~1.5 kb and ~2.6 kb (Figure V-12, lanes 2-6). The ~1.5 kb band is the expected size for the border segment containing the 3′ end of T-DNA I along with the adjacent genomic DNA flanking the 3′ end of T-DNA I (Figure V-1). The ~2.6 kb band is the expected size for the border segment containing the 5′ end of T-DNA I along with the adjacent genomic DNA flanking the 5′ end of T-DNA I (Figure V-1). The fingerprint of the Southern blot signals from multiple generations, R_2, R_4, R_5, and R_6 (Figure V-12, lanes 2, 4, 5, and 6), of MON 87708 is consistent with the fully characterized generation R_3 (Figure V-3, lanes 2 and 6; Figure V-12, lane 3). No unexpected bands were detected, indicating that MON 87708 contains one copy of T-DNA I that is stably maintained across multiple generations.

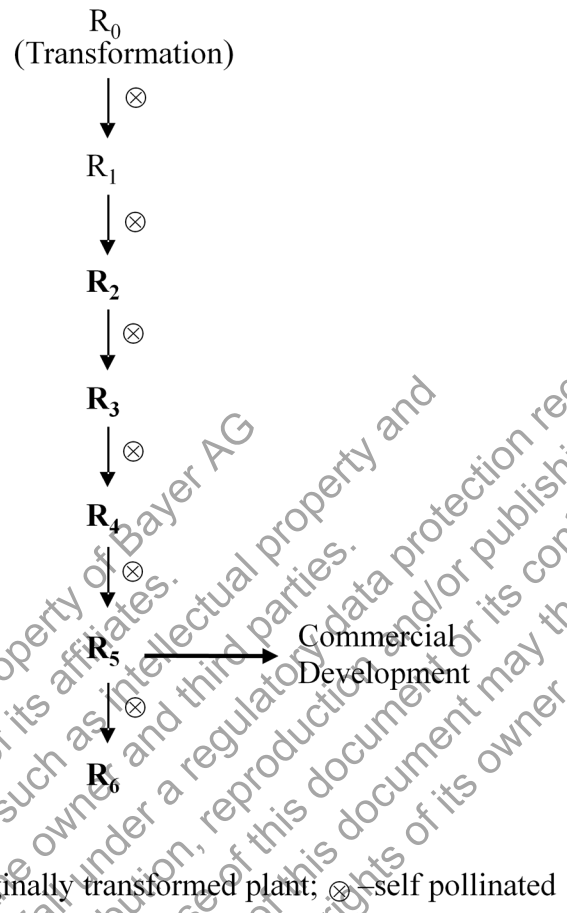


Figure V-11. Breeding History of MON 87708

The R_3 generation was used for the molecular analyses reported in Figures V-2 through V-8 and is referred to as MON 87708 in all Southern blot figures. The R_5 generation was used for development of all commercial products. MON 87708 from generations R_2, R_3, R_4, R_5, and R_6 (bolded in the breeding tree) were used for analyzing the stability of T-DNA I in MON 87708 across generations (Figure V-12).

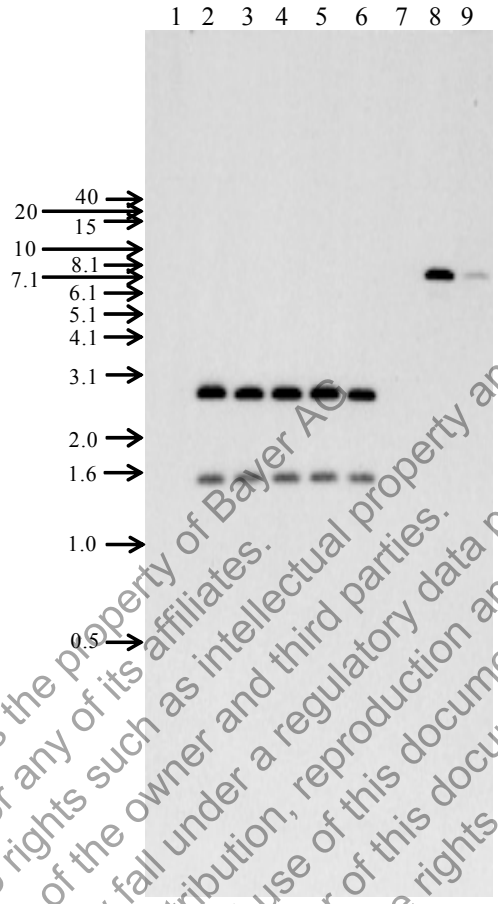


Figure V-12. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87708: Probe 9

The blot was hybridized with a ^{32}P labeled T-DNA I probe that spans the coding region of the T-DNA I (Probe 9, Figure IV-1). Each lane contains approximately 10 µg of digested genomic DNA isolated from leaf tissue. Lane designations are as follows:

Lane	Description
1.	Conventional control (*Bsp1286* I/*Pvu* II)
2.	R_2 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
3.	R_3 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
4.	R_4 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
5.	R_5 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
6.	R_6 generation of MON 87708 (*Bsp1286* I/*Pvu* II)
7.	Blank
8.	Conventional control (*Bsp1286* I and *Pvu* II) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~1 genome equivalent]
9.	Conventional control (*Bsp1286* I and *Pvu* II) spiked with PV-GMHT4355 (*Aat* II/*Nde* I) [~0.1 genome equivalent]

Arrows denote sizes of DNA, in kilobase pairs, obtained from molecular weight markers on ethidium bromide stained gel.

V.G. Inheritance of the Genetic Insert in MON 87708

During development of MON 87708, segregation data were generated to assess the heritability and stability of the T-DNA I present in MON 87708. Chi square analysis was performed over several generations to confirm the segregation and stability of T-DNA I in MON 87708. The Chi square analysis is based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 87708 breeding path, from which segregation data were generated, is described in Figure V-13. The transformed R_0 plant was self-pollinated to produce R_1 seed. An individual plant (#2, designated as MON 87708), that was homozygous for a single copy of the *dmo* expression cassette, was identified from the R_1 segregating population via Invader® and Southern blot analysis. Invader is a non-PCR based assay that can be used to accurately quantify transgene copy number in plant genomes (Gupta, et al., 2008).

The selected R_1 MON 87708 plant was self-pollinated to give rise to a population of R_2 plants that were repeatedly self-pollinated through the R_4 generation. At each generation, the fixed homozygous plants were tested for the expected segregation pattern of 1:0 (positive:negative) for the *dmo* expression cassette using the Invader analysis, Southern blot analysis, and/or PCR.

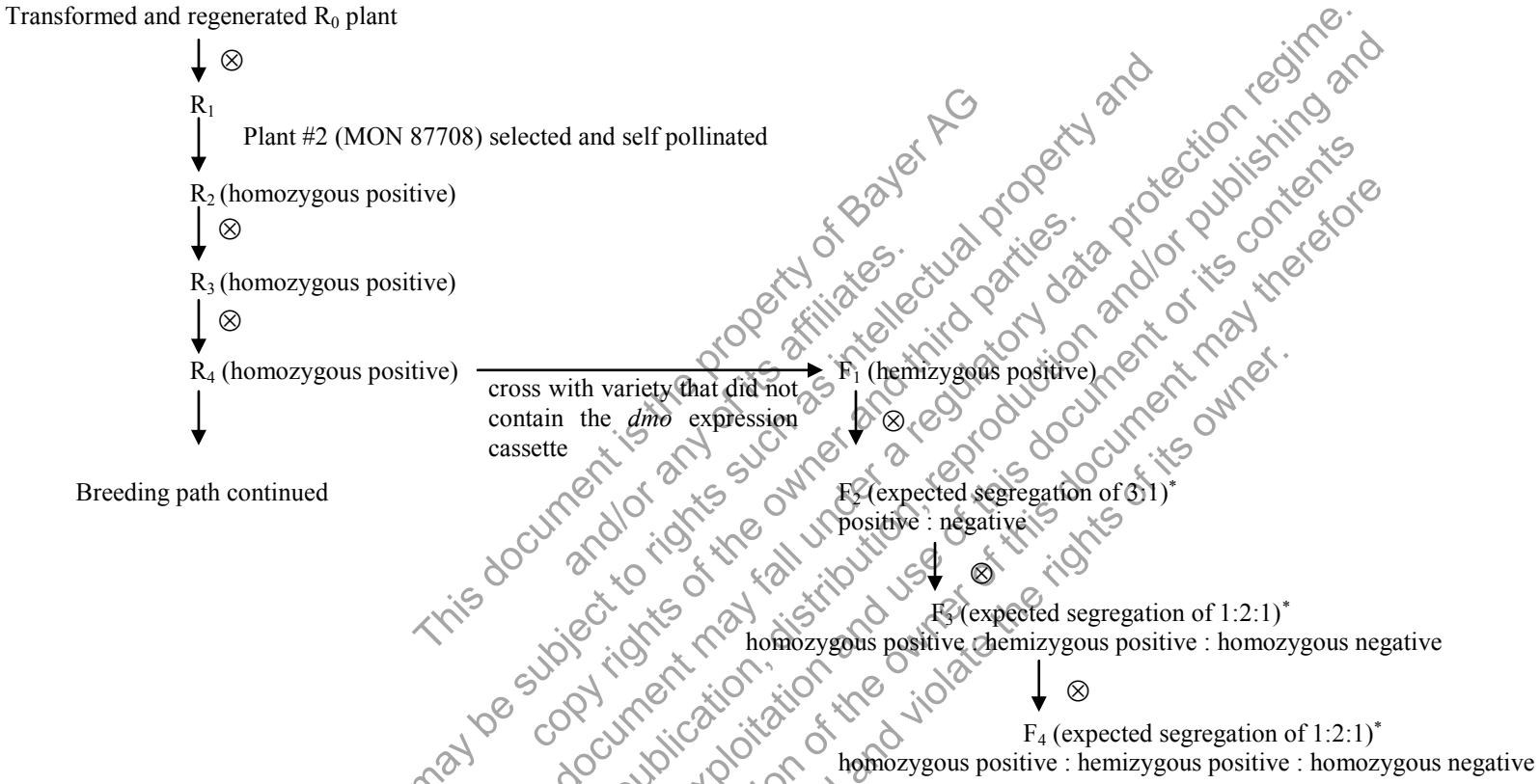
At the R_4 generation, homozygous MON 87708 plants were bred via traditional breeding with a soybean variety that did not contain the *dmo* expression cassette to produce F_1 hemizygous seed. The resulting F_1 plants were then self-pollinated to produce F_2 seed. The F_2 plants were tested for the presence of the *dmo* expression cassette by Invader analysis, and hemizygous F_2 plants were selected and self-pollinated to produce F_3 seed. This process was repeated through the F_4 generation. The heritability and stability of the *dmo* expression cassette in MON 87708 was assessed in the F_2, F_3, and F_4 generations. A total of 2413 out of 3223 plants were positive for the presence of the *dmo* expression cassette in the F_2 generation; however, the zygosity of 200 of those 2413 plants could not be determined from the assay. Exclusion of these *dmo*-positive plants from the analysis likely would have skewed the distribution of homozygous positive: hemizygous positive:homozygous negative plants. Therefore, the segregation assessment in the F_2 generation was based on the presence or absence of the *dmo* expression cassette which was expected to segregate at a 3:1 (positive:negative) ratio according to Mendelian inheritance principles. Subsequently, assessment of segregation in the F_3 and F_4 generations was based on zygosity, and the *dmo* expression cassette was predicted to segregate at a 1:2:1 (homozygous positive:hemizygous positive :homozygous negative) ratio according to Mendelian inheritance principles.

A Chi square ($\chi 2$) analysis was used to compare the observed segregation ratios to the expected ratios according to Mendelian inheritance principles. The $\chi 2$ was calculated as:

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

where o = observed frequency of the phenotype and e = expected frequency of the phenotype. The level of statistical significance was predetermined to be 5%.

The results of the $\chi 2$ analysis of the segregating progeny of MON 87708 are presented in Table V-3. The $\chi 2$ value for the F_2, F_3, and F_4 generations indicated no significant difference between the observed and expected segregation ratios. These results support the conclusion that the *dmo* expression cassette in MON 87708 resides at a single locus within the soybean genome and is inherited according to expected Mendelian inheritance principles. These results are also consistent with the molecular characterization data that indicate MON 87708 contains a single, intact copy of the *dmo* expression cassette that was inserted into the soybean genome at a single locus.



⊗ = Self pollinated

Figure V-13. Breeding Path for Generating Segregation Data for MON 87708

* Chi-square analysis conducted on segregation data from the F_2, F_3, and F_4 generations.
Note: Hemizygous positive plants in the F_1, F_2, F_3, and F_4 generations were selected and self-pollinated to produce seed of the subsequent generation.

Table V-3. Segregation of the *dmo* Gene During the Development of MON 87708

Generation[1]	Total Plants Tested[2]	Observed # Plants Positive	Observed # Plants Negative	3:1 Segregation[3] Expected # Plants Positive	Expected # Plants Negative	χ^2	Probability
F_2	3223	2413	810	2417.25	805.75	0.03	0.863

Generation[1]	Total Plants Tested[2]	Observed # Plants Homozygous Positive	Observed # Plants Hemizygous Positive	Observed # Plants Homozygous Negative	1:2:1 Segregation Expected # Plants Homozygous Positive	Expected # Plants Hemizygous Positive	Expected # Plants Homozygous Negative	χ^2	Probability
F_3	118	29	52	37	29.5	59	29.5	2.7	0.2534
F_4	343	83	171	89	85.75	171.5	85.75	0.2	0.8991

[1]F_2, F_3, and F_4 progeny were from self-pollinated F_1, F_2, and F_3 plants hemizygous positive for the *dmo* expression cassette, respectively.
[2]Plants were tested for the presence of the *dmo* expression cassette by Invader analysis.
[3]Assessment of segregation in the F_2 generation was based on the presence or absence of the *dmo* expression cassette due to an unacceptable number of *dmo*-positive plants for which zygosity could not be determined from the assay.

V.H. Genetic Modification Characterization Conclusion

Molecular characterization of MON 87708 by Southern blot analyses demonstrated that a single copy of the T-DNA I sequences from the plasmid vector PV-GMHT4355 was integrated into the soybean genome at a single locus. There were no additional genetic elements from the T-DNA II or backbone sequences of the plasmid vector PV-GMHT4355 detected, linked or unlinked to the intact T-DNA I present in MON 87708.

The PCR and DNA sequence analyses performed on MON 87708, which confirmed the organization of the elements within T-DNA I, demonstrated the 5′ and 3′ insert-to-plant junctions and determined the complete DNA sequence of T-DNA I and adjacent DNA sequence flanking the insert in MON 87708. Analysis of the T-DNA I insertion site indicates that there was an 899 bp deletion of genomic DNA at the insert-to-plant DNA junction. Additionally, a 128 bp insertion was identified in the 5′ adjacent flanking sequence of MON 87708 and a 35 bp insertion was identified in the 3′ adjacent flanking sequence of MON 87708.

Generational stability analysis by Southern blot demonstrated that MON 87708 has been maintained through five breeding generations, thereby confirming the stability of T-DNA I in MON 87708. Results from segregation analyses show heritability and stability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA I in MON 87708 at a single chromosomal locus.

VI. SAFETY ASSESSMENT OF EXPRESSED PRODUCTS

In this section, a multistep approach is used to assess the safety of MON 87708 DMO. These steps include: 1) characterization of the physicochemical and functional properties of DMO; 2) quantification of DMO expression in MON 87708 plant tissues; 3) examination of the similarity of DMO to known allergens, toxins or other biologically active proteins known to have adverse effects on mammals; 4) evaluation of the digestibility of DMO in simulated gastrointestinal fluids; 5) documentation of the history of safe consumption of DMO or its structural and functional homology to proteins that lack adverse effects on human or animal health; 6) evaluation of the stability of DMO after heat treatment; 7) investigation of potential mammalian toxicity in an acute mouse gavage and calculation of margins of exposure; and 8) assessment of the potential for allergenicity, toxicity, and adverse biological activity of putative polypeptides encoded by the insert and flanking sequences.

Most assessments described in this section utilized MON 87708 DMO, isolated from seed of MON 87708. In a few instances, DMO produced from an alternative source was used; in which case, the protein is clearly described for that assessment. The DMO protein descriptions are found in Appendix B, along with the purification process for MON 87708 DMO and methods used for characterization.

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.

VI.A. Description of MON 87708 DMO

In MON 87708, the introduced DMO proteins are active in the chloroplast, a plastid organelle, where it can interact with other proteins needed for its function (Section VI.B; Behrens et al, 2007). In the construction of the plasmid vector used in the development of MON 87708, PV-GMHT4355, a transit peptide coding sequence (*RbcS,* Table V-2) was joined to the *dmo* coding sequence to transport the produced protein to the soybean chloroplast; this coding sequence results in the production of a precursor protein consisting of the DMO protein, a chloroplast transit peptide (CTP), and an intervening sequence (IS), and is referred to as the MON 87708 DMO precursor protein. 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 DMO precursor protein produced in MON 87708.

MON 87708 contains a *dmo* expression cassette that encodes for a single MON 87708 DMO precursor protein targeted to the plant's chloroplasts. The MON 87708 DMO precursor protein contains 84 amino acids at the N-terminus of the protein that were added to target the protein to the chloroplast. These additional amino

acids correspond to a 57 amino acid chloroplast transit peptide (CTP) from pea Rubisco small subunit and the first 24 amino acids from the N-terminus of the mature Rubisco small subunit, which are incorporated to improve the targeting of the precursor protein to the chloroplast (Behrens et al., 2007; Comai, et al., 1988). Finally, three amino acids encoded by an intervening sequence were used for cloning purposes (Tables IV-1 and V-2).

Analysis of mature seed extracts from MON 87708 by western blot demonstrated the presence of two immunoreactive bands (Figure VI-4). Characterization of these two bands (Section VI.D) revealed that the precursor protein had been processed into two forms of the protein according to Figure VI-1. Analysis of these two bands determined that the lower molecular weight band corresponded to the full-length protein that was the result of the removal of the CTP, the 24 amino acids from pea Rubisco small subunit and the amino acids from the intervening sequence (Figure VI-1 and Section VI.D.1.2). Additional processing at the N-terminus by methionine aminopeptidase removed the N-terminal methionine residue (Section VI.D.1.2), a common occurence in all organisms (Arfin and Bradshaw, 1988; Bradshaw, et al., 1998). This form of the protein will be referred to as the MON 87708 DMO protein; it has an apparent molecular weight of 39.8 kDa and is a single polypeptide chain of 339 amino acids (Figure VI-1 and Appendix B, Figure B-1). Alternative processing of the MON 87708 DMO precursor protein resulted in the production of a second higher molecular weight (approximately 42 kDa) protein. The 42 kDa protein corresponds to the MON 87708 DMO protein plus 27 amino acids on the N-terminus originating from the pea Rubisco small subunit and intervening sequence that were not cleaved. Since the N-terminal methionine of the MON 87708 DMO protein is not exposed in this processed form of the protein it was not cleaved, resulting in a 367 amino acid polypeptide (Figures VI-1 and Appendix B, Figure B-1). This form of the protein will be referred to as the MON 87708 DMO+27 protein. This alternative processing was not unexpected since alternative processing of a DMO precursor protein was also observed in other dicamba-tolerant plants transformed with a similar cassette containing the *dmo* gene (Behrens et al., 2007). Both the MON 87708 DMO protein and MON 87708 DMO+27 protein were characterized by the methods described in Appendix B.

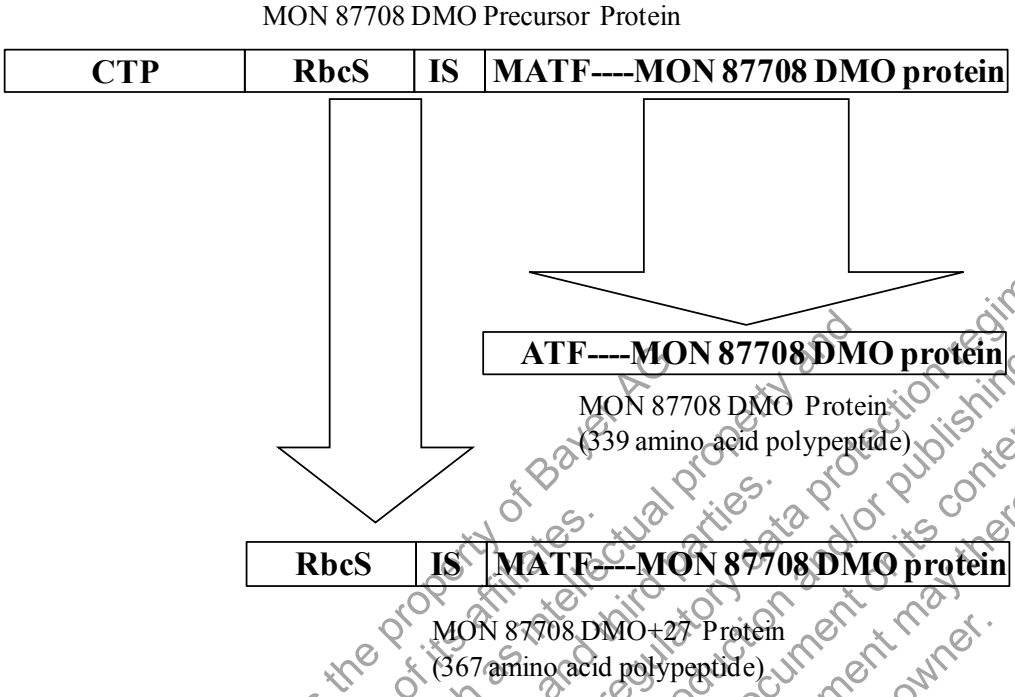


Figure VI-1. Processing of MON 87708 DMO Precursor Protein
The MON 87708 DMO precursor protein produced in MON 87708 contains a 57 amino acid chloroplast transit peptide (CTP), the first 24 amino acids from the N-Terminus of the small sub-unit of Rubisco (*RbcS*), and three amino acids from an intervening sequence (IS). Processing in the chloroplast removes the *RbcS*, IS, and the N-terminal methionine resulting in the MON 87708 DMO protein (339 amino acids). Alternative processing occurs when only the CTP is cleaved to produce the MON 87708 DMO+27 protein (367 amino acids). MATF represents the N-terminal amino acids of the *dmo* coding sequence. The methionine (M) has been removed in the MON 87708 DMO protein by methionine amino-peptidase.

The MON 87708 DMO protein has an identical sequence to the wild-type DMO protein (Herman et al., 2005), except for an additional alanine in position two added for cloning purposes and a cysteine instead of tryptophan at position 112 (Appendix B, Figure B-1). The MON 87708 DMO+27 protein is identical to the MON 87708 DMO protein, and has the same amino acid differences when compared to the wild-type DMO, with the exception of the additional 27 amino acids and the methionine at the N-terminal portion of MON 87708 DMO protein (Appendix B, Figure B-1). The differences in the amino acid sequence between the wild-type DMO protein and MON 87708 DMO protein and the MON 87708 DMO+27 protein are not expected to have an effect on structure, activity, or specificity because they are sterically distant from the catalytic site (Appendix B, Figure B-2).

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 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). In MON 87708, the trimer can be comprised of the MON 87708 DMO protein, the MON 87708 DMO+27 protein, or a combination of both. This document will refer to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. MON 87708 DMO was purified from soybean of MON 87708 and was characterized (Section VI.D). Under denaturing conditions, for example when separating on SDS-PAGE, the proteins were analyzed individually (*e.g.*, N-terminal sequencing, Section VI.D.1.2). In these cases each protein will be referred to individually as either the MON 87708 DMO protein or the MON87708 DMO+27 protein.

VI.B. Mode-of-Action and Specificity of DMO

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 soybean, soil, and livestock 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 thousand 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 nicotinamide adenine dinucleotide (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-2.

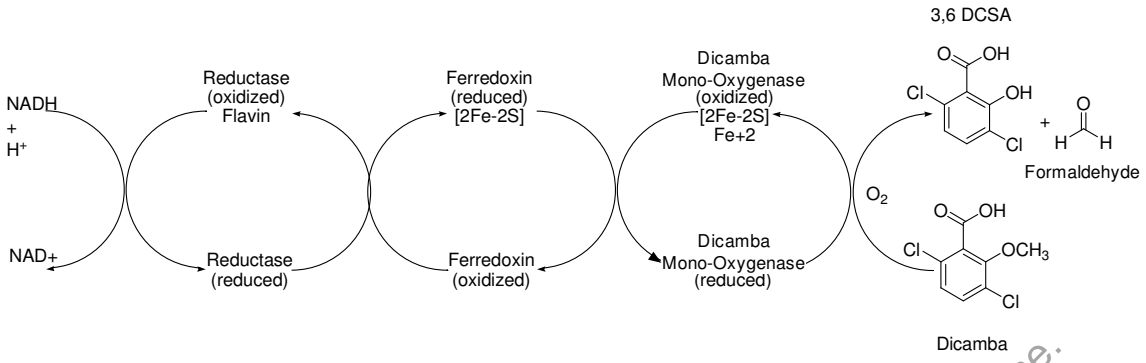


Figure VI-2. Three Components of the DMO Oxygenase System
Depicted is the electron transport chain that starts with NADH and ends with DMO resulting in the demethylation of dicamba to form DCSA.

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 poly-histidine 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-2). 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).

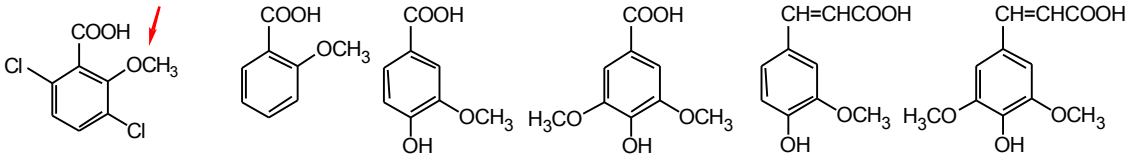
Therefore, in order for MON 87708 to be tolerant to dicamba, a functional trimeric MON 87708 DMO must be formed. The active trimeric form of MON 87708 DMO, as purified from MON 87708, confers dicamba tolerance to MON 87708, and its

demethylase activity on dicamba was confirmed during characterization (Section VI.D. and Appendix B) supporting the conclusion that the trimer required for functional activity was likely formed in MON 87708.

VI.B.1. Specificity of MON 87708 DMO

The substrate specificity of MON 87708 DMO was evaluated to understand potential interactions DMO may have with potential substrates present in MON 87708 soybean. As stated previously, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. 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). These interactions were clearly observed in the crystals of DMO when dicamba was present in the catalytic site indicating that these chemical groups are very important in correctly orienting the substrate 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 87708 DMO will catalyze the conversion of other endogenous substrates.

The potential for MON 87708 DMO to metabolize endogenous plant substrates was evaluated in *in vitro* experiments using a purified N-terminal histidine tagged DMO that is identical to wild-type DMO, except for a histidine tag at the N-terminus added to aid in protein purification (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 soybean (Janas, et al., 2000). 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-3). 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 was metabolized by the DMO in these *in vitro* experiments (Appendix B, Figure B-4). To assess whether MON 87708 DMO has the same specificity as the DMO used in the *in vitro* experiments, MON 87708 DMO isolated from seed of MON 87708 was incubated with *o*-anisic acid, the endogenous compound that has the greatest structural similarity to dicamba. *o*-anisic acid was not metabolized by MON 87708 DMO (Appendix B, Figure B-5), indicating the importance of the chlorine atoms in positioning the substrate in the catalytic site as described by D'Ordine et al. (2009). These results demonstrate that DMO, including MON 87708 DMO, is specific for dicamba as a substrate. These results also support that the minor changes in amino acid sequence among the different DMO proteins tested did not affect the specificity of DMO.



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

Figure VI-3. Dicamba and Potential Endogenous Substrates Tested in *In Vitro* Experiments with DMO
The arrow indicates methyl group removed by DMO.

VI.C. History of Safe Use of MON 87708 DMO

MON 87708 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-Anolles, et al., 2009; Illergard, et al., 2009). This conservation of structure is predominant within important functional and structural domains of proteins in similar classes (Illergard 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 87708 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 87708 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).

As discussed previously, the crystal structure of DMO (Appendix B, Figure B-2) has been solved (D'Ordine et al., 2009; Dumitru et al., 2009). 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 87708 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 87708 DMO+27 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 87708 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 26% (Table VI-1). The highest homology was observed to proteins that are involved in chlorophyll metabolism. Chlorophyllide A oxygenase is 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 is also a Rieske-type oxygenase that plays a key role in the overall regulation of chlorophyll degradation in plants (Rodoni, et al., 1997). The protein 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 it should 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 87708 DMO has occurred through consumption of these crops.

Therefore, MON 87708 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 the MON 87708 DMO protein, the MON 87708 DMO+27 protein and other proteins present in plants and bacteria

Protein	Accession Number	Source	Sequence Identity (%) MON 87708 DMO Protein	Sequence Identity (%) MON 87708 DMO+27 Protein
Chlorophyllide A oxygenase	ACG42449	Zea mays	26.6	26.2
Rieske iron-sulfur protein Tic55	CAA04157	Pisum sativum	26.2	25.6
Pheophorbide A oxygenase	ABD60316	Brassica napus	25.3	24.8
Lethal leaf spot-1 like protein*	ABA40832	Glycine max	25.3	24.3
Pheophorbide A oxygenase	CAR82238	Pisum sativum	24.3	24.3
Pheophorbide A oxygenase	ACG28057	Zea mays	24.1	23.2
Rieske domain containing protein	ABF99438	Oryza sativa	23.8	22.9
Flavonoid-3-hydroxylase	AAV74195	Sorghum bicolor	20.9	20.2
Choline mono-oxygenase	CAE17617	Oryza sativa	19.1	18.8
Choline mono-oxygenase	AAB52509	Spinacia oleracea	18.2	17.6
Sparse inflorescencel	ACI43576	Zea mays	18.0	17.5
Beta-carotene hydroxylase	AAX45523	Zea mays	15.6	15.5
Rieske domain containing protein	ACG43734	Zea mays	13.1	13.1

*Later identified as Pheophorbide A Oxygenase (Yang et al., 2004)

Protein sequences were extracted from publicly available databases. Each sequence was aligned to the MON 87708 DMO protein and the MON 87708 DMO+27 protein 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.D. Characterization of MON 87708 DMO

The safety assessment of crops derived through biotechnology includes characterization of the functional and physicochemical properties, and confirmation of the safety of the introduced protein. As stated previously, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. MON 87708 DMO was purified in sufficient quantities directly from the seed of MON 87708 and used in the safety assessment. Typically protein safety assessments are conducted on proteins produced in heterologous expression systems, such as *E. coli* (Harrison, et al., 1996). Since the MON 87708 DMO used in the safety assessment was purified directly from MON 87708, equivalence evaluations between plant-produced and bacterial-produced MON 87708 DMO were not necessary. The physicochemical characteristics and functional activity of MON 87708 DMO were determined by a panel of analytical techniques. When the proteins were separated by denaturation (*e.g.*, SDS-PAGE) for analysis each protein will

be referred to individually as either the MON 87708 DMO protein or the MON87708 DMO+27 protein. These analytical techniques included: 1) immunoblot analysis to establish identity and immunoreactivity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein using an anti-DMO antibody, 2) N-terminal sequence analysis of the MON 87708 DMO protein and the MON 87708 DMO+27 protein, 3) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to generate a tryptic peptide map of the MON 87708 DMO protein and the MON 87708 DMO+27 protein, 4) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to establish the apparent molecular weight of the MON 87708 DMO protein and the MON 87708 DMO+27 protein, 5) glycosylation status of the MON 87708 DMO protein and the MON 87708 DMO+27 protein, and 6) MON 87708 DMO activity analysis to demonstrate functional activity. The details of the materials and methods are described in Appendix B, while the results and conclusions of the MON 87708 DMO characterizations are summarized below.

The identities of both the MON 87708 DMO protein and MON 87708 DMO+27 protein produced in MON 87708 were confirmed by western blot, N-terminal sequencing, and MALDI-TOF MS. The antibody specifically detected both the MON 87708 DMO protein and MON 87708 DMO+27 protein on an immunoblot. The N-terminal sequence of the first 15 amino acid residues of both the MON 87708 DMO protein and MON 87708 DMO+27 protein was identical to the predicted amino acid sequence, with the exception of the N-terminal methionine residue. MALDI-TOF MS analyses of the trypsin digested MON 87708 DMO protein and MON 87708 DMO+27 protein yielded peptide masses consistent with their expected sequence. The apparent molecular weights of the MON 87708 DMO protein and MON 87708 DMO+27 protein were 39.8 and 42.0 kDa, respectively and neither were glycosylated. The activity of MON 87708 DMO, in it's active trimeric form, was determined by measuring the production of DCSA using dicamba as the substrate, resulting in a specific activity of 62.21 nmoles/min/mg of MON 87708 DMO. Taken together, these data provide a detailed characterization of MON 87708 DMO isolated from the seed of MON 87708.

VI.D.1. MON 87708 DMO Identity and Characterization

VI.D.1.1 Immunoblot Analysis

MON 87708 DMO purified from seeds of MON 87708 was analyzed by immunoblot. MON 87708 DMO was first separated under denaturing conditions by SDS-PAGE and then transferred to a PVDF membrane for immuno detection. On the immunoblot, the goat anti-DMO antibody recognized two bands migrating at the expected apparent molecular weights of approximately 39.8 kDa (MON 87708 DMO protein) and 42.0 kDa (MON 87708 DMO+27 protein), respectively (Figure VI-4). As expected, the intensity of the immunoreactive bands increased with increasing amount of total protein loaded. No additional immunoreactive bands were observed in MON 87708 DMO. This immunoblot analysis confirmed the identities of the MON 87708 DMO protein and MON 87708 DMO+27 protein.

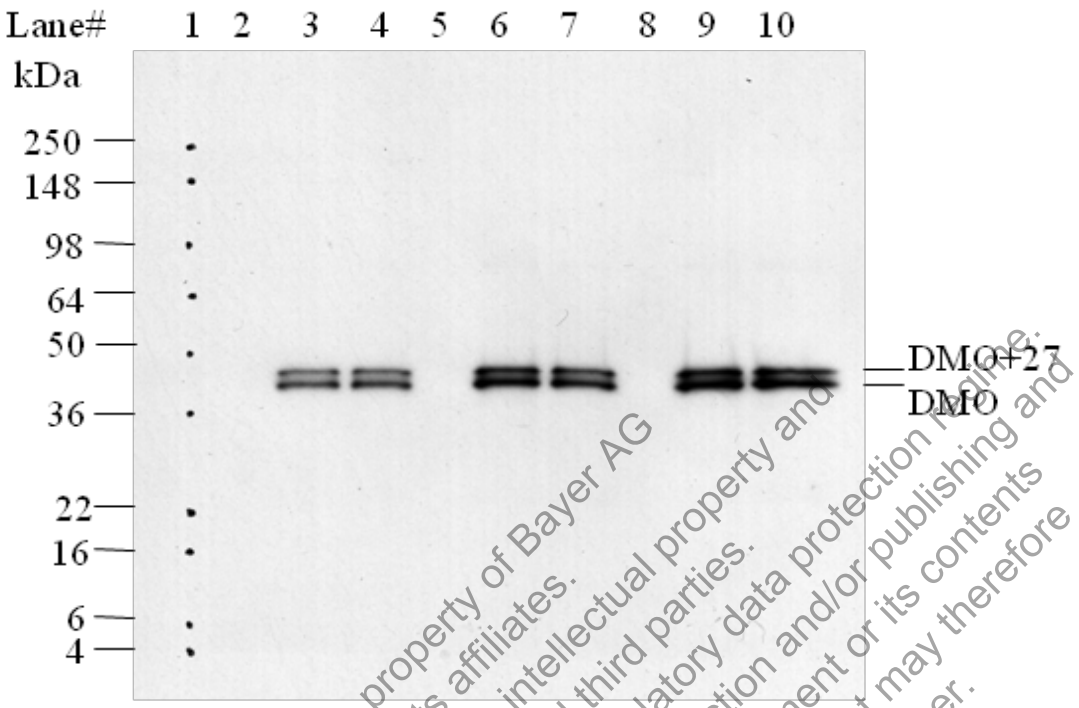


Figure VI-4. Immunoblot Analysis of the MON 87708 DMO
An aliquot of MON 87708 DMO and molecular weight markers were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with goat anti-DMO antibody and immunoreactive bands were visualized using an ECL system. Approximate MWs (kDa) are shown on the left and correspond to the markers loaded in Lane 1. Amount loaded corresponds to total protein. The 20 second exposure is shown.

Lane	Sample	Amount (ng)
1	See Blue Plus2 Pre-Stained MW markers	—
2	empty	
3	MON 87708 DMO	20
4	MON 87708 DMO	20
5	empty	
6	MON 87708 DMO	30
7	MON 87708 DMO	30
8	empty	
9	MON 87708 DMO	40
10	MON 87708 DMO	40

VI.D.1.2. N-Terminal Sequence Analysis

N-terminal sequence analysis of MON 87708 DMO was done by first separating MON 87708 DMO on SDS-PAGE and transferring the protein bands to a PVDF membrane. The two protein bands, similar to those observed in Figure VI-4, returned a sequence of 15 amino acids per band that matched the expected N-terminal sequences of the MON 87708 DMO protein and the MON 87708 DMO+27 protein (Figures VI-5 and VI-6, respectively), which were deduced from the *dmo* and *Rbcs* coding regions present in the seed of MON 87708 (Section IV).

The N-terminal methionine residue in the MON 87708 DMO 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). The 15 amino acids correspond to the expected sequence, after the methionine, of the MON 87708 DMO protein.

In the case of the MON 87708 DMO+27 protein, the first cycle of N-terminal sequence analysis resulted in an amino acid that corresponds to a methylated modification of the N-terminal methionine. It is well-known that the N-terminal methionine of the Rubisco small subunit is post-translationally modified to N-methyl methionine *in vivo* in pea and other plant species (Grimm, et al., 1997; Whitney and Andrews, 2001). The amino acids identified in the next 14 cycles corresponded to the expected sequence of the N-terminus of the small subunit of Rubisco confirming the alternative processing of the MON 87708 DMO+27 protein.

The N-terminal sequencing results confirm the identity of the MON 87708 DMO protein and MON 87708 DMO+27 protein.

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	A	T	F	V	R	N	A	W	Y	V	A	A	L	P	E
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence	-	A	T	F	V	R	N	A	W	Y	V	A	A	L	P	E

Figure VI-5. N-Terminal Sequence of the MON 87708 DMO Protein
The expected amino acid sequence of the N-terminus of the MON 87708 DMO protein was deduced from the *dmo* coding region present in MON 87708. The experimental sequence obtained from the MON 87708 DMO protein was compared to the expected sequence. (-) indicates the residue was not observed.

Amino acid residue # from the N-terminus →	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Expected Sequence	M	Q	V	W	P	P	I	G	K	K	K	F	E	T	L
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence	M*	Q	V	W	P	P	I	G	K	K	K	F	E	T	L

Figure VI-6. N-Terminal Sequence of the MON 87708 DMO+27 Protein
The expected amino acid sequence of the N-terminus of the MON 87708 DMO+27 protein was deduced from the *dmo* and *RbcS* coding region present in MON 87708. The experimental sequence obtained from the MON 87708 DMO+27 protein was compared to the expected sequence. M*, indicates methylated methionine.

VI.D.1.3. Tryptic Peptide Mapping

The identity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein was confirmed by tryptic mapping using MALDI-TOF MS analysis of the fragments produced by trypsin digestion. MON 87708 DMO was first denatured and separated on SDS-PAGE. The protein bands corresponding to the MON 87708 DMO protein and the MON 87708 DMO+27 protein (Figure VI-9 were excised from the gel, reduced, alkylated and digested with trypsin. 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 the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen, et al., 1997).

There were 26 unique peptides identified for the MON 87708 DMO protein that matched the expected masses of the MON 87708 DMO protein trypsin-digested peptides while 29 unique peptides from the MON 87708 DMO+27 protein were found to match the expected masses of the MON 87708 DMO+27 protein trypsin-digested peptides (Tables VI-2 and VI-3, respectively). The identified peptides were used to assemble a coverage map indicating the matched peptide sequences for the entire MON 87708 DMO protein and MON 87708 DMO+27 protein sequences, resulting in 77.4% (263/340) and 82.0% (301/367) coverage of the amino acid sequence, respectively (Figures VI-7 and VI-8, respectively). The N-terminal peptides were also identified by MALDI-TOF analysis and confirmed the N-terminal sequencing data that demonstrated the N-terminal methionine was missing in the MON 87708 DMO protein and methylated in the MON 87708 DMO+27 protein (Tables VI-2 and VI-3; Figures VI-7 and VI-8). These results confirm the identity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein.

Table VI-2. Summary of the Tryptic Masses Identified for the MON 87708 DMO Protein Using MALDI-TOF MS

Matrix						Expected Mass[1]	Difference[2]	AA position[3]	Fragment
α-Cyano		DHB		Sinapinic acid					
Ext.1	Ext.2	Ext.1	Ext.2	Ext.1	Ext.2				
			331.20			331.22	0.02	304-305	RR
		391.34				391.18	0.16	293-295	EDK
		435.38	435.38			435.27	0.11	206-208	FLR
593.61	593.61	593.51	593.53			593.34	0.27	2-6	ATFVR
720.67	720.68	720.60	720.60			720.37	0.30	131-136	VDPAYR
833.78	833.80	833.74	833.77			833.45	0.33	99-105	SFPVVER
856.77	856.78	856.72	856.75			856.43	0.34	242-248	EQSIHSR
914.89	914.91	914.84	914.88			914.53	0.36	296-303	VVVEAIER
1030.96	1030.97	1030.92	1030.92			1030.57	0.39	284-292	SWQAQALVK
1108.93	1108.95	1108.89	1108.94			1108.50	0.43	167-176	ANAQTDAEDR
		1171.08				1170.63	0.45	194-205	IPGGTPSVEMAK
1276.17	1276.20	1276.19	1276.21	1276.19		1275.73	0.44	26-36	TILDTPLALYR
1287.14		1287.19				1286.70	0.44	293-303	EDKVVVEAIER
1429.18	1429.20	1429.23	1429.26	1429.20		1428.69	0.49	209-221	GANTPVDAWNDIR
		1502.35	1502.37	1502.34		1501.79	0.56	180-193	EVIVGDGEIQALMK
1507.27	1507.27	1507.32				1506.73	0.54	167-179	ANAQTDAFDRLER
1578.24	1578.27	1578.32	1578.33	1578.30		1577.73	0.51	270-283	NFGIDDPEMDGVLR
1745.42	1745.51	1745.59		1745.56		1744.93	0.49	225-241	VSAMLNFIAVAPEGTPK
1762.48	1762.48	1762.62		1762.54		1761.90	0.58	37-52	QPDGVVAALLDICPHR
1994.65	1994.67	1994.76		1994.78		1994.03	0.62	150-166	LLVDNLMDLGHAQYVHR
2143.78	2143.84	2143.94		2143.96	2143.95	2143.12	0.66	7-25	NAWYVAALPEELSEKPLGR
2294.97				2294.93		2294.09	0.88	306-326	AYVEANGIRPAMLSCDEAAVR
2398.86	2398.77			2399.15		2398.08	0.78	249-269	GTHILTPETEASCHYFFGSSR
2581.87				2582.22		2582.34	0.47	225-248	VSAMLNFIAVAPEGTPKEQSIHSR
2700.08				2700.31		2699.25	0.83	106-130	DALIWICPGDPALADPGAIPDFGCR
				4218.47[4]		4217.77[4]	0.70	99-136	SFPVVERDALIWICPGDPALADPGAIPDFGCRVDPAYR

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The number represents the difference between the expected mass and the first column, which has the corresponding numbers.
[3]AA position refers to amino acid position within the predicted MON 87708 DMO protein sequence as depicted in Figure VI-7.
[4]Mass average.

Table VI-3. Summary of the Tryptic Masses Identified for the MON 87708 DMO+27 Protein Using MALDI-TOF MS

Matrix						Expected Mass[1]	Difference[2]	AA position[3]	Fragment
α-Cyano		DHB		Sinapinic acid					
Ext.1	Ext.2	Ext.1	Ext.2	Ext.1	Ext.2				
			331.19			331.22	0.03	331-332	RR
		435.30				435.27	0.03	233-235	FLR
720.55	720.64	720.47	720.55			720.37	0.18	158-163	VDPAYR
795.61	795.71	795.54	795.66			795.42	0.19	27-33	AMATFVR
833.65	833.73	833.59	833.69			833.45	0.20	126-132	SFPVVER
856.64	856.72	856.58	856.64			856.43	0.21	269-275	EQSIHSR
914.74		914.69				914.53	0.21	323-330	VVVEAIER
1030.80	1030.89	1030.75				1030.57	0.23	311-319	SWQAQALVK
1069.78	1069.92					1069.57	0.21	1-9	M*QVWPPIGK
1108.75	1108.90	1108.71				1108.50	0.25	194-203	ANAQTDAFDR
			1170.98			1170.66	0.32	221-232	IPGGTPSVLMAK
1275.97	1276.12	1275.98	1276.14	1275.97		1275.73	0.24	53-63	TILDTPLALYR
1286.95		1286.97				1286.70	0.25	320-330	EDKVVVEAIER
1428.95	1429.10	1429.00	1429.26	1428.93		1428.69	0.26	236-248	GANTPVDAWNDIR
		1470.93		1469.94		1470.63	0.30	164-176	TVGGYGHVDCNYK
				1502.10		1501.79	0.31	207-220	EVIVGDGEIQALMK
1507.01	1507.18	1507.03				1506.73	0.28	194-206	ANAQTDAFDRLER
1565.13	1565.30	1565.22	1565.34			1564.87	0.26	11-23	KFETLSYLPPLTR
1578.02	1578.14	1578.06	1578.29	1578.04		1577.73	0.29	297-310	NFGIDDPEMDGVLR
1693.26	1693.38	1693.29				1692.97	0.29	10-23	KKFETLSYLPPLTR
1745.17	1745.36	1745.28	1745.51	1745.22		1744.93	0.24	252-268	VSAMLNFIAVAPEGTPK
1762.17	1762.37	1762.29				1761.90	0.27	64-79	QPDGVVAALLDICPHR
1994.34	1994.55	1994.48		1994.42		1994.03	0.31	177-193	LLVDNLMDLGHAQYVHR
2143.46	2143.63	2143.57	2143.98	2143.57		2143.12	0.34	34-52	NAWYVAALPEELSEKPLGR
				2294.62		2294.09	0.53	333-353	AYVEANGIRPAMLSCDEAAVR
	2398.72	2398.49		2398.52		2398.08	0.64	276-296	GTHILTPETEASCHYFFGSSR
				2581.78		2582.34	0.56	252-275	VSAMLNFIAVAPEGTPKEQSIHSR
		2699.73		2699.84		2699.25	0.48	133-157	DALIWICPGDPALADPGAIPDFGCR
				4215.70		4215.03	0.67	126-163	SFPVVERDALIWICPGDPALADPGAIPDFGCRVDPAYR

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The number represents the difference between the expected mass and the first column, which has the corresponding numbers.
[3]AA position refers to amino acid position within the predicted MON 87708 DMO+27 protein sequence as depicted in Figure VI-8.
*Methylated methionine.

001 MATFVRNAWY VAALPEELSE KPLGRTILDT PLALYRQPDG VVAALLDICP

051 HRFAPLSDGI LVNGHLQCPY HGLEFDGGGQ CVHNPHGNGA RPASLNVRSF

101 PVVERDALIW ICPGDPALAD PGAIPDFGCR VDPAYRTVGG YGHVDCNYKL

151 LVDNLMDLGH AQYVHRANAQ TDAFDRLERE VIVGDGEIQA LMKIPGGTPS

201 VLMAKFLRGA NTPVDAWNDI RWNKVSAMLN FIAVAPEGTP KEQSIHSRGT

251 HILTPETEAS CHYFFGSSRN FGIDDPEMDG VLRSWQAQAL VKEDKVVVEA

301 IERRRAYVEA NGIRPAMLSC DEAAVRVSRE IEKLEQLEAA

Figure VI-7. MALDI-TOF MS Coverage Map of the MON 87708 DMO Protein.
The amino acid sequence of the MON 87708 DMO protein was deduced from the *dmo* coding region present in MON 87708. Boxed regions correspond to tryptic peptides that were identified from MON 87708 DMO protein using MALDI-TOF MS. In total, 77.4% (263 of 340 total amino acids) of the expected protein sequence was identified.

001 MQVWPPIGKK KFETLSYLPP LTRDSRAMAT FVRNAWYVAA LPEELSEKPL

051 GRTILDTPLA LYRQPDGVVA ALLDICPHRF APLSDGILVN GHLQCPYHGL

101 EFDGGGQCVH NPHGNGARPA SLNVRSFPVV ERDALIWICP GDPALADPGA

151 IPDFGCRVDP AYRTVGGYGH VDCNYKLLVD NLMDLGHAQY VHRANAQTDA

201 FDRLEREVIV GDGEIQALMK IPGGTPSVLM AKFLRGANTP VDAWNDIRWN

251 KVSAMLNFIA VAPEGTPKEQ SIHSRGTHIL TPETEASCHY FFGSSRNFGI

301 DDPEMDGVLR SWQAQALVKE DKVVVEAIER RRAYVEANGI RPAMLSCDEA

351 AVRVSREIEK LEQLEAA

Figure VI-8. MALDI-TOF MS Coverage Map of the MON 87708 DMO+27 Protein
The amino acid sequence of the MON 87708 DMO+27 protein was deduced from the *dmo* coding region, *RbcS*, and intervening sequence present in MON 87708. Boxed regions correspond to tryptic peptides that were identified from MON 87708 DMO+27 protein using MALDI-TOF MS. In total, 82.0% (301 of 367 total amino acids) of the expected protein sequence was identified.

VI.D.1.4. Molecular Weight and Purity

The apparent molecular weight of MON 87708 DMO protein and the MON 87708 DMO+27 protein was determined by analyzing the proteins using denaturing SDS-PAGE and then gel stained using Brilliant Blue G Colloidal stain (Sigma). Purity and apparent molecular weight of the MON 87708 DMO protein and

MON 87708 DMO+27 protein was determined using densitometric analysis of the gel (Figure VI-9). As summarized in Table VI-4, apparent molecular weight values were averaged from duplicated loads of 0.5, 1.0, and 1.5 µg of total protein (Figure VI-9, lanes 2-7). The predominant bands identified as the MON 87708 DMO protein and MON 87708 DMO+27 protein were estimated to have apparent molecular weights of 39.8 kDa and 42.0 kDa, respectively. Purity of MON 87708 DMO was calculated as the average purity of the MON 87708 DMO protein plus the average purity of the MON 87708 DMO+27 protein. This was done to reflect the purity of the purified MON 87708 DMO, *i.e.*, both processed forms of the proteins and all forms of the trimer. The purity of MON 87708 DMO was 81%.

Table VI-4. Molecular Weight and Purity of MON 87708 DMO

Total Protein Loaded	Apparent Molecular Weight (kDa)		Purity (%)		
	MON 87708 DMO Protein	MON 87708 DMO+27 Protein	MON 87708 DMO Protein	MON 87708 DMO+27 Protein	MON 87708 DMO[1]
0.5 µg in lane 2	39.2	41.6	34	44	
0.5 µg in lane 3	39.2	41.5	33	43	
1.0 µg in lane 4	39.5	41.7	36	46	
1.0 µg in lane 5	39.8	42.0	34	46	
1.5 µg in lane 6	40.3	42.4	37	47	
1.5 µg in lane 7	40.7	42.8	35	47	
Average	39.8	42.0	35	46	81

[1]Calculated as the sum of the average purity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein.

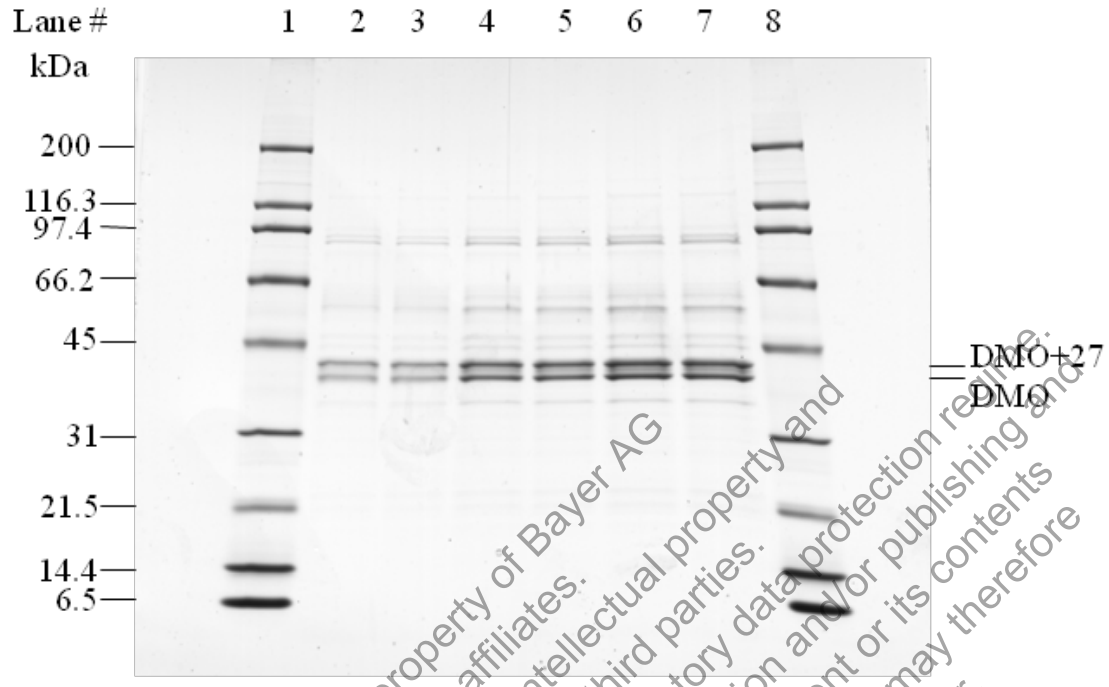


Figure VI-9. Molecular Weight and Purity Analysis of MON 87708 DMO
An aliquot of MON 87708 DMO was separated on a 4 - 20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G Colloidal stain. Approximate apparent molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Amount loaded corresponds to total protein. Empty lanes were cropped.

Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	MON 87708 DMO	0.5
3	MON 87708 DMO	0.5
4	MON 87708 DMO	1.0
5	MON 87708 DMO	1.0
6	MON 87708 DMO	1.5
7	MON 87708 DMO	1.5
8	Broad Range MW markers	4.5

VI.D.1.5. Functional Activity

MON 87708 DMO functional activity was determined by measuring the production of DCSA. The reaction mixture contained all the necessary compounds required for catalysis including the additional proteins (reductase and ferredoxin) involved in the electron transport from NADH (Appendix B, Section B.3.9). The specific activity was determined to be 62.21 nmol/min/mg of MON 87708 DMO (Table VI-5). The value represents an average of three independent assays. This result demonstrates that MON 87708 DMO isolated from the seed of MON 87708 is functionally active.

Table VI-5. MON 87708 DMO Functional Assay

Assay#	Specific activity (nmol/min/mg)	Average (nmol/min/mg) ±Standard Deviation
1	61.92	62.21 ± 11.03
2	51.33	
3	73.39	

VI.D.1.6. Glycosylation Analysis

To test whether MON 87708 DMO is glycosylated, MON 87708 DMO was first separated on SDS-PAGE and then transferred to a PVDF membrane. The two protein bands, similar to those observed in Figure VI-4, corresponding to the MON 87708 DMO protein and MON 87708 DMO+27 protein were analyzed for glycosylation using a GE Glycoprotein Detection Module (GE Healthcare). Transferrin, a naturally glycosylated protein, was used as a positive control in the assay. The results of this analysis are presented in Figure VI-10. The positive control was clearly detected at the expected molecular weight and the bands increased with increasing protein concentration (Figure VI-10, lanes 2-4). No bands were observed for the MON 87708 DMO protein or MON 87708 DMO+27 protein at their expected molecular weight positions (39.8 and 42.0 kDa) (Figure VI-10, lanes 5 and 6) indicating that neither are glycosylated.

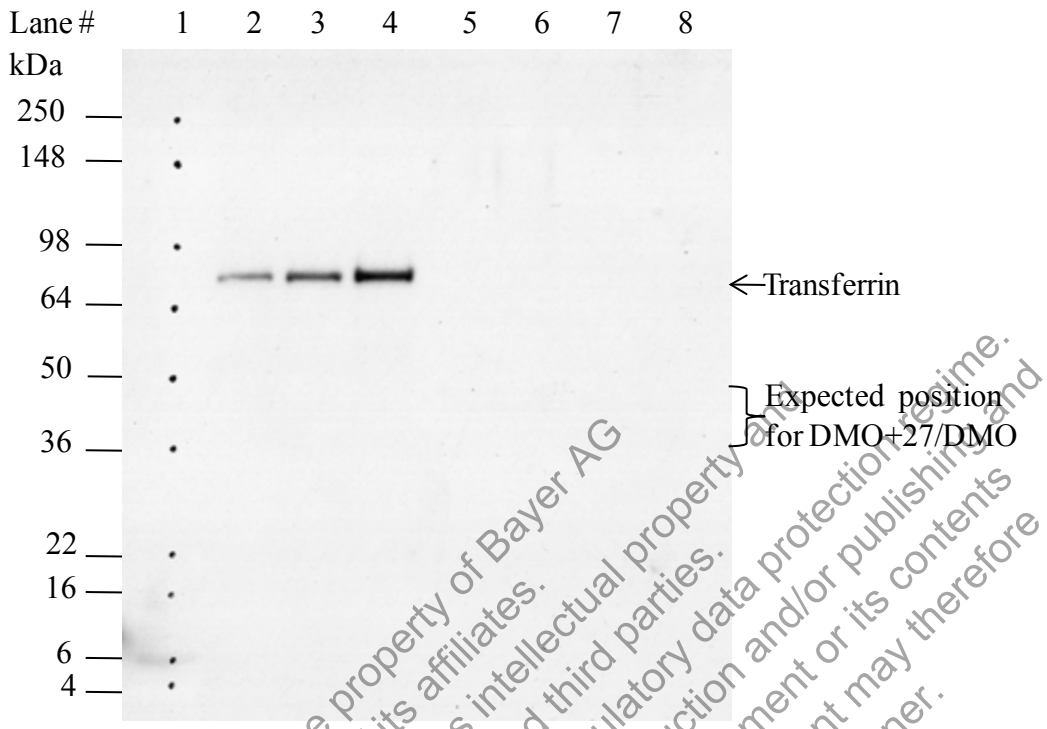


Figure VI-10. Glycosylation Analysis of the MON 87708 DMO
Molecular weight markers, transferrin (positive control) and an aliquot of MON 87708 DMO was separated by SDS-PAGE and electrotransferred to a PVDF membrane. The image was captured using a Bio-Rad GS800 with Quantity One software (version 4.4.0). Amount loaded corresponds to total protein. The 30 second exposure is shown.

Lane	Sample	Amount (ng)
1	See Blue Plus2 Pre-Stained MW markers	—
2	Transferrin	50
3	Transferrin	100
4	Transferrin	200
5	MON 87708 DMO	100
6	MON 87708 DMO	200
7	Empty lane	
8	Empty lane	

VI.E. Expression Levels of MON 87708 DMO

The levels of MON 87708 DMO in various tissues of MON 87708 that are relevant to the risk assessment were determined using a validated ELISA. Tissues of MON 87708 and the near isogenic conventional soybean control A3525 were collected during the 2008 growing season from five field sites in the U.S.: Jefferson County, Iowa; Stark County, Illinois; Clinton County, Illinois; Parke County, Indiana; and Berks County, Pennsylvania. These field sites were representative of soybean producing regions suitable for commercial production. At each site, three replicated plots containing MON 87708, as well as the conventional control, were planted using a randomized complete block field design. Over-season leaf (OSL 1 - 4), root, forage, and seed tissues were collected from each replicated plot at all field sites (except for the conventional control for forage tissue from Berks County, Pennsylvania where only two replicates were collected). A description of tissues collected is provided in Table VI-6.

Table VI-6. Tissues Collected and Analyzed for MON 87708 DMO

Tissue	Soybean Development Stage[1]	Days After Planting
OSL-1	V3-V4	21-30
OSL-2	V5-V8	31-42
OSL-3	R2-V12	43-58
OSL-4	R5-V16	55-78
Root	R6	70-91
Forage	R6	70-91
Seed	R8	109-147

[1]Soybean plant growth stages described in Soybean Growth and Development (Pedersen, 2004).

The levels of MON 87708 DMO were determined in all seven tissue types as described in Table VI-6. As previously described, this document will refer to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. The ELISA assay detected MON 87708 DMO (*i.e.*, both processed forms of the protein and all forms of the trimer), therefore the levels represent the total MON 87708 DMO. The results obtained from the ELISA analysis are summarized in Table VI-7 and the details of the materials and methods are described in Appendix C. In summary, expression analysis of the samples from the 2008 U.S. field trial showed that MON 87708 DMO was detected in all tissue types across all five sites ranging from 3.9-180 µg/g dry weight (dwt). The mean levels of MON 87708 DMO across the five sites were highest in leaf (ranging from OSL-1 at 17 µg/g dwt, to OSL-4 at 69 µg/g dwt), followed by forage (53 µg/g dwt), seed (47 µg/g dwt), and root (6.1 µg/g dwt). As expected for the conventional control, the ELISA values for MON 87708 DMO were less than the limit of quantitation (LOQ) of the assay in all tissue types.

Table VI-7. Summary of the Levels of MON 87708 DMO in Leaf, Root, Forage, and Seed from MON 87708 Grown in 2008 U.S. Field Trials

Tissue Type	MON 87708 DMO[1] Mean (SD)[2] (µg/g fwt)[3]	Range[4] (µg/g fwt)	MON 87708 DMO Mean (SD) (µg/g dwt)[5]	Range (µg/g dwt)	LOQ/LOD (µg/g fwt)[6,7]
OSL-1	3.1 (1.9)	0.87 – 6.8	17 (7.7)	6.2 – 29	0.63/0.20
OSL-2	5.2 (2.6)	1.4 – 9.8	31 (13)	12 – 54	0.63/0.20
OSL-3	6.0 (2.2)	3.5 – 11	44 (14)	25 – 71	0.63/0.20
OSL-4	16 (12)	4.6 – 43	69 (46)	23 – 180	0.63/0.20
Root	1.9 (0.73)	1.2 – 3.6	6.1 (2.1)	3.9 – 11	0.031/0.015
Forage	12 (2.5)	7.0 – 17	53 (18)	25 – 84	0.63/0.10
Seed	43 (7.7)	31 – 55	47 (8.7)	34 – 59	1.3/0.21

[1]Represents total for MON 87708 DMO (*i.e.*, both processed forms of the protein and all forms of the trimer).
[2]The mean and standard deviation (SD) were calculated (n=15). The "n" values for the calculated mean and standard deviations represent the number of samples figured into the calculation.
[3]Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
[4]Minimum and maximum values were determined for each tissue type.
[5]Protein levels are expressed as µg/g dwt. The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factors obtained from moisture analysis data.
[6]The limit of quantitation (LOQ) was calculated based on the lowest *E. coli*-produced DMO standard concentration. The "ng/ml" value was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.
[7]The limit of detection (LOD) was calculated as the mean value of a conventional control plus three SD using the data generated with conventional control sample extracts for each tissue type. The LOD value in "ng/ml" was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.

VI.E.1. Generational Stability of MON 87708 DMO Expression in MON 87708

In order to confirm the presence of MON 87708 DMO across multiple generations, western blot analysis of MON 87708 DMO was conducted on leaf tissue collected from generations R_2, R_3, R_4, R_5, and R_6 (Figure VI-11) of MON 87708, and on leaf tissue of the near isogenic conventional soybean control A3525. Materials and methods are detailed in Appendix D. As previously described, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO.

MON 87708 DMO was first separated on a denaturing SDS-PAGE and then transferred to a PVDF membrane for immunoblot analysis. The presence of the MON 87708 DMO protein and MON 87708 DMO+27 protein in harvested leaf tissue of the R_2, R_3, R_4, R_5, and R_6 generations of MON 87708 was demonstrated (Figure VI-11). An *E. coli*-produced DMO standard (10 ng and 5 ng) was used as a reference for the identification of MON 87708 DMO, and the conventional leaf tissue extract was used as the negative control. The presence of MON 87708 DMO in leaf tissue was determined by visual comparison of the bands produced in the multiple generations (Figure VI-11, lanes 5-9) to the *E. coli*-produced DMO reference standard (Figure VI-11, lanes 2-3). The MON 87708 DMO protein is clearly observed in all generations migrating to the same position on the immunoblot as the *E.coli*-produced DMO reference standard. The MON 87708 DMO+27 protein is not present in the *E.coli*-produced DMO reference standard for comparison, however, a band was observed for all MON 87708 samples at the expected molecular weight of ~42.0 kDa indicating the presence of the MON 87708 DMO+27 protein in all five generations of MON 87708 harvested leaf tissue samples. A more intense band was observed for the R_3 generation (Figure VI-11, lane 6) when compared to the other four generations (R_2, R_4, R_5, and R_6; Figure VI-11, lanes 5, 7-9, respectively). This difference was likely due to the R_3 generation being grown separately from and sampled at a later growth stage than the R_2, R_4, R_5, and R_6 generations (see Appendix D). As expected, MON 87708 DMO was not detected in the conventional control leaf tissue (Figure VI-11, lane 4).

On the western blot an additional band was observed at approximately 50 kDa in both MON 87708 and control leaf tissue samples for all generations (Figure VI-11, lanes 5-9 and 4, respectively). This is likely the result of non-specific binding of either the primary or secondary antibody to a protein endogenous to soybean leaves. This band appeared more intense in the R_3 generation of MON 87708 and the conventional control (Figure VI-11, lanes 6 and 4, respectively). This may be explained by the fact that the conventional control and R_3 generation material were from the same greenhouse production, while material from the R_2, R_4, R_5, and R_6 generations was collected from a different greenhouse production (Appendix D). Additional bands are also visible, and it is also likely that they are the result of non-specific binding of either the primary or secondary antibody to a protein endogenous to soybean leaves.

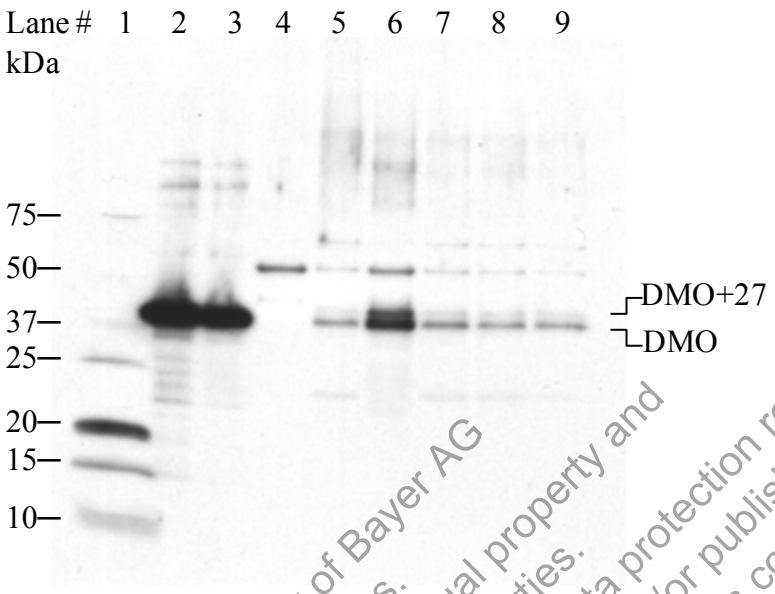


Figure VI-11. Presence of MON 87708 DMO in Multiple Generations of MON 87708

Aliquots of extracts from five generations of MON 87708 leaf tissues and molecular weight markers were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with goat anti-DMO antibody. The image represents a 30 second exposure.

Lane	Description	Amount Loaded on Gel
1	Molecular Weight Marker	-
2	*E. coli*-produced DMO Protein Standard	10ng
3	*E. coli*-produced DMO Protein Standard	5 ng
4	A3525 Conventional Control	20 µl
5	R_2 Generation	20 µl
6	R_3 Generation	20 µl
7	R_4 Generation	20 µl
8	R_5 Generation	20 µl
9	R_6 Generation	20 µl

VI.F. Assessment of the Potential Allergenicity, Toxicity, and Dietary Safety of the MON 87708 DMO Protein

The history of safe use of the introduced protein (Section VI.C) is one important consideration in the assessment for potential allergenicity, toxicity, and dietary safety.

Additionally, according to guidelines adopted by the Codex Alimentarius Commission for the assessment of potential allergenicity of introduced proteins, the allergenic potential of an introduced protein is assessed by comparing the biochemical characteristics of the introduced protein to characteristics of known allergens (Codex Alimentarius, 2003; Codex Alimentarius, 2009). The biochemical characteristics are assessed by determining if: 1) the protein is from an allergenic source; 2) the protein represents only a very small portion of the total plant protein; 3) the protein shares any amino acid sequence similarities to known allergens; 4) the protein is rapidly digested in mammalian gastrointestinal systems; and 5) the protein is stable to heat treatment. MON 87708 DMO has been assessed for its potential allergenicity according to these safety assessment guidelines, and was determined to pose no significant risk of allergenicity.

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. These biochemical characteristics are assessed by determining if: 1) the protein has amino acid sequence 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; 3) the protein is stable to heat treatment; 4) the protein exerts any acute toxic effects in mammals; and 5) the anticipated dietary exposure levels for humans and animals. MON 87708 DMO has been assessed for its potential toxicity based on these criteria and was determined to pose no significant toxicological risk. As previously described, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO.

VI.F.1. Assessment of Potential Allergenicity of MON 87708

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 mininmal 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. MON 87708 DMO as a Proportion of Total Protein

MON 87708 DMO was detected in all plant tissues assayed at a number of time points during the growing season (Table VI-6). Harvested seed is the most relevant tissue analyzed for an allergenicity assessment because it can be consumed directly. The mean level of MON 87708 DMO in harvested seed is 47 µg/g dwt (Table VI-7). The mean percent dry weight of total protein in harvested seed from MON 87708 is 40.9% (or 409,000 µg/g) (Table VII-1). The percent of MON 87708 DMO in harvested seed from MON 87708 is 0.011% and is calculated as follows:

(47 µg/g ÷ 409,000 µg/g) × 100% = 0.011% of total soybean protein

This low percent of MON 87708 DMO in relation to the total protein reduces the potential for the protein to be an allergen.

VI.F.1.3. Structural Similarity of MON 87708 DMO to Known Allergens

In 2003, the Codex Alimentarius Commission published guidelines for the evaluation of the potential allergenicity of introduced proteins (Codex Alimentarius, 2003). This guideline is based on the comparison of amino acid sequences between introduced proteins and allergens, where potential allergenic cross-reactivity may exist if the introduced protein is found to have at least 35% amino acid identity with an allergen over any segment of at least 80 amino acids. The Codex guideline also 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 amino acid sequence similarities between MON 87708 DMO and known allergens, gliadins, and glutenins was assessed using the FASTA sequence alignment tool and an eight-amino acid sliding window search (Codex Alimentarius, 2003; Thomas, et al., 2005). The methods used are summarized below and detailed in Appendix E. The results demonstrated that MON 87708 DMO, which refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, 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) and the alignment of the data may be used to infer shared higher order structural similarities between two sequences (*i.e.*, secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. Homologous proteins usually have common secondary structures, common three-dimensional configuration, and may share similar functions (Caetano-Anolles et al., 2009; Illergard et al., 2009). The allergen, gliadin, and glutenin sequence database (AD_2010) used for the evaluation was obtained from the Food Allergy Research and Resource Program Database (FARRP, 2010). The AD_2010 database contains 1,471 sequences. The sequence similarity evaluation was conducted using the MON 87708 DMO+27 protein, which contains the MON 87708 DMO protein sequence, and protein sequences contained in AD_2010. A FASTA algorithm, used to search AD_2010, produces an *E*-score (expectation score) which is a statistical measure of the likelihood that the observed similarity could have occurred by chance. 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 need to have an *E*-score of 1×10^{-5} or smaller to be considered to have sufficient sequence similarity to infer homology (Silvanovich, et al., 2009). The FASTA analysis yielded no *E*-scores less than or equal to 1×10^{-5}, demonstrating a lack of sequence similarity between the MON 87708 DMO+27 protein (and the MON 87708 DMO protein whose sequence is fully contained in the MON 87708 DMO+27 protein sequence) and sequences in the allergen database. Moreover, no alignment met or exceeded the threshold of 35% identity over 80 amino acids as recommended by Codex Alimentarius (Codex Alimentarius, 2003).

A second bioinformatic tool, an eight-amino acid sliding window search, was used to specifically identify short linear polypeptide matches to known or suspected allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may still contain smaller immunologically significant epitopes. An amino acid sequence may be considered to 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 significant 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 sequence alignments of eight contiguous amino acids were detected when the MON 87708 DMO+27 protein sequence was compared to the AD_2010 sequence database.

In conclusion, the bioinformatic results demonstrated there were no biologically relevant sequence similarities to allergens when the MON 87708 DMO+27 protein sequence was used as a query for a FASTA search of the AD_2010 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the MON 87708 DMO+27 protein sequence and proteins in the AD_2010 database. Since the MON 87708 DMO+27 protein sequence contains the MON 87708 DMO protein sequence, these data demonstrate the lack of both structurally and immunologically relevant similarities between the protein sequences of MON 87708 DMO and known allergens, gliadins, and glutenins.

VI.F.1.4. Digestive Fate of MON 87708 DMO

One characteristic of many protein allergens is their ability to withstand proteolytic digestion by enzymes present in the mammalian gastrointestinal tract (Astwood, et al., 1996; Moreno, et al., 2005; Vassilopoulou, et al., 2006; Vieths, et al., 1999). When resistant to digestion, allergens, or their fragments, are presented to the intestinal immune system, which 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 87708 DMO, which refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, was assessed using assays with both simulated gastric fluid (SGF) containing pepsin and simulated intestinal fluid (SIF) containing pancreatin.

A correlation between the digestibility in SGF and the likelihood of an introduced protein being an allergen has been previously reported with a group of proteins consisting of both allergens and non-allergens (Astwood et al., 1996), but this correlation is not absolute (Fu, et al., 2002). The SGF assay protocol has been standardized by ILSI 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 are reproducible when standard protocols were followed. Using this protocol, the pepsin digestion assay was used to assess the susceptibility of MON 87708 DMO to *in vitro* pepsin digestion (Appendix F) and a summary of the results is below. Materials and methods are detailed in Appendix F.

The digestibility of a protein in SIF is used as an independent test system to assess the *in vitro* digestibility of food components (Okunuki, et al., 2002; Yagami, et al., 2000). The relationship between protein allergenicity and protein stability in the standalone *in vitro* SIF assay is limited, because the protein has not been first exposed to the acidic, denaturing conditions of the stomach, as would be the case *in vivo* (Helm, 2001). Using an established protocol, the pancreatin digestion assay was used to assess the susceptibility of MON 87708 DMO to *in vitro* pancreatin digestion (Appendix F) and a summary of the result is below. Materials and methods are detailed in Appendix F.

VI.F.1.4.1. Digestibility of MON 87708 DMO in SGF and SIF

Digestibility of MON 87708 DMO in SGF was assessed using SDS-PAGE and immunoblot methods (Appendix F). The extent of MON 87708 DMO digestion was evaluated by visual analysis of stained polyacrylamide gels (Figures VI-12 and VI-13) or by visual analysis of western blots (Figures VI-14 and VI-15). In both cases MON 87708 DMO was first separated on a denaturing SDS-PAGE and analyzed, or

transferred to a PVDF membrane for immunoblot analysis. In each case the degradation of the MON 87708 DMO protein and the MON 87708 DMO+27 protein was evaluated.

Initially, the digestibility of MON 87708 DMO in SGF was evaluated by visual analysis of a colloidal Brilliant Blue G stained Tricine 10-20% polyacrylamide gradient gel where MON 87708 DMO was completely digested within 30 seconds (Figure VI-12). However, pepsin and the MON 87708 DMO protein migrate to similar positions in this gel system. To further confirm that the MON 87708 DMO protein is being digested and not being masked by pepsin, a visual analysis of a colloidal Brilliant Blue G stained Tris-glycine 8% polyacrylamide gel was also conducted (Figure VI-13, panel A), confirming the previous results that MON 87708 DMO was completely digested within 30 seconds. The migration of pepsin relative to MON 87708 DMO is different in each gel system. Changes in protein mobility in different gel systems are due to a variety of factors including changes in acrylamide percentage and pH of each gel system (Makowski and Ramsby, 1997).

Due to the improved resolution of pepsin and MON 87708 DMO, a separate Tris-glycine 8% polyacrylamide gel was used to determine the limit of detection (LOD) of the MON 87708 DMO (Figure VI-13, panel B). The LOD of MON 87708 DMO by Colloidal Brilliant Blue G staining was 0.02 µg or approximately 2% of the total protein loaded (0.02 µg divided by 1.0 µg of total protein loaded in each lane of the gel; Figure VI-13, panel B, lane 6).

Visual examination of the colloidal Brilliant Blue G stained Tris-glycine 8% gel (Figure VI-13, panel A) showed that MON 87708 DMO was digested to less than 2% of total protein loaded in SGF within 30 seconds (Figure VI-13, panel A, lane 5). No fragments corresponding to MON 87708 DMO were observed in the 30 second digestion sample. A diffuse, faint band with an approximate molecular weight of 21 kDa was observed for all time points from 30 seconds to 60 minutes in the colloidal Brilliant Blue stained Tricine 10-20% polyacrylamide gradient gel. The N-terminal sequence of this band was determined and it did not match any of the MON 87708 DMO sequences. It is likely that this fragment originated from soybean proteins that co-purified with MON 87708 DMO.

The digestibility of MON 87708 DMO in SGF was also evaluated by western blot (Appendix F). A two gel system was employed and proteins were separated by SDS-PAGE using a Tricine 10-20% polyacrylamide gradient gel (Figure VI-14) to determine if any fragments were detected, and a Tris-glycine 8% polyacrylamide gel (Figure VI-15) to confirm that the MON 87708 DMO protein was digested and not masked due to co-migration with pepsin. In both cases, the results demonstrated that MON 87708 DMO is digested within 30 seconds of exposure to SGF. The western blot of the Tris-glycine 8% polyacrylamide gel (Figure VI-15, panel A) was run concurrently with a western blot to determine the LOD for MON 87708 DMO (Figure VI-15, panel B). The LOD was determined to be 0.3 ng or approximately 1.5% of the total protein loaded (0.3 ng divided by 20 ng of the protein loaded in each lane of the gel; Figure VI-15, panel B, lane 7). Visual examination of the western blot confirmed that MON 87708 DMO was digested to less than 1.5% of the total protein loaded in SGF

within 30 seconds (Figure VI-15, Panel A, lane 5). No fragments corresponding to MON 87708 DMO were observed. A faint band is visible at approximately 25 kDa in the MON 87708 DMO only controls and the SGF T0 sample, which is likely the result of non-specific binding of either the primary or secondary antibody to a protein that co-purified with MON 87708 DMO.

SIF was also used to test digestibility of MON 87708 DMO. The assay was performed according to methods described in the United States Pharmacopeia (USP, 1995) (Appendix F). The digestion of MON 87708 DMO in SIF was separated by SDS-PAGE using a Tricine 10-20% polyacrylamide gradient gel and then evaluated by western blot (Figure VI-16). A western blot to determine the LOD (Figure VI-16, panel B) of MON 87708 DMO was performed concurrently with the SIF assay (Figure VI-16, panel A). The LOD was determined to be 1.0 ng or approximately 5.0% of the total protein loaded (1.0 ng divided by 20 ng of loaded protein loaded in each lane of the gel; Figure VI-16, panel B, lane 6). Visual examination of the western blot confirmed that MON 87708 DMO was digested to less than 5% of the total protein loaded in SIF within five minutes (Figure VI-16, panel A, lane 5). No proteolytic fragments of MON 87708 DMO were detected at any digestion time points.

In conclusion, these results show that MON 87708 DMO was readily digestible in SGF and SIF. The rapid digestion of the MON 87708 DMO in SGF and SIF indicates that it is highly unlikely that MON 87708 DMO will pose any safety concern to human and animal health.

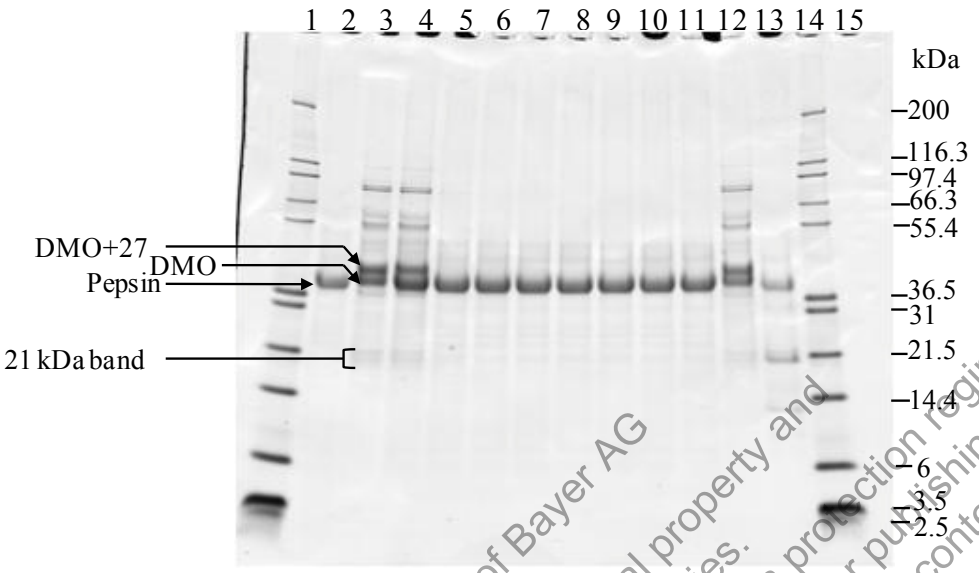


Figure VI-12. Colloidal Blue Stained 10-20% SDS-PAGE Gel Showing the Digestion of MON 87708 DMO in Simulated Gastric Fluid

Colloidal Brilliant Blue G stained Tricine 10-20% polyacrylamide gel was used to analyze the digestibility of MON 87708 DMO in SGF. Based on pre digestion total protein concentrations, 1.0 µg (total protein) was loaded in each lane containing MON 87708 DMO (SGF T0-SGF T7). Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. N0 and N7 correspond to control samples that did not contain MON 87708 DMO. P0 and P7 correspond to controls that did not contain pepsin.

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Mark 12 MWM	-
15	Blank	-

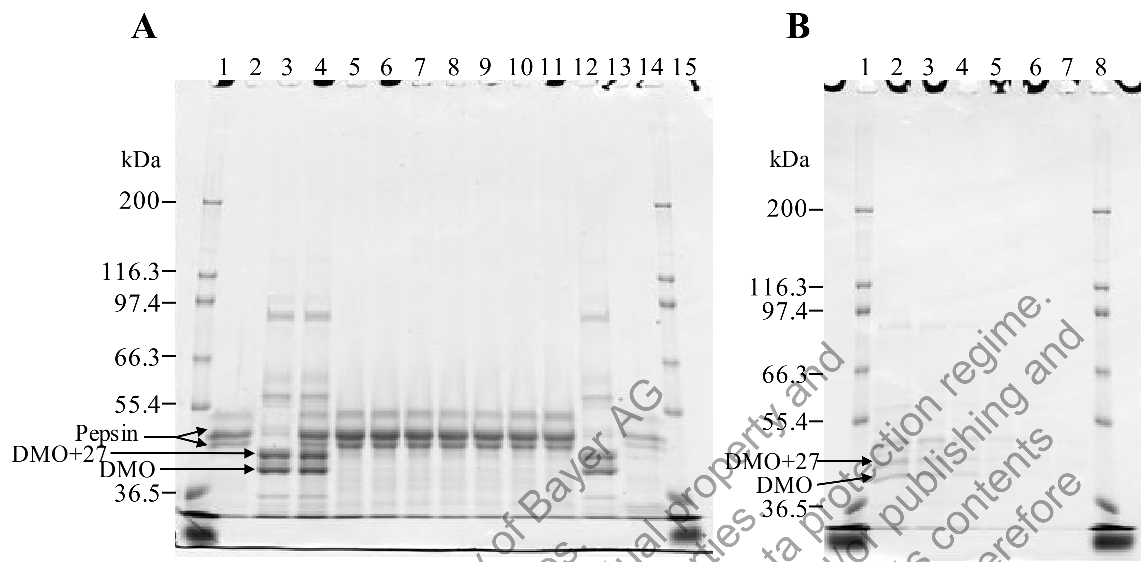


Figure VI-13. Colloidal Blue Stained 8% SDS-PAGE Gel Showing the Digestion of MON 87708 DMO in Simulated Gastric Fluid

Colloidal Brilliant Blue G stained 8% Tris-glycine polyacrylamide gels were used to analyze the digestibility of MON 87708 DMO in SGF. Panel A corresponds to the MON 87708 DMO digestion in SGF. Based on predigestion protein concentrations, 1.0 µg (total protein) was loaded in each lane containing MON 87708 DMO (SGF T0-SGF T7). Panel B corresponds to the analysis to determine the limit of detection of MON 87708 DMO. The amount loaded corresponds to the total protein amount of MON 87708 DMO. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. N0 and N7 correspond to control samples that did not contain MON 87708 DMO. P0 and P7 correspond to controls that did not contain pepsin.

Panel A

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	-
2	SGF N0	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Mark 12 MWM	-
15	Blank	-

Panel B

Lane	Sample	Amount (µg)
1	Mark 12 MWM	-
2	T0, protein+SGF	0.25
3	T0, protein+SGF	0.13
4	T0, protein+SGF	0.06
5	T0, protein+SGF	0.03
6	T0, protein+SGF	0.02
7	T0, protein+SGF	0.01
8	Mark 12 MWM	-

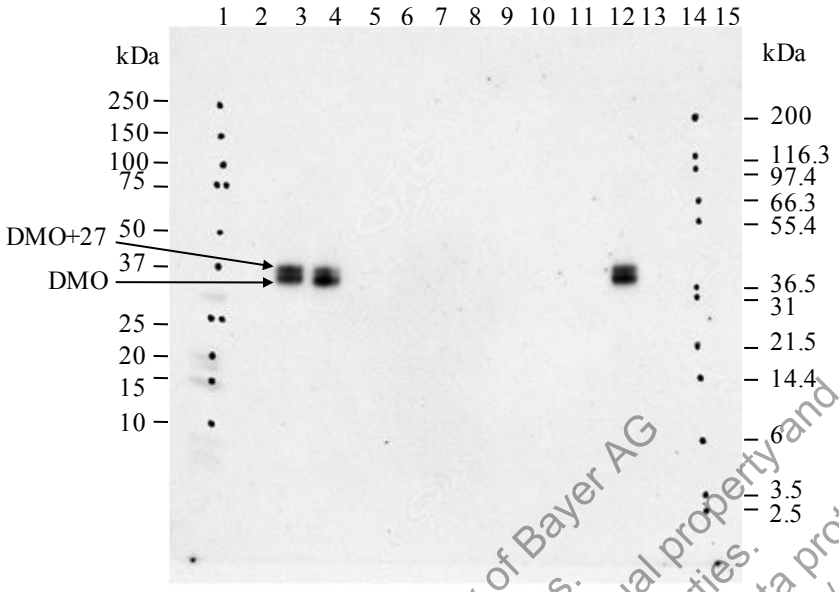


Figure VI-14. Western Blot Analysis Using 10-20% SDS-PAGE of MON 87708 DMO in Simulated Gastric Fluid

The figure corresponds to MON 87708 DMO digestion in SGF separated by SDS-PAGE using a Tricine 10-20% polyacrylamide gradient gel. Based on pre-digestion total protein concentrations, 20 ng (total protein) was loaded in the lanes containing MON 87708 DMO (SGF T0-SGF T7). Approximate molecular weights (kDa) are shown on the left and right of the blot. A 30 second exposure is shown. N0 and N7 correspond to control samples that did not contain MON 87708 DMO. P0 and P7 correspond to controls that did not contain pepsin.

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	-
2	SGF N0	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Precision Plus MWM	-
15	Blank	-

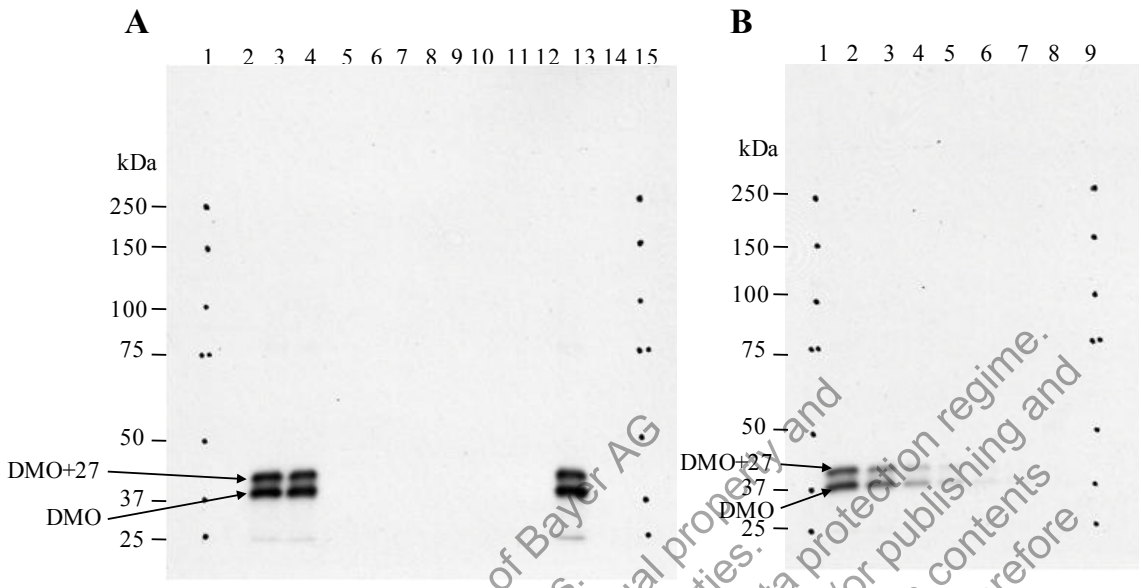


Figure VI-15. Western Blot Analysis Using 8% SDS-PAGE of MON 87708 DMO in Simulated Gastric Fluid

Panel A corresponds to MON 87708 DMO digestion in SGF separated by SDS-PAGE using Tris-glycine 8% polyacrylamide gels. Based on pre-digestion total protein concentrations, 20 ng (total protein) was loaded in the lanes containing MON 87708 DMO (SGF T0-SGF T7). Panel B corresponds to the analysis to determine the limit of detection of MON 87708 DMO. The amount loaded corresponds to the total protein amount of MON 87708 DMO. The lanes have been cropped and re-numbered. Approximate molecular weights (kDa) are shown on the left, and correspond to the markers loaded in each gel. A 15 second exposure is shown. N0 and N7 correspond to control samples that did not contain MON 87708 DMO. P0 and P7 correspond to controls that did not contain pepsin.

Panel A			Panel B		
Lane	**Sample**	**Incubation Time (min)**	**Lane**	**Sample**	**Amount (ng)**
1	Precision Plus MWM	-	1	Precision Plus MWM	-
2	SGF N0	0	2	T0, protein+SGF	8.0
3	SGF P0	0	3	T0, protein+SGF	4.0
4	SGF T0	0	4	T0, protein+SGF	2.0
5	SGF T1	0.5	5	T0, protein+SGF	1.0
6	SGF T2	2	6	T0, protein+SGF	0.5
7	SGF T3	5	7	T0, protein+SGF	0.3
8	SGF T4	10	8	T0, protein+SGF	0.1
9	SGF T5	20	9	Precision Plus MWM	-
10	SGF T6	30			
11	SGF T7	60			
12	SGF P7	60			
13	SGF N7	60			
14	Precision Plus MWM	-			
15	Blank	-			

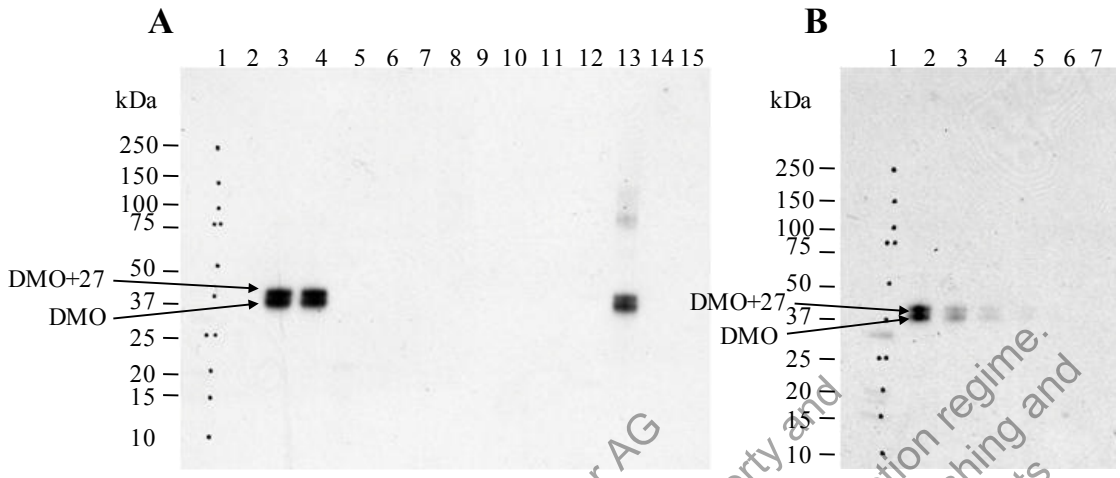


Figure VI-16. Western Blot Analysis of MON 87708 DMO in Simulated Intestinal Fluid

Panel A corresponds to MON 87708 DMO digestion in SIF. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in the lanes containing MON 87708 DMO (SIF T0-SIF T8). Panel B corresponds to the limit of detection of MON 87708 DMO. The amount loaded corresponds to the total protein amount of MON 87708 DMO. The lanes were cropped and re-numbered. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. A 15 second exposure is shown. N0 and N8 correspond to control samples that did not contain MON 87708 DMO. P0 and P8 correspond to controls that did not contain pancreatin. MWM denotes molecular weight marker.

Panel A			Panel B		
Lane	**Sample**	**Incubation Time**	**Lane**	**Sample**	**Amount (ng)**
1	Precision Plus MWM	-	1	Precision Plus MWM	-
2	SIF N0	0	2	T0, protein+SIF	15
3	SIF P0	0	3	T0, protein+SIF	10
4	SIF T0	0	4	T0, protein+SIF	5
5	SIF T1	5 minutes	5	T0, protein+SIF	2.5
6	SIF T2	15 minutes	6	T0, protein+SIF	1
7	SIF T3	30 minutes	7	Mark 12 MWM	-
8	SIF T4	1 hour			
9	SIF T5	2 hours			
10	SIF T6	4 hours			
11	SIF T7	8 hours			
12	SIF T8	24 hours			
13	SIF P8	24 hours			
14	SIF N8	24 hours			
15	Mark 12 MWM	-			

VI.F.1.5. Heat Stability of MON 87708 DMO

Temperature can have a profound effect on the structure and function of proteins. Soybean processing involves treatment of soybean with different temperatures for varying periods of time (Lusas, 2000; Lusas and Riaz, 1995). It is reasonable then to assume that the conditions encountered during the processing of soybean from MON 87708 will have an effect on the functional activity and structure of MON 87708 DMO when consumed in different food products.

The effect of heat treatment on the activity of MON 87708 DMO was evaluated using a functional activity assay (Appendix B, Section B.3.9, and Appendix G). Aliquots of MON 87708 DMO were heated to 25, 37, 55, 75, and 95°C for 15 and 30 minutes, while a separate aliquot of MON 87708 DMO was maintained on ice for the duration of the heat treatments to serve as a temperature control. The heated and temperature control MON 87708 DMO samples were denatured and separated and analyzed by SDS-PAGE using Colloidal Blue staining to assess integrity of the MON 87708 DMO protein and the MON 87708 DMO+27 protein.

The effects of heating on the functional activity of MON 87708 DMO are presented in Tables VI-8 and VI-9. The functional activity of MON 87708 DMO was unaffected at 25ºC and 37ºC for 15 and 30 minutes. After incubation at 55 ºC or higher for 15 minutes or more, the functional activity was below the LOQ of the assay, indicating that the majority of the functional activity of MON 87708 DMO had been lost during heating. These results suggest that temperature has a significant effect on the activity of MON 87708 DMO. MON 87708 DMO samples analyzed by SDS-PAGE showed no significant change in band intensity of the heat-treated samples at temperatures up to 55 °C (Figure VI-17 and VI-18). Heating at 75 °C for 30 minutes did result in a visually detectable loss of the MON 87708 DMO protein and the MON 87708 DMO+27 protein (Figure VI-18). Heating at 95 °C for 15 minutes did result in a visually detectable loss of the MON 87708 DMO protein and the MON 87708 DMO+27 protein (Figure VI-17), and the proteins were almost completely visually undetectable after 30 minutes (Figure VI-18).

Soybean processing involves treatment of soybean with different temperature regimes, many of which are higher than 55 °C and of variable duration (Lusas, 2000; Lusas and Riaz, 1995). Additionally, many steps, especially deactivation of anti-nutrient components, are carried out at considerably higher temperature (*e.g.*, greater than 100 °C) leading to a loss in active MON 87708 DMO in products such as soybean meal (Lusas, 2000; Lusas and Riaz, 1995). Therefore, it is reasonable to conclude that MON 87708 DMO would not be consumed as an active protein in food products.

Table VI-8. Specific Activity of MON 87708 DMO Following 15 Minute Heat Treatment

Temperature	Specific Activity[1] (nmole/min/mg MON 87708 DMO)	Activity DMO Remaining (% of control treatment)
0 ºC (Control Treatment)	16.6	100 %[2]
25 ºC	17.9	95 %
37 ºC	17.0	90 %
55 ºC	Below LOQ[3]	<25 %
75 ºC	Below LOQ[3]	<25 %
95 ºC	Below LOQ[3]	<25 %

[1] The specific activity was determined by measuring the production of DCSA. Mean specific activity determined from n=3.
[2]DMO activity of control treatment was assigned 100 % active.
[3] The LOQ is 4.4 nmoles/min/mg MON 87708 DMO.

Table VI-9. Specific Activity of MON 87708 DMO Following 30 Minute Heat Treatment

Temperature	Specific Activity[1] (nmole/min/mg MON 87708 DMO)	Activity DMO Remaining (% of control treatment)
0 ºC (Control Treatment)	16.6	100 %[2]
25 ºC	17.8	95 %
37 ºC	12.0	85 %
55 ºC	Below LOQ[3]	<25 %
75 ºC	Below LOQ[3]	<25 %
95 ºC	Below LOQ[3]	<25 %

[1] The specific activity was determined by measuring the production of DCSA. Mean specific activity determined from n=3.
[2]DMO activity of control treatment was assigned 100 % active DMO.
[3] The LOQ is 4.4 nmoles/min/mg MON 87708 DMO.

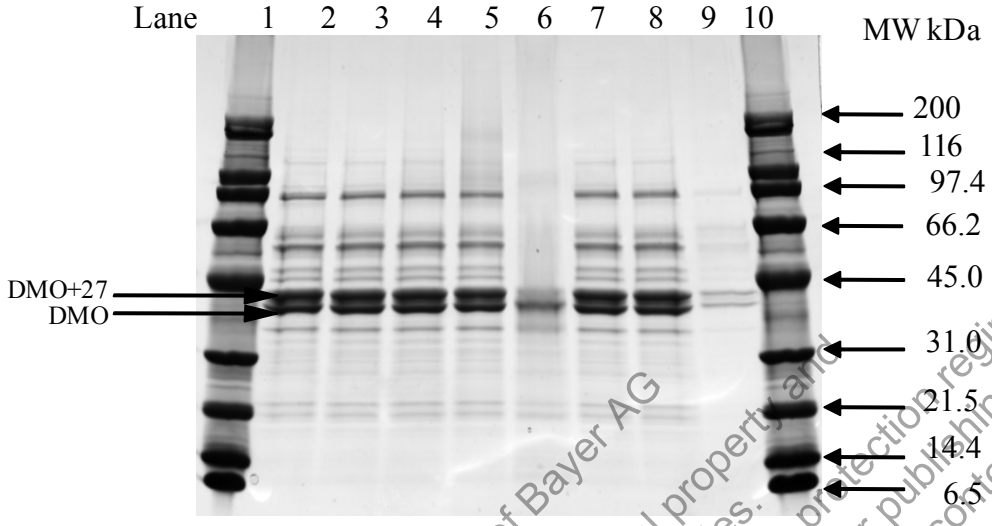


Figure VI-17. Colloidal Blue Stained SDS-PAGE of MON 87708 DMO Demonstrating the Effect after 15 Minutes at Elevated Temperatures on Protein Structural Stability

Heated samples of MON 87708 DMO (2.8 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were 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. Non-treated MON 87708 DMO samples were also mixed with loading buffer and loaded at 2.8 µg (100% equivalence) and 0.28 µg (10% equivalence) total protein.

Lane	Description	Amount (µg)
1	Broad Range Molecular Weight Markers	40.5
2	MON 87708 DMO 25ºC	2.8
3	MON 87708 DMO 37ºC	2.8
4	MON 87708 DMO 55ºC	2.8
5	MON 87708 DMO 75ºC	2.8
6	MON 87708 DMO 95ºC	2.8
7	MON 87708 DMO Temperature Control	2.8
8	MON 87708 DMO Reference 100% Equivalence	2.8
9	MO N87708 DMO Reference 10% Equivalence	0.28
10	Broad Range Molecular Weight Markers	40.5

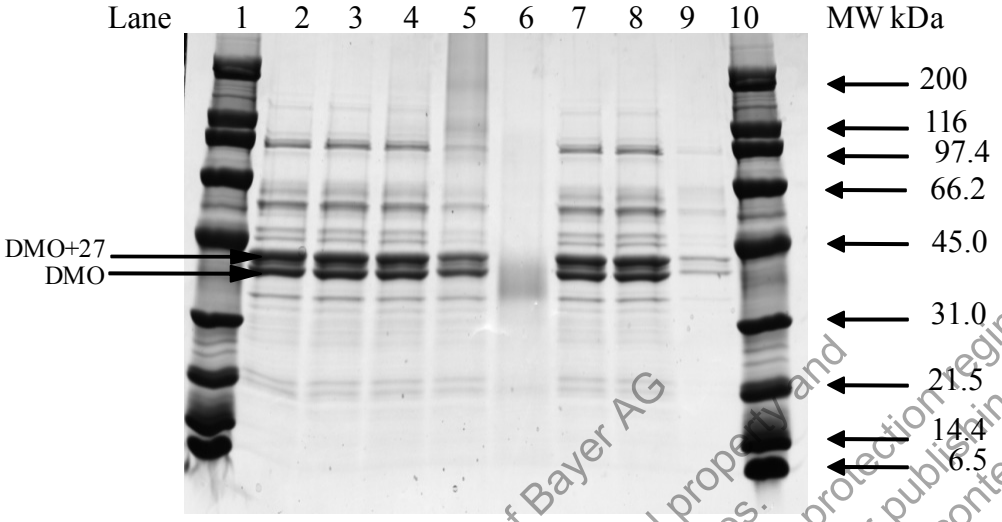


Figure VI-18. Colloidal Blue Stained SDS-PAGE of MON 87708 DMO Demonstrating the Effect after 30 Minutes at Elevated Temperatures on Protein Structural Stability

Heated samples of MON 87708 DMO (2.8 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were 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. Fresh MON 87708 DMO samples were also mixed with loading buffer and loaded at 2.8 µg (100% equivalence) and 0.28 µg (10% equivalence) total protein.

Lane	Description	Amount (µg)
1	Broad Range Molecular Weight Markers	40.5
2	MON 87708 DMO 25ºC	2.8
3	MON 87708 DMO 37ºC	2.8
4	MON 87708 DMO 55ºC	2.8
5	MON 87708 DMO 75ºC	2.8
6	MON 87708 DMO 95ºC	2.8
7	MON 87708 DMO Temperature Control	2.8
8	MON 87708 DMO Reference 100% Equivalence	2.8
9	MO N87708 DMO Reference 10% Equivalence	0.28
10	Broad Range Molecular Weight Markers	40.5

VI.F.1.6. Assessment of the Endogenous Allergenicity of MON 87708

Soybean is one of eight allergenic foods that, together, are responsible for approximately 90% of all food allergies (Cordle, 2004). Soybean is less allergenic than other foods in this group and is rarely responsible for severe, life-threatening reactions (Cordle, 2004). Allergy to soybean is more prevalent in children than adults and is considered a transient allergy of infancy/childhood (Sicherer et al., 2000). Since soybean is a known allergenic food crop, there is a need to ensure that the levels of endogenous allergenic proteins in MON 87708 are similar to the levels of these proteins in commercially available soybean varieties that are currently consumed. To determine this, MON 87708 binding values were compared to the binding values observed in commercial soybean varieties. Determining the levels of direct IgE binding using an ELISA has been shown to be an appropriate method to perform such comparisons (Sten, et al., 2004), especially when the assay is validated and calibrated prior to the production of the data (Ahlstedt, 2002; Holzhauser, et al., 2008).

The purpose of this assessment was to quantitatively evaluate the binding potential of soybean-specific IgE antibody from soybean-allergic subjects to aqueous protein extracts prepared from ground soybean seeds of MON 87708, near-isogenic conventional soybean control A3525, and commercial reference varieties. A quantitative evaluation of soybean-specific IgE provides an estimate of the endogenous allergens present in soybean seed. Protein extracts prepared from ground soybean seeds of MON 87708, conventional control, and 17 commercial reference varieties were evaluated (Appendix H). The commercial reference varieties were used to establish the range of soybean-specific IgE binding using sera from clinically diagnosed soybean allergic individuals and included high protein, high oil, and food-grade (tofu) soybean varieties that are already on the market and are being used for human consumption.

Sera from 13 clinically documented soybean-allergic subjects and five non-allergic subjects were used to assess IgE binding to each soybean extract. Only soybean-allergic subjects with a documented case history of soybean allergy with anaphylaxis and a positive Double-Blind Placebo Controlled Food Challenge (DBPCFC) were included as soybean positive subjects.

Aqueous protein extracts were prepared from the ground soybean seed of MON 87708, the conventional control, and the commercial reference varieties. These extracts were then analyzed for soybean-specific IgE antibody binding using a validated ELISA. Each soybean extract was tested in triplicate. Soybean specific IgE binding was quantified by interpolation against a soybean-specific IgE standard curve and was expressed as ng of IgE/ml of serum. The standard curve was created by loading serial dilutions of human serum PEI 163 that contains a known amount of soybean-specific IgE into wells coated with internal reference soybean extract. The concentration of soybean-specific IgE in serum PEI 163 was 36 kU/l (kilo units per liter) as measured by Capsulated Hydrolic Carrier Polymer-FluoroEnzyme Immunoassay (CAP-FEIA).

The IgE binding values obtained for the 17 commercial reference varieties extracts were used to calculate a 99% tolerance interval for each subject's serum. The IgE binding

values obtained for extracts prepared from MON 87708 and the conventional control were compared to the tolerance interval derived for each serum. All of the IgE binding values for MON 87708 and the control were within the commercial reference varieties tolerance limits for each subject's serum (Figure VI-19). None of the MON 87708, conventional control, or commercial reference varieties showed IgE binding to sera from non-allergic subjects.

The results of this assessment demonstrate that soybean-specific IgE binding to endogenous allergens in MON 87708 and the control are comparable with the IgE binding to commercially available conventional varieties. Therefore, MON 87708 and products derived from MON 87708 do not pose an increased endogenous allergenicity concern to humans over currently consumed soybean foods.

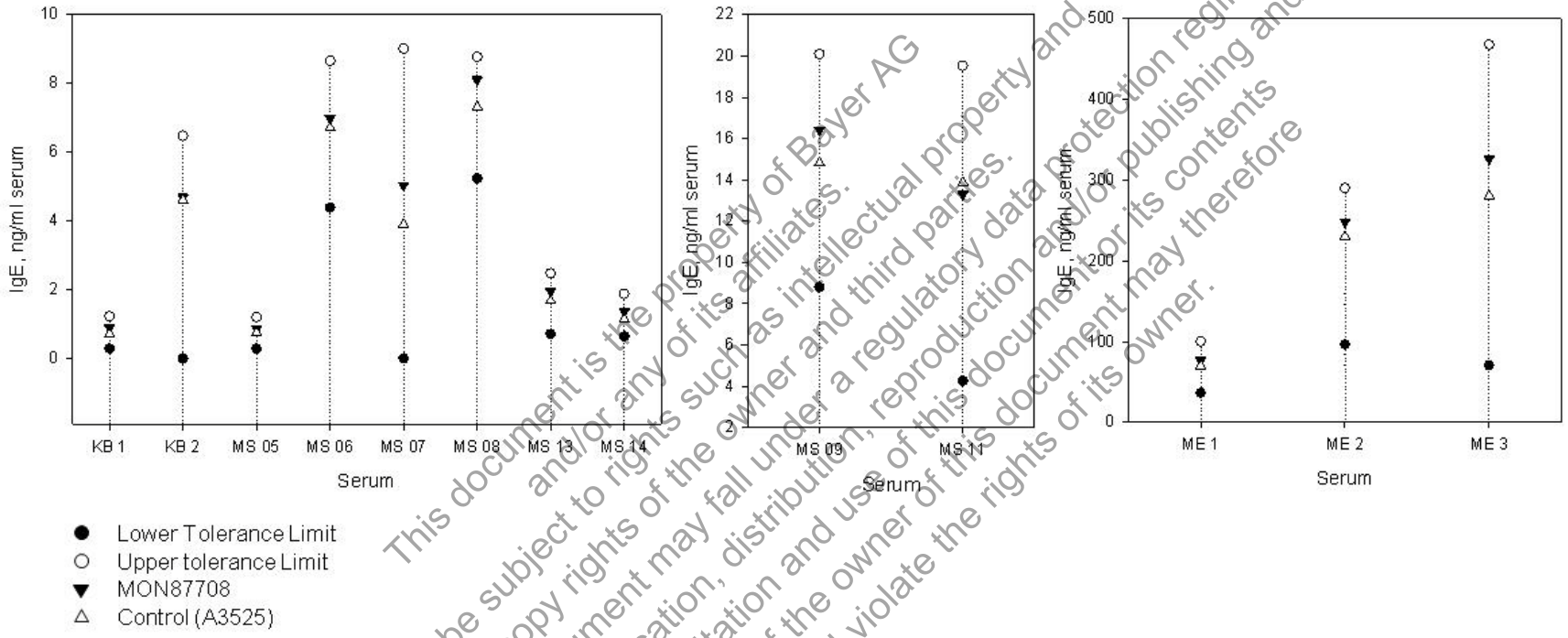


Figure VI-19. Serum IgE Binding Values for MON 87708, Conventional Control (A3525), and the Tolerance Limits for 17 Commercial Reference Varieties

Only those sera that met the set acceptance criteria were analyzed (see Appendix H). The lower and upper tolerance limits for 99 % tolerance intervals with 95 % confidence for each serum are the result of a tolerance interval analysis for 17 commercial reference varieties. Lower limits of the tolerance intervals that were calculated as less than zero were reported as zero in the analysis. Data are presented in three graphs due to the difference in IgE concentration range between sera. Abbreviations KB, MS, and ME are subject designations.

VI.F.2. Assessment for the Potential for Toxicity of MON 87708

VI.F.2.1. Structural Similarity of MON 87708 DMO 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 87708 DMO+27 protein, the MON 87708 DMO protein, and sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (*i.e.*, primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (*i.e.*, secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous (Caetano-Anolles et al., 2009; Illergard et al., 2009). Homologous proteins usually have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions.

FASTA bioinformatic alignment searches using the MON 87708 DMO+27 protein amino acid sequence, that contains the full MON 87708 DMO protein sequence, were performed with the TOX_2010 database to identify possible homology with proteins that may be harmful to human and animal health. The TOX_2010 database is a subset of sequences derived from the GenBank protein database (PRT_2010), release 175 (December, 15, 2009). Sequences were selected using a keyword search and filtered to remove likely non-toxin proteins. The TOX_2010 database contains 8,448 sequences.

An *E*-score acceptance criterion of 1×10^{-5} or less for any alignment was used to identify proteins from the TOX_2010 database with potential for significant shared structural similarity and function with MON 87708 DMO+27 protein. The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences require an *E*-score of 1×10^{-5} or smaller to be considered to have sufficient sequence similarity to infer homology (Silvanovich et al., 2009). The results of the search comparisons showed that no relevant alignments were observed against proteins in the TOX_2010 database. No FASTA alignment displayed an *E*-score of less than 1×10^{-5}.

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between MON 87708 DMO and any known toxin that would be harmful to human or animal health.

VI.F.2.2. Heat Stability and Digestability of MON 87708 DMO

The stability of a protein to heat or its degradation in simulated mammalian gastrointestinal fluids is a key consideration in the assessment of its potential toxicity. 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 87708 DMO was evaluated using a functional activity assay, and the results show that MON 87708 DMO was completely deactivated by heating at temperatures above 55°C (Section VI.F.1.5.). The digestability of MON 87708 DMO in simulated gastrointestinal fluids was evaluated by incubation with SGF and SIF, and the results show that MON 87708 DMO was readily digested (Section VI.F.1.4.). Therefore, it is anticipated that exposure to functionally active MON 87708 DMO from the consumption of MON 87708 or foods derived from MON 87708 will be negligible.

VI.F.2.3. Acute Oral Toxicity Analysis of MON 87708 DMO

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, that manifest toxicity in a short term (three week) feeding study (Liener, 1994). The amino acid sequence of the MON 87708 DMO+27 protein is not similar to any of these anti-nutritional proteins or to any other known protein toxins (Section VI.F.2.1). Therefore, an acute oral mouse toxicity study was considered sufficient to evaluate the toxicity of MON 87708 DMO.

MON 87708 DMO, which refers to the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, was administered as a single dose by oral gavage to a group of five male and five female CD-1 mice at a dose level of 140 mg/kg body weight (bw). Additional groups of mice were administered comparable levels of bovine serum albumin (BSA) to serve as a protein control. Following dosing, all mice were observed twice daily for mortality or signs of toxicity. Food consumption was measured weekly. Body weights were measured prior to dosing (study day 0) and on study days 7 and 14. All animals were sacrificed on day 14 and subjected to a gross necropsy. There were no treatment-related effects on survival, clinical observations, body weight, body weight gain, food consumption or gross pathology. Therefore, the No Observable Adverse Effect Level (NOAEL) for MON 87708 DMO was considered to be 140 mg/kg bw.

VI.F.3. Dietary Risk Assessment of MON 87708 DMO

VI.F.3.1. Estimated Human Exposure to MON 87708 DMO

Estimates of acute dietary exposure to MON 87708 DMO from consumption of foods derived from MON 87708 were determined using the Dietary Exposure Evaluation Model (DEEM-FCID version 2.03, Exponent Inc.) and food consumption data from the 1994, 1996, and 1998 USDA Continuing Survey of Food Intakes by Individuals (CSFII). DEEM-FCID differentiates soybean consumption into four fractions: seed, flour, soymilk, and soybean oil. However, since soybean oil contains negligible amounts of protein (Martin-Hernandez, et al., 2008; Tattrie and Yaguchi, 1973), it would not be a significant source of dietary exposure to MON 87708 DMO and was thus excluded from this assessment. Estimated human exposure to MON 87708 DMO in the U.S. was

considered using a reasonable worst case scenario of the 95^{th} percentile estimate of acute soybean consumption estimated on an "eater-only" basis.

MON 87708 is intended primarily for use as a broad-acre commodity (or field) soybean and not for vegetable, garden, or food-grade soybean that are generally used to produce tofu, soybean sprouts, soymilk, green soybean (*e.g.*, edamame) or other similar food items. Vegetable and food-grade soybean generally have a different size, flavor, texture and other characteristics than field soybean, and are more easily cooked. Field soybean is a blended commodity that is highly processed before being consumed by humans. Thus, most food products derived from MON 87708 would likely be blended with those derived from other commercial soybean varieties before entering the human food supply. However, estimating the percentage of consumed soybean products that would specifically be derived from MON 87708 is challenging. Therefore, for the purposes of this dietary risk assessment, the conservative assumption was made that 100% of all soybean products (excluding oil) consumed in the U.S. will be derived from MON 87708.

Because soybean is a blended commodity, the mean level of MON 87708 DMO in each of the consumed food fractions (seed, flour, and soymilk) should be used when estimating total intake of MON 87708 DMO from consumption of MON 87708. However, specific values for each of these fractions are not available. Thus, the concentration of MON 87708 DMO in soybean seed and milk was assumed to be equal to the mean concentration in whole MON 87708 seed grown in 2008 field trials (43 µg/gram fresh weight) (Table VI-7). Protein content of soybean meal is concentrated approximately 1.35-fold relative to protein levels in soybean seed (Lundry, et al., 2008). This value was derived from the ratio of total protein in processed meal to total protein in soybean seed. It was reasonable to estimate that this same concentration occurs in soybean flour and, therefore, the concentration of MON 87708 DMO in soybean flour was estimated to be 58 µg/g fwt, 1.35 times the mean concentration in whole MON 87708 fresh soybean seed harvested in 2008 field trials.

These estimates for MON 87708 DMO levels in soybean fractions are very conservative, since they assume that there is no loss of MON 87708 DMO during storage, processing, and/or cooking. Soybean contains certain components, such as trypsin inhibitors, which may act as antinutrients if the soybean is not properly heated during preparation (Rackis, 1974). Extensive heating processes are employed in extraction of soybean oil and in production of soybean meal and flour, soybean protein concentrates, soybean protein isolates, hydrolyzed vegetable protein, textured soybean protein, soy milk, and tofu. Therefore, virtually all protein-containing soybean fractions are heated during processing prior to consumption by humans. The functional activity of MON 87708 DMO is not heat stable (Section VI.F.1.5.). Thus, the amounts of functionally active MON 87708 DMO present in consumed soybean products will be substantially lower than the levels assumed for this evaluation.

Based on the above assumptions, the 95^{th} percentile acute intake (eater-only) for MON 87708 DMO, which refers to the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, was estimated to be 0.0056 mg/kg bw/day for the overall U.S. population. The 95^{th} percentile estimate of

acute intake (eater-only) for non-nursing infants in the U.S., the most highly exposed sub-population, was 0.2314 mg/kg bw/day.

VI.F.3.1.1. Dietary Exposure Assessment: Margin of Exposure for MON 87708 DMO

A common approach used to assess potential health risks from chemicals or other potentially toxic products is to calculate a Margin of Exposure (MOE) between the lowest NOAEL from an appropriate animal toxicity study and an estimate of human exposure. No adverse health effects were observed when male or female mice were administered a dose of 140 mg/kg bw of MON 87708 DMO. Based on a lack of toxicity (no hazard), the history of safe use of MON 87708 DMO, its digestibility and heat inactivation, dietary risk assessment for this protein would normally not be considered necessary. Nevertheless, a dietary risk assessment for MON 87708 DMO was still conducted in order to provide further assurance of the safety of MON 87708 DMO.

Potential health risks from the acute dietary intake of MON 87708 DMO from consumption of food derived from MON 87708 were evaluated by calculating the MOE based on the acute mouse NOAEL for MON 87708 DMO and the 95th percentile "eater-only" estimates of acute dietary exposure from DEEM-FCID. The MOEs for acute dietary intake of MON 87708 DMO were estimated to be 24,800 and 600 for the general population and for non-nursing infants, respectively (Table V1-10). These large MOEs indicate that there are no meaningful risks to human health from dietary exposure to MON 87708 DMO.

Table VI-10. Acute (95th Percentile, "eater-only") Dietary Intake and Margins of Exposure for MON 87708 DMO from Consumption of MON 87708 Soybean Meal-Derived Food Products in the U.S.[1]

Population	Protein Intake[2] (mg/kg/day)	Margin of Exposure[3]
General Population	0.0056	24,800
Non-nursing Infants	0.2314	600

[1]Estimated using DEEM-FCID version 2.03, Exponent Inc., utilizing food consumption data from the 1994-1996 and 1998 USDA Continuing Surveys of Food Intakes by Individuals (CSFII). Includes soybean seed, flour, and soymilk. Assumes 100% of soybean products (excluding soybean oil) consumed in the U.S. are derived from MON 87708.

[2]Based on average expression levels of 43.0 µg/gram fresh weight for MON 87708 DMO in whole seed (Section VI.E). As described above, MON 87708 DMO content in soybean flour was assumed to be 1.35x the level in whole seed from MON 87708.

[3]Calculated by dividing the NOAEL from the acute mouse gavage study (140 mg/kg bw) by estimated dietary intake of MON 87708 DMO from MON 87708. MOEs were rounded to the nearest hundred.

VI.F.3.2. Estimated Animal Exposure to MON 87708 DMO

In 2007, 59% of U.S. grown soybean was crushed domestically, 38% was exported and 3% was used directly as feed or seed (USDA-ERS, 2007). About 85% of the world's soybean are crushed to produce soybean meal and oil (Soyatech, 2010), as a result most soybean is fed as soybean meal. Of the soybean meal produced in the U.S., approximately 98% is consumed by animals (Soyatech, 2010). Poultry consume 48%, swine 26%, beef cattle 12%, dairy cows 9%, companion animals 2%, and other animals 3% (ASA, 2010a). Other soybean-derived products such as full-fat (whole) soybean and soybean forage are also fed to animals. The feeding level of full-fat soybean for dairy cattle is typically 2.7 (2.3 -3.1) kg/cow/day (Harris, 1990; Hutjens, 1999). In swine, the feeding of heat treated full-fat soybean is limited to a maximum of 20% of the swine diet due to its high oil content (Yacentiuk, 2008). Full-fat soybean is not commonly fed to poultry due to its high oil content and low availability of the oil (North and Bell, 1990). Soybean forage can only be fed to ruminants such as beef and dairy cattle and is limited to 50% of the total ration dry matter (Brown, 1999).

Animals might be exposed to MON 87708 DMO through dietary intake of feed derived from MON 87708 seed, and MON 87708 forage in the case of the lactating dairy cow. The primary exposure of poultry and livestock to MON 87708 DMO will be from the feeding of soybean meal, with some animals being fed heat-treated, full-fat soybean. Since livestock diets typically contain a much higher level of the protein from soybean meal than from the full-fat soybean, dietary intake estimates of soybean (except forage) will assume consumption as soybean meal. It was also assumed that lactating dairy cows would consume both forage and soybean meal derived from MON 87708. Table VI-11 details the estimated poultry and livestock dietary intake of soybean, which is then used to estimate the animal dietary exposure of MON 87708 DMO.

Table VI-11. Poultry and Livestock Dietary Intake of Soybean

Species	Soybean Source in Diet	% Diet	Daily Consumption (g dw/day)	% Dry Matter (DM)	Body Weight (kg)	Daily Dietary Intake (DDI) [1] (g DM/kg bw/day)
Chicken broiler[2]	SBM	30.2	161	89	1.6	27.0
Young pig[3]	SBM	24.3	2020	89	40	10.9
Finishing pig[3]	SBM	14	3040	89	100	3.8
Lactating dairy	SBM	18.6	27,400	na[6]	655	7.8
cow[4,5]	Forage	50	27,400	na[6]	655	20.9

SBM= soybean meal

[1]DDI = Daily Consumption x % DM x % Diet ÷ BW

[2](Popescu and Criste, 2003).

[3](Cromwell, et al., 2002).

[4](Bal, et al., 2000).

[5](Brown, 1999).

[6] All diets are on a wet weight basis except dairy cow diet, which is on dry weight basis.

VI.F.3.2.1. Animal Dietary Intake of MON 87708 DMO

The potential animal dietary exposure of MON 87708 DMO, resulting from the consumption of MON 87708 soybean meal or forage, can be estimated by multiplying the consumption of each commodity by the level of MON 87708 DMO in that feedstuff. For estimating animal dietary exposure of MON 87708 DMO, a worst-case exposure scenario was used along with the mean and maximum levels of MON 87708 DMO reported for MON 87708 seed and forage. The mean and maximum values of MON 87708 DMO levels in soybean seed and forage used in this assessment were from MON 87708 grown in the U.S. in 2008 (Table VI-7). In order to calculate the highest animal exposure to MON 87708 DMO, several conservative assumptions were made. First, soybean meal used for animal feed would be expected in most instances to have gone through a series of commingling steps with soybean meal derived from non-MON 87708 soybean as it makes its way through commerce. However, since estimating the percentage of consumed meal that would specifically be derived from MON 87708 is challenging, this assessment assumes that the only source of soybean in the diet is MON 87708. Second, statistics from USDA for 2009 indicate that during the crushing process 19.9 kg of soybean meal was produced from each bushel of soybean (27.2 kg), which is equivalent to 0.73 kg soybean meal/kg of soybean. It is assumed that there is no degradation of MON 87708 DMO during the crushing process. The mean level of MON 87708 DMO in MON 87708 seed is 47 µg/g dry weight (dwt) with a maximum of 59 µg/g dwt. Therefore, assuming a crushing yield of 73%, the calculated mean and maximum levels of MON 87708 DMO in soybean meal derived from MON 87708 seed would be 64.38 µg/g dwt and 80.82 µg/g dwt, respectively. The mean level of MON 87708 DMO in MON 87708 forage is 53 µg/g dwt with a maximum of 84 µg/g dwt.

The estimate of daily dietary intake of MON 87708 DMO (DDI-DMO) is calculated as follows:

$$\text{DDI-DMO} = [\text{DDI-SBM} \times \text{MON 87708 DMO SBM}] + [\text{DDI-FOR} \times \text{MON 87708 DMO Forage}]$$

Where DDI-SBM is the daily dietary intake of SBM and DDI-FOR is the daily dietary intake of forage (dairy cattle only) from Table VI-11, and MON 87708 DMO SBM and MON 87708 DMO forage are the levels of MON 87708 DMO in SBM and forage, respectively. As stated previously, MON 87708 DMO refers, collectively, to the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer.

The estimated mean and maximum poultry and livestock dietary exposure estimates for MON 87708 DMO are shown in Table VI-12.

Table VI-12. Poultry and Livestock Dietary Exposure to MON 87708 DMO

Species	Soybean Source in Diet	Daily Dietary Intake (DDI) (g DM/kg of bw/day)	Levels of MON 87708 DMO in Soybean Source (µg/g dw)		MON 87708 DMO Intake (DDI-DMO) (g/kg bw/day) [1]	
			Mean	Maximum	Mean	Maximum
Chicken broiler	SBM	27.0	64.38	80.82	0.001738	0.002182
Young pig	SBM	10.9	64.38	80.82	0.000702	0.000881
Finishing pig	SBM	3.8	64.38	80.82	0.000245	0.000307
Lactating dairy cow	SBM	7.8	64.38	80.82	0.001610[2]	0.002386[2]
	Forage	20.9	53	84		

SBM= soybean meal

[1] Values expressed as g/kg bw/day were obtained by applying a conversion factor of 10^{-6} to convert units from µg/kg bw/day to g/kg bw/day.

[2] Total considers intake from SBM and forage

VI.F.4. Potential Allergenicity or Toxicity of MON 87708 DMO Summary and Conclusion

A strong safety profile has been established for MON 87708 DMO. Its donor organism, *S. maltophilia*, is ubiquitous in the environment and is found on a variety of foods. MON 87708 DMO shares strong functional and structural homology with a variety of oxygenases that themselves have a history of safe use. MON 87708 DMO is present at a very low level in the harvested seed of MON 87708, and therefore, constitutes a very small portion of the total protein present in food and feed derived from MON 87708. MON 87708 DMO lacks structural similarity to known allergen or toxins known to have adverse effects on mammals. MON 87708 DMO was rapidly digested in SGF and SIF. MON 87708 DMO loses activity upon heating and did not demonstrate acute oral toxicity in mice at the level tested. Large MOEs have been demonstrated for the consumption of MON 87708 DMO derived from MON 87708 for the U.S. general population and for non-nursing infants, the highest exposed sub-population. In addition, no meaningful risks to animal health from dietary exposure to MON 87708 DMO are anticipated as a result of very low exposure to MON 87708 DMO from the consumption of MON 87708 soybean meal or forage.

Based on the above information, the consumption of MON 87708 DMO from MON 87708 seed or products derived from MON 87708 is considered safe for humans and animals.

VI.G. Bioinformatic Assessment of Putative Open Reading Frames of MON 87708 Insert and Flanking Sequences

The 2003 Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2003) 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 87708 are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

In addition to the bioinformatic analysis conducted on MON 87708 DMO (Sections VI.F.1.3 and VI.F.2.1), bioinformatic analyses were also performed on the MON 87708 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 87708 insert DNA as well as ORFs present in the 5' and 3' inserted DNA-5' and 3' flanking sequence junctions (Table V-2 and Figure VI-20). These various bioinformatic evaluations are depicted in Figure VI-20. ORFs spanning the 5' flanking sequence DNA-inserted DNA junctions, 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). Putative peptides/polypeptides from each reading frame were then compared to toxin, allergen and all proteins databases using bioinformatic tools. Similarly, the entire MON 87708 insert DNA sequence was translated in all six reading frames (three forward reading frames and three reading frames in reverse orientation) and the resulting deduced amino acid sequence was subjected to bioinformatic analyses. There are no analytical data that indicate any putative polypeptides/proteins subjected to bioinformatic evaluation other than the MON 87708 DMO which is 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 87708 DMO was 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 putative polypeptides for MON 87708 relatedness 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 87708 (Figure VI-20).

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2010, TOX_2010, and PRT_2010 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 alignment length as 35% or greater identity in 80 or greater amino acids (to ascertain if alignments exceeded Codex (Codex Alimentarius, 2003) thresholds for FASTA searches of the AD_2010 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_2010 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_2010) or toxin (TOX_2010) 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 search the PRT_2010 database, translations of frames 1-4 yielded alignments with *E*-scores less than or equal to a 1×10^{-5} threshold. Inspections of frame 1, 2, and 4 alignments revealed that they were punctuated with numerous stop codons in the query sequence and required numerous gaps to optimize the alignment. As a result, it is unlikely these alignments reflect conserved structure. When used as a query in a FASTA search of the PRT_2010 database, the translation of frame 3 yielded numerous alignments with *E*-scores less than or equal to the 1×10^{-5} threshold. The top three alignments displayed 99.7% identity over 338 amino acids with an oxygenase from *S. maltophilia*. In addition, a second group of alignments between frame 3 and ribulose 1,5-bisphosphate were observed. These frame 3 alignments positively identify MON 87708 DMO and the associated chloroplast targeting peptide, and the alignments are consistent with the known structure of protein coding sequence contained in the MON 87708 inserted DNA.

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 87708. As a result, in the unlikely event that a translation product other than MON 87708 DMO and the associated chloroplast targeting peptide was 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 87708 inserted DNA were performed using a bioinformatic

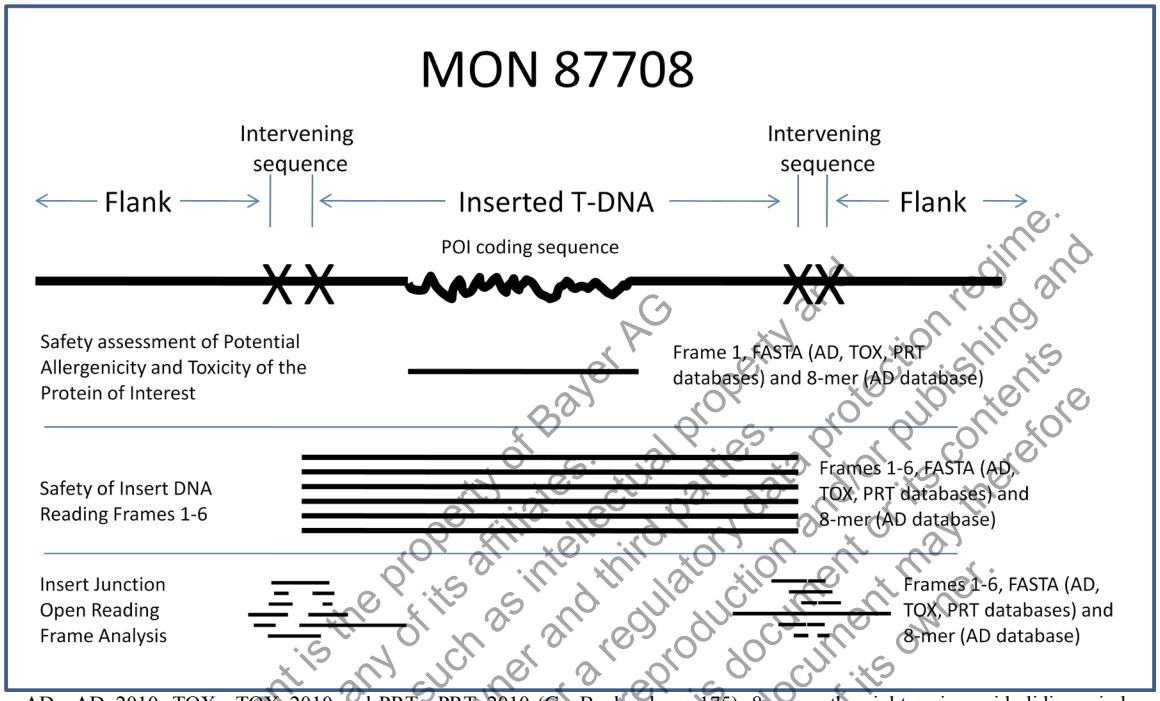
comparison strategy (Figure VI-20). The purpose of the assessment is to evaluate the potential for novel open reading frames (ORFs) that may have homology to known allergens, toxins, or proteins that display adverse biological activity. Sequences spanning the 5′ flanking sequence DNA-inserted DNA and the inserted DNA-3′ flanking sequence DNA (Figure VI-20) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. The resulting putative polypeptides from each reading frame, that were eight amino acids or greater in length, were compared to AD_2010, TOX_2010, and PRT_2010 databases using FASTA and to the AD_2010 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_2010, TOX_2010, and PRT_2010 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 Alimentarius (2003) thresholds for FASTA searches of the AD_2010 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_2010database.

No biologically relevant structural similarity to known allergens or toxins 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 DNA junctions of MON 87708, 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 87708 Polypeptides Putatively Encoded by the Insert and Flanking Sequences Summary and Conclusions

A conservative bioinformatic assessment of potential allergenicity, toxicity and adverse biological activity for putative polypeptides that span the 5’ and 3’ insert junctions or were derived from different reading frames of the entire insert was conducted for MON 87708. The data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than MON 87708 DMO and its associated chloroplast targeting peptide was 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 87708.



AD= AD_2010; TOX= TOX_2010 and PRT= PRT_2010 (GenBank release 175): 8-mer = the eight amino acid sliding window search. POI Coding sequence corresponds to the *dmo* coding sequence.

Figure VI-20. Schematic Summary of MON 87708 Bioinformatic Analyses

VI.H. Safety Assessment of Expression Products Summary and Conclusion

MON 87708 DMO is an oxygenase derived from *S. maltophilia*. *S. maltophilia* is an environmentally ubiquitous bacterium that does not pose a health risk to healthy individuals. MON 87708 DMO is a Rieske-type mono-oxygenase that has homology with other oxygenases present in bacteria and plants that share many of the typical structural and functional characteristics of these types of oxygenases, while maintaining specificity for its substrate, dicamba. A history of safe use has been established for homologs of MON 87708 DMO which lack structural similarity to known allergens or toxins known to have adverse effects on mammals. MON 87708 DMO, which collectively refers to the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, was fully characterized confirming both the N-terminal and a large portion of the amino acid sequence, and the lack of glycosylation. Since the MON 87708 DMO used in the described safety studies was purified directly from MON 87708 seed and was enzymatically active, equivalence evaluations between plant-produced and bacterial-produced MON 87708 DMO were not required. Expression studies using ELISA demonstrated that MON 87708 DMO was expressed in all assayed

tissues at levels ranging from 3.9-180 µg/g dwt, and was shown to be stably expressed across five different generations. MON 87708 DMO is present at a very low level in the harvested seed of MON 87708, was not structurally similar to known allergens, was rapidly digested in SGF and SIF, and loses functional activity when exposed to heat. Additionally, the IgE binding to endogenous allergens in MON 87708 was comparable to that of commercially available conventional varieties. MON 87708 DMO had no sequence similarity to known toxins and was not stable to heating, dramatically losing activity at temperatures above 55 °C. MON 87708 DMO did not exhibit toxicity when evaluated in an acute oral toxicity study in mice. Given the very low level of MON 87708 DMO in the harvested seed of MON 87708 it will constitute a very small portion of the total protein present in food and feed derived from MON 87708. Large MOEs were demonstrated for the consumption of MON 87708 DMO derived from MON 87708 for the general population and for non-nursing infants, the highest exposed sub-population. Furthermore, there was no evidence for concern regarding health implications of putative polypeptides for MON 87708.

Taken together, the available data and information provided in this safety assessment on MON 87708 DMO support a conclusion that there is no meaningful risk to human or animal health from dietary exposure to MON 87708 DMO, and food and feed products containing MON 87708 or derived from MON 87708 are as safe as soybean currently on the market for human and animal consumption.

VII. COMPOSITIONAL ASSESSMENT OF MON 87708

Safety assessments of biotech crops typically include comparisons of the composition of forage and whole grain of the GM crop to that of conventional counterparts (Codex Alimentarius, 2003). Compositional assessments are performed using the principles and analytes outlined in the OECD consensus documents for soybean composition (OECD, 2001).

A recent review of compositional assessments conducted according to OECD guidelines that encompassed a total of seven GM 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). 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). Compositional equivalence between biotechnology-derived and conventional crops provides an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2001). The OECD consensus documents emphasize quantitative measurements of essential nutrients and known anti-nutrients. This is based on the premise that such comprehensive and detailed analyses will most effectively discern any compositional changes that imply potential safety and nutritional concerns. Levels of the components in seed and forage of the biotechnology-derived crop are compared to: 1) corresponding levels in a conventional comparator, the non-biotechnology near isogenic line, grown concurrently, under identical 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 latter comparison 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.

VII.A. Compositional Equivalence of MON 87708 Seed and Forage to Conventional Soybean

Seed and forage samples were collected from MON 87708 and the near isogenic conventional soybean control A3525 grown in a 2008 U.S. field production. Four different commercial reference varieties 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 five sites: Jefferson County, Iowa; Stark County, Illinois; Clinton County, Illinois; Parke County, Indiana; and Berks County, Pennsylvania. All soybean plants including MON 87708, the conventional control, and the commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition, MON 87708 plots were treated at the V2-V3 growth stage with dicamba herbicide at the maximum in-crop label rate (0.5 lb acid equivalence [a.e.]/acre).

Compositional analyses were conducted to assess whether levels of key nutrients and anti-nutrients in MON 87708 were equivalent to levels in the conventional control and to the composition of the commercial reference varieties. A description of nutrients and anti-nutrients present in soybean is provided in the OECD consensus document on compositional considerations for soybean (OECD, 2001). Nutrients assessed included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), fiber, amino acids (18 components), fatty acids (FA, C8-C22), and vitamin E (α- tocopherol) in seed, and proximates (ash, carbohydrates by calculation, moisture, protein, and fat) and fiber in forage. Anti-nutrients assessed in seed included raffinose, stachyose, lectin, phytic acid, trypsin inhibitors, and isoflavones (daidzein, genistein, and glycitein).

In all, 64 different components were measured (seven in forage and 57 in seed). Of those 64 components, 14 had more than 50% of the observations below the assay limit of quantitation (LOQ) and subsequently were excluded from statistical analysis. Therefore, 50 components were statistically assessed using a mixed-model analysis of variance method. Values for all assessed components were reported on a dry weight basis with the exception of moisture, which was reported as % fresh weight (fwt) and fatty acids, which were reported as % of total FA.

For MON 87708, six statistical comparisons to the conventional control were conducted. One comparison was based on compositional data combined across all five field sites (combined-site analysis) and five separate comparisons were conducted on data from each of the individual field sites. Statistically significant differences were identified at a 5% level of significance. Data from the commercial reference varieties were combined across all sites and used to calculate a 99% tolerance interval for each compositional component to define the natural variability of each component in soybean varieties that have a history of safe consumption and that were grown concurrently with MON 87708 and the conventional control in the same trial.

For the combined-site analysis, statistically significant differences in nutrient and anti-nutrient components were further evaluated using considerations relevant to the safety and nutritional quality of MON 87708 when compared to the conventional control A3525, the conventional counterpart with a history of safe consumption: 1) the relative magnitude of the difference in the mean values of nutrient and anti-nutrient components of MON 87708 and the conventional control, 2) whether the MON 87708 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of the commercial varieties grown concurrently in the same trial, 3) analyses of the reproducibility of the statistically significant combined-site component differences at individual sites, and 4) assessing the differences within the context of natural variability of commercial soybean composition published in the scientific literature and in the ILSI Crop Composition Database (ILSI, 2009; Ridley, et al., 2004).

This analysis provides a comprehensive comparative assessment of the levels of key nutrients and anti-nutrients in seed, and of key nutrients in forage of MON 87708 and the conventional control, discussed in the context of natural variability in commercial soybean. Results of the comparison indicate that the composition of the seed and forage

of MON 87708 is equivalent to that of the near isogenic conventional soybean control A3525 and within the range of natural variability of the commercial reference varieties.

VII.A.1. Nutrient Levels in Soybean Seed

In the combined-site analysis of nutrient levels in seed, the following components showed no statistically significant differences in mean values between MON 87708 and the conventional control: moisture, total fat, six amino acids (alanine, lysine, methionine, serine, threonine, and tryptophan), and three fatty acids (18:0 stearic acid, 20:0 arachidic acid, and 20:1 eicosenoic acid) (Table VII-2).

The components that showed statistically significant differences in mean values between MON 87708 and the conventional control in the combined-site analysis were: three proximates (ash, carbohydrates by calculation, and protein), 12 amino acids (arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, phenylalanine, proline, tyrosine, and valine), three types of fiber (acid detergent fiber [ADF], neutral detergent fiber [NDF], and crude fiber), five fatty acids (16:0 palmitic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic acid, and 22:0 behenic acid), and vitamin E (Tables VII-1 and VII-2).

These statistically significant differences in nutrients were evaluated using considerations relevant to the safety and nutritional quality of MON 87708 when compared to the conventional control:

1) All nutrient component differences observed in the combined-site analysis, whether reflecting increased or decreased MON 87708 mean values with respect to the conventional control were small. Relative magnitude of differences ranged from 2.65 to 7.91% for amino acids, 1.51 to 8.19% for fatty acids, 15.13% for vitamin E, and 2.41 to 12.37% for proximates and fibers.

2) Mean values for all of these statistically different nutrient components from the combined-site analysis of MON 87708 were within the 99% tolerance interval established from the commercial reference varieties grown concurrently and were, therefore, within the range of natural variability of that component in commercial soybean varieties with a history of safe consumption (Tables VII-1 and VII-2).

3) Assessment of the reproducibility of the combined-site differences at the five individual sites showed: statistically significant differences for carbohydrates by calculation, crude fiber, cystine, and glycine at one site; aspartic acid, phenylalanine, proline, tyrosine, valine, 16:0 palmitic acid, and 18:2 linoleic acid at two sites; protein, arginine, glutamic acid, histidine, isoleucine, leucine, and 22:0 behenic acid at three sites; vitamin E at four sites; and 18:1 oleic acid and 18:3 linolenic acid differed across all five sites. Although they were different in the combined site analysis, no differences were observed for ash, ADF or NDF at any of the individual sites. Individual site mean values of MON 87708 for all nutrient components with statistically significant differences fell within the 99% tolerance interval established from the commercial reference varieties

grown concurrently and were, therefore, within the range of natural variability of that component in commercial soybean varieties with a history of safe consumption.

4) All mean values of MON 87708 for all nutrient components were within the context of the natural variability of commercial soybean composition as published in the scientific literature and available in the ILSI Crop Composition Database (ILSI, 2009; Ridley et al., 2004).

Thirteen of the 24 differences between MON 87708 and the conventional control observed in the combined-site data analysis were attributable to small differences in protein and 12 individual amino acids (all expressed as % dwt). The relative magnitude of the difference between the mean protein values for MON 87708 and the conventional control was small (a decrease of 3.65% in the combined-site analysis for MON 87708) and reached statistical significance at only three of the five individual sites. Correspondingly, differences in all amino acids were small and not observed consistently as statistically significant differences at all individual sites. Eleven of the 12 amino acids observed to be different in the combined-site analysis were decreased (2.65 7.91%) relative to the conventional control and, as with protein, statistically significant differences were not consistently observed at all individual sites. Cystine showed a relative increase of 3.01% but was statistically significantly different at only one site. Four of the six amino acids (alanine, lysine, serine, and threonine) not observed to be statistically different in the combined-site analysis also showed modest decreases ranging from ~ 1.5 2.3% (Table VII-2) consistent with the directionality of the changes observed in protein content. Overall, observed differences in protein and amino acid levels are not considered to be meaningful from a food and feed safety and nutritional perspective because they were small, and the mean MON 87708 values were within the 99% tolerance interval established by the commercial reference varieties grown concurrently in the same trial.

Five of the combined-site differences between MON 87708 and the conventional control were attributable to fatty acid levels (all expressed as % total FA) in seed, whereas total fat content was not statistically significantly different. For 18:1 oleic acid and 18:3 linolenic acid, the relative magnitude of differences between the mean values for MON 87708 and conventional control were small in the combined-site analysis (a decrease of 8.19% and an increase of 6.65% compared to the conventional control, respectively) and at the five individual sites (levels were <11% decreased for 18:1 oleic acid and <10% increased for 18:3 linolenic acid at all sites compared to conventional control) (Tables VII-2, I-4, I-7, I-10, I-13, and I-16).

By comparison, the observed differences between MON 87708 and conventional control for 18:1 oleic and 18:3 linolenic acids are markedly less than differences in soybean varieties developed through conventional breeding (Clemente and Cahoon, 2009; Fehr, 2007). The average relative levels of 18:3 linolenic acid in commercial soybean are approximately 10% total FA, while the average relative level of 18:1 oleic acid in commercial soybean is approximately 18-25% total FA. In the compositional analysis presented here, the values of FA components in the conventional control, when assessed as individual replicates across all five individual sites, ranged from 19.6 to 22.4% total

FA for 18:1 oleic acid and from 8.4 to 10.1% total FA for 18:3 linolenic acid (Table VII-2). The values from the commercial reference varieties ranged from 17.9 to 25.3% total FA for 18:1 oleic acid and 7.4 to 11.4% total FA for 18:3 linolenic acid (Table VII-2). Additionally, literature data from Lundry et al. (2008) and Berman et al. (2009) and the ILSI Crop Composition Database (Berman, et al., 2009; ILSI, 2009; Lundry et al., 2008; Ridley et al., 2004) highlight the extensive natural variability in fatty acid levels in soybean, as presented in Table VII-5. The small relative magnitudes of the differences in 18:3 linolenic acid and 18:1 oleic acid compared to the conventional control as well the broad range of these fatty acids present in commercial soybean varieties, suggest that the differences are not meaningful to food and feed safety and nutritional quality in MON 87708.

The relative magnitudes of differences between the mean values for MON 87708 and the conventional control for the other three fatty acids observed in the combined-site analysis were small (2.29% increase for 16:0 palmitic acid, 1.51% increase for 18:2 linoleic acid and a 4.70% decrease for 22:0 behenic acid). The small magnitude of differences as well as the lack of statistical differences across all individual sites (Tables VII-2, I-4, I-7, I-10, I-13, and I-16) further confirmed that the differences observed in fatty acid composition are not meaningful to food and feed safety and nutritional quality.

One of the combined-site differences observed between MON 87708 and the conventional control was attributable to vitamin E (expressed as mg/100g dwt). The relative magnitude of difference between the mean values of MON 87708 and conventional control for vitamin E in the combined-site analysis was an increase of 15.1% with respect to the conventional control (Tables VII-1).

Levels of vitamin E are known to be affected by environmental growing conditions (E) and germplasm (G) as demonstrated in results from recent assessments on soybean varieties grown at three locations in the U.S. over a period of four years (Britz, et al., 2008) and across six environments in Eastern Canada in a single year (Seguin, et al., 2009). Britz et al. (2008) showed more than a two-fold variation in levels across their study (units expressed as the ratio of α tocopherol [vitamin E] to total tocopherol content). Vitamin E values in Seguin et al. (2009) ranged from 0.87 to 3.32 mg/100g dwt. Both assessments showed that G and E effects as well as G × E interaction effects influenced vitamin E content. In the compositional analysis presented here, values of vitamin E in the conventional control, when assessed as individual replicates across all sites, ranged by as much as 0.89 to 2.11 mg/100g dwt (Table VII-2). Ranges of vitamin E values from the concurrently grown commercial reference varieties were even greater and ranged from 0.69 to 2.91 mg/100g dwt (Table VII-2). Literature data from other compositional assessments (Berman et al., 2009; ILSI, 2009; Lundry et al., 2008; Ridley et al., 2004) that further highlight the extensive natural variability in vitamin E levels in soybean are presented in Table VII-5. Therefore, given this established variability of vitamin E levels in conventional soybean and the fact that soybean is not an important nutritional source of vitamin E in human or animal diets, this increase in vitamin E levels in MON 87708 compared to the conventional control supports the conclusion that this observed difference is not meaningful to food and feed safety and nutritional quality.

The remaining combined-site differences between MON 87708 and the conventional control were attributable to two proximates (ash and carbohydrates by calculation) and three fibers (ADF, NDF, and crude fiber). The relative magnitude of these increases were small (2.41% to 12.37%) and there was no consistency of these combined-site differences at the individual sites (carbohydrates by calculation and crude fiber were different at only one site, whereas ash, ADF and NDF were not different at any of the individual sites). The combined-site mean values for these nutrient components also were within the 99% tolerance interval established from the commercial reference varieties grown concurrently establishing that these differences are not meaningful to food and feed safety and nutrition.

In summary, statistical analyses found no consistent differences across sites in the levels of nutrient components in seed from MON 87708 and the conventional control, except for differences in 18:1 oleic acid, 18:3 linoleic acid, and vitamin E levels that were of small magnitude and were within the natural variability of the concurrently grown commercial soybean varieties. These data support the conclusion that MON 87708 is compositionally equivalent to conventional soybean.

VII.A.2. Anti-Nutrient Levels in Soybean Seed

In the combined-site analysis, no statistically significant differences were observed in four of the eight anti-nutrient component comparisons (lectin, trypsin inhibitors, genistein, and glycitein) between MON 87708 and the conventional control. Statistically significant differences were observed between MON 87708 and the conventional control in the other four anti-nutrient components that were measured (Tables VII-1 and VII-3). The differences included decreased mean values for phytic acid, raffinose, stachyose, and an increased mean level of daidzein, compared to the conventional control.

The statistically significant differences in anti-nutrients were evaluated using considerations relevant to the safety and nutritional quality of MON 87708 when compared to the conventional control:

1) All anti-nutrient component differences observed in the combined-site analysis, whether reflecting increased or decreased MON 87708 mean values with respect to the conventional control were small. Relative magnitude of differences in the combined-site analysis for the anti-nutrients that were decreased in MON 87708 ranged from 6.1% (phytic acid) to 7.73% (raffinose). The relative magnitude of difference (increase) in daidzein was 11.5%.

2) MON 87708 mean values for these anti-nutrient components from the combined-site analysis were within the 99% tolerance interval established from the commercial reference varieties concurrently grown in the same trial and, therefore were within the range of natural variability of these components in commercial soybean varieties with a history of safe consumption (Tables VII-1 and VII-3).

3) Assessment of the reproducibility of the combined-site differences at the five individual sites showed no consistent pattern across sites. A statistically significant

decrease was observed for stachyose at one site and phytic acid at two sites, whereas a significant increase was seen for daidzein at two sites. No differences for raffinose were observed at any of the individual sites. Mean values for all of the above anti-nutrient components in MON 87708 at the individual sites were within the 99% tolerance interval established from the concurrently grown commercial reference varieties.

4) All mean values of MON 87708 for all anti-nutrients were within the context of the natural variability of commercial soybean composition as published in the scientific literature and available in the ILSI Crop Composition Database (ILSI, 2009; Ridley et al., 2004).

In summary, statistical analyses found no consistent differences across sites in the levels of anti-nutrient components in seed from MON 87708 and the conventional control. Thus, a comprehensive evaluation of anti-nutrient components in seed support the conclusion that MON 87708 is compositionally equivalent to conventional soybean.

VII.A.3. Nutrient Levels in Soybean Forage

In the combined-site analysis of forage, six of the seven nutrient component comparisons did not have a statistically significant difference between MON 87708 and the conventional control (Tables VII-1 and VII-4). The only statistical difference was for the ADF mean value and it was evaluated using considerations relevant to the safety and nutritional quality of MON 87708 when compared to the conventional control.

1) The relative magnitude of difference in ADF, with respect to the conventional control, was small with an increase of 10.45%.

2) The mean value for ADF from the combined-site analysis of MON 87708 was within the 99% tolerance interval established from the commercial reference varieties grown concurrently in the same trial and, therefore within the range of natural variability of that component in commercial soybean varieties with a history of safe consumption (Tables VII-1 and VII-4).

3) Assessment of the reproducibility of the combined-site difference of ADF across the individual sites showed no statistically significant differences at any of the five individual sites.

4) The level of ADF was within the natural variability observed for commercial soybean varieties as published in the scientific literature and available in the ILSI Crop Composition Database (ILSI, 2009; Ridley et al., 2004).

In summary, statistical analyses found no consistent differences across sites in the levels of nutrient components in forage from MON 87708 and the conventional control. Thus, a comprehensive evaluation of nutrient components in forage supports the conclusion that MON 87708 is compositionally equivalent to conventional soybean.

Table VII-1. Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Combined-Site Analysis						
Seed Proximate (% dwt)						
Ash	5.24	5.12	2.41	0.031	4.94 - 5.69	4.74, 6.01
Carbohydrates	37.93	36.64	3.50	0.012	35.65 - 39.21	32.07, 40.08
Protein	40.86	42.41	-3.65	0.016	39.00 - 42.53	35.50, 45.19
Seed Fiber (% dwt)						
Acid Detergent Fiber	13.55	12.86	5.30	0.009	12.45 - 15.57	10.06, 18.04
Crude Fiber	8.29	7.37	12.37	<0.001	6.23 - 9.65	5.76, 10.76
Neutral Detergent Fiber	15.29	14.34	6.63	0.028	13.11 - 17.83	11.36, 19.38
Seed Amino Acid (% dwt)						
Arginine	3.30	3.58	-7.91	0.006	3.09 - 3.50	2.55, 3.83

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Combined-Site Analysis						
Seed Amino Acid (% dwt)						
Aspartic Acid	4.63	4.78	-3.18	0.016	4.44 - 4.80	4.04, 5.13
Cystine	0.61	0.59	3.01	<0.001	0.58 - 0.63	0.50, 0.68
Glutamic Acid	7.38	7.69	-4.03	0.010	7.05 - 7.73	6.28, 8.30
Glycine	1.76	1.81	-2.65	0.020	1.67 - 1.83	1.53, 1.92
Histidine	1.06	1.09	-3.07	0.017	1.02 - 1.10	0.93, 1.16
Isoleucine	1.88	1.95	-3.58	0.006	1.75 - 1.97	1.65, 2.06
Leucine	3.06	3.17	-3.37	0.008	2.93 - 3.19	2.72, 3.39
Phenylalanine	2.06	2.13	-3.33	0.034	1.92 - 2.18	1.80, 2.30

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87708 minus Control) Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Combined-Site Analysis						
Seed Amino Acid (% dwt)						
Proline	1.99	2.05	-3.24	0.017	1.90 - 2.09	1.65, 2.26
Tyrosine	1.37	1.42	-3.47	0.048	1.28 - 1.46	1.24, 1.50
Valine	1.98	2.06	-3.89	0.006	1.82 - 2.09	1.72, 2.20
Seed Fatty Acid (% Total FA)						
16:0 Palmitic	11.59	11.33	2.29	0.002	11.25 - 12.16	8.44, 12.56
18:1 Oleic	19.20	20.91	-8.19	<0.001	17.85 - 19.94	15.73, 27.19
18:2 Linoleic	54.40	53.59	1.51	0.010	53.42 - 55.67	48.61, 59.37
18:3 Linolenic	10.12	9.49	6.65	<0.001	8.99 - 10.88	6.01, 12.58
22:0 Behenic	0.27	0.28	-4.70	0.001	0.25 - 0.29	0.24, 0.40

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control)			
			Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Combined-Site Analysis						
Seed Vitamin (mg/100g dwt)						
Vitamin E	1.41	1.23	15.13	0.001	1.08 - 2.17	0, 3.49
Seed Anti-nutrient (% dwt)						
Phytic Acid	1.30	1.39	-6.14	0.043	1.08 - 1.51	0.77, 1.91
Raffinose	0.43	0.47	-7.73	0.045	0.32 - 0.59	0.13, 0.70
Stachyose	3.36	3.62	-7.24	0.011	3.07 - 4.02	2.30, 4.07
Seed Isoflavone (µg/g dwt)						
Daidzein	1494.97	1340.71	11.51	0.046	899.83 - 2305.26	0, 2271.38
Forage Fiber (% dwt)						
Acid Detergent Fiber	30.58	27.69	10.45	0.021	23.30 - 45.11	16.54, 41.80

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Five Individual Sites						
Seed Fatty Acid (% Total FA)						
18:1 Oleic Site IARL	19.38	21.67	-10.58	0.001	19.07 - 19.73	15.73, 27.19
18:1 Oleic Site ILCY	19.74	21.57	-8.46	0.011	19.44 - 19.94	15.73, 27.19
18:1 Oleic Site ILWY	19.52	21.14	-7.66	0.010	19.34 - 19.64	15.73, 27.19
18:1 Oleic Site INRC	18.78	20.19	-6.96	<0.001	18.58 - 18.95	15.73, 27.19
18:1 Oleic Site PAHM	18.58	20.01	-7.13	0.015	17.85 - 19.42	15.73, 27.19
18:3 Linolenic Site IARL	10.64	10.04	5.94	0.033	10.58 - 10.74	6.01, 12.58
18:3 Linolenic Site ILCY	9.07	8.58	5.78	0.007	8.99 - 9.16	6.01, 12.58
18:3 Linolenic Site ILWY	10.54	10.05	4.92	0.026	10.51 - 10.59	6.01, 12.58

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Five Individual Sites						
Seed Fatty Acid (% Total FA)						
18:3 Linolenic Site INRC	10.03	9.31	7.65	<0.001	9.89 - 10.10	6.01, 12.58
18:3 Linolenic Site PAHM	10.33	9.47	9.02	0.006	9.91 - 10.88	6.01, 12.58
Statistically Significant Differences Observed in Four Individual Sites						
Seed Vitamin (mg/100g dwt)						
Vitamin E Site IARL	1.15	0.94	22.25	0.033	1.10 - 1.22	0, 3.49
Vitamin E Site ILCY	2.13	1.86	14.43	0.038	2.10 - 2.17	0, 3.49
Vitamin E Site ILWY	1.18	0.94	24.64	0.011	1.08 - 1.26	0, 3.49
Vitamin E Site PAHM	1.32	1.23	7.90	0.010	1.21 - 1.54	0, 3.49
Statistically Significant Differences Observed in Three Individual Sites						
Seed Proximate (% dwt)						
Protein Site ILCY	40.17	41.72	-3.72	0.047	39.44 - 40.96	35.50, 45.19

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Three Individual Sites						
Seed Proximate (% dwt)						
Protein Site ILWY	40.88	41.99	-2.64	0.042	40.56 - 41.37	35.50, 45.19
Protein Site PAHM	40.25	43.69	-7.86	0.002	39.00 - 41.05	35.50, 45.19
Seed Amino Acid (% dwt)						
Arginine Site ILWY	3.30	3.57	-7.58	0.002	3.24 - 3.33	2.55, 3.83
Arginine Site INRC	3.44	3.72	-7.37	0.011	3.39 - 3.50	2.55, 3.83
Arginine Site PAHM	3.25	3.88	-16.13	0.001	3.09 - 3.36	2.55, 3.83
Glutamic Acid Site ILCY	7.43	7.61	-2.38	0.032	7.27 - 7.54	6.28, 8.30
Glutamic Acid Site ILWY	7.29	7.51	-2.86	0.002	7.20 - 7.35	6.28, 8.30
Glutamic Acid Site PAHM	7.28	8.00	-9.08	0.003	7.06 - 7.40	6.28, 8.30

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Three Individual Sites						
Seed Amino Acid (% dwt)						
Histidine Site ILCY	1.06	1.08	-1.84	0.022	1.04 - 1.07	0.93, 1.16
Histidine Site ILWY	1.05	1.07	-1.62	0.019	1.05 - 1.05	0.93, 1.16
Histidine Site PAHM	1.05	1.13	-7.52	0.002	1.02 - 1.06	0.93, 1.16
Isoleucine Site ILCY	1.89	1.97	-3.98	0.010	1.87 - 1.93	1.65, 2.06
Isoleucine Site ILWY	1.87	1.90	-1.22	0.004	1.85 - 1.89	1.65, 2.06
Isoleucine Site PAHM	1.85	2.00	-7.59	0.014	1.79 - 1.90	1.65, 2.06
Leucine Site ILCY	3.09	3.17	-2.42	0.002	3.04 - 3.14	2.72, 3.39
Leucine Site ILWY	3.02	3.10	-2.49	<0.001	3.00 - 3.04	2.72, 3.39

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Three Individual Sites						
Seed Amino Acid (% dwt)						
Leucine Site PAHM	3.03	3.28	-7.42	0.002	2.96 - 3.09	2.72, 3.39
Seed Fatty Acid (% Total FA)						
22:0 Behenic Site IARL	0.26	0.28	-5.49	0.022	0.25 - 0.27	0.24, 0.40
22:0 Behenic Site ILWY	0.26	0.28	-6.67	0.008	0.26 - 0.27	0.24, 0.40
22:0 Behenic Site INRC	0.28	0.29	-4.85	0.038	0.27 - 0.29	0.24, 0.40
Statistically Significant Differences Observed in Two Individual Sites						
Seed Proximate						
Moisture (% fwt) Site ILWY	6.96	6.16	12.99	0.022	6.80 - 7.17	4.27, 9.58
Moisture (% fwt) Site PAHM	7.84	10.50	-25.30	<0.001	7.38 - 8.47	4.27, 9.58
Seed Amino Acid (% dwt)						
Aspartic Acid Site ILWY	4.59	4.67	-1.90	0.011	4.55 - 4.61	4.04, 5.13

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Two Individual Sites						
Seed Amino Acid (% dwt)						
Aspartic Acid Site PAHM	4.56	4.94	-7.65	0.002	4.45 - 4.63	4.04, 5.13
Phenylalanine Site ILWY	2.01	2.07	-2.95	0.046	1.96 - 2.06	1.80, 2.30
Phenylalanine Site PAHM	2.04	2.21	-7.96	0.010	2.00 - 2.07	1.80, 2.30
Proline Site ILWY	1.94	2.05	-5.09	0.020	1.93 - 1.96	1.65, 2.26
Proline Site PAHM	1.98	2.10	-5.98	0.016	1.94 - 2.00	1.65, 2.26
Threonine Site ILWY	1.52	1.55	-1.69	0.005	1.51 - 1.53	1.40, 1.69
Threonine Site PAHM	1.55	1.62	-4.23	0.029	1.52 - 1.57	1.40, 1.69
Tyrosine Site INRC	1.38	1.44	-4.49	0.044	1.35 - 1.43	1.24, 1.50

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Two Individual Sites						
Seed Amino Acid (% dwt)						
Tyrosine Site PAHM	1.35	1.49	-9.43	0.011	1.28 - 1.43	1.24, 1.50
Valine Site ILCY	1.96	2.05	-4.37	0.013	1.94 - 2.01	1.72, 2.20
Valine Site PAHM	1.95	2.13	-8.17	0.012	1.89 - 2.00	1.72, 2.20
Seed Fatty Acid (% Total FA)						
16:0 Palmitic Site IARL	11.49	11.00	4.47	0.001	11.44 - 11.54	8.44, 12.56
16:0 Palmitic Site ILWY	11.26	11.04	2.02	0.017	11.25 - 11.27	8.44, 12.56
18:2 Linoleic Site ILCY	54.54	53.26	2.40	0.021	54.45 - 54.70	48.61, 59.37
18:2 Linoleic Site INRC	54.98	54.43	1.00	0.019	54.80 - 55.14	48.61, 59.37

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Two Individual Sites						
Seed Anti-nutrient (% dwt)						
Phytic Acid Site IARL	1.36	1.53	-11.28	0.018	1.33 - 1.38	0.77, 1.91
Phytic Acid Site ILWY	1.40	1.55	-9.34	0.030	1.33 - 1.46	0.77, 1.91
Seed Isoflavone (µg/g dwt)						
Daidzein Site ILWY	1458.08	1271.60	14.67	0.004	1416.31 - 1535.98	0, 2271.38
Daidzein Site INRC	1683.50	1419.40	18.61	0.049	1593.24 - 1777.49	0, 2271.38
Glycitein Site ILWY	111.77	79.70	40.23	<0.001	109.88 - 113.86	31.24, 233.60
Glycitein Site INRC	111.51	98.42	13.31	0.016	110.91 - 112.28	31.24, 233.60
Forage Proximate (% dwt)						
Protein Site IARL	25.21	23.00	9.63	0.043	24.71 - 25.52	15.69, 26.63

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in Two Individual Sites						
Forage Proximate (% dwt)						
Protein Site INRC	21.78	23.33	-6.63	0.019	20.99 - 22.51	15.69, 26.63
Statistically Significant Differences Observed in One Individual Site						
Seed Proximate (% dwt)						
Carbohydrates Site PAHM	38.30	35.23	8.71	0.008	37.69 - 38.65	32.07, 40.08
Seed Fiber (% dwt)						
Crude Fiber Site INRC	8.06	6.89	17.03	0.009	7.76 - 8.47	5.76, 10.76
Seed Amino Acid (% dwt)						
Alanine Site PAHM	1.75	1.86	-5.81	0.010	1.74 - 1.77	1.56, 1.91
Cystine Site PAHM	0.62	0.59	4.79	0.024	0.60 - 0.63	0.50, 0.68
Glycine Site PAHM	1.73	1.86	-6.78	0.004	1.69 - 1.75	1.53, 1.92

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Soybean Component Levels for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87708 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87708 Range	Commercial Tolerance Interval[5]
Statistically Significant Differences Observed in One Individual Site						
Seed Amino Acid (% dwt)						
Lysine Site PAHM	2.60	2.75	-5.39	0.009	2.53 - 2.65	2.33, 2.84
Serine Site ILWY	1.98	2.06	-3.83	0.003	1.97 - 2.00	1.78, 2.27
Tryptophan Site ILCY	0.51	0.48	6.21	0.024	0.49 - 0.53	0.38, 0.52
Seed Anti-nutrient (% dwt)						
Lectin (H.U./mg dwt) Site ILWY	1.10	2.33	-52.88	0.045	0.59 - 1.51	0, 7.73
Stachyose Site INRC	3.14	3.46	-9.18	0.043	3.12 - 3.17	2.30, 4.07
Forage Proximate (% dwt)						
Carbohydrates Site PAHM	70.95	65.81	7.81	0.015	69.23 - 73.31	60.69, 73.46
Moisture (% fwt) Site PAHM	74.27	74.91	-0.86	0.021	73.40 - 75.40	62.08, 89.80

[1]dwt = dry weight; fwt = fresh weight; FA = fatty acid; H.U. = Hemagglutinating Units.
[2]MON 87708 was treated with dicamba.
[3]Mean = least-square mean.
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table VII-2. Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	5.24 (0.067) (4.94 - 5.69)	5.12 (0.067) (4.73 - 5.47)	0.12 (0.055) (-0.28 - 0.45)	0.011, 0.24	0.031	4.74, 6.01 (4.93 - 5.88)
Carbohydrates	37.93 (0.50) (35.65 - 39.21)	36.64 (0.50) (34.11 - 38.45)	1.28 (0.40) (-0.38 - 4.07)	0.36, 2.20	0.012	32.07, 40.08 (33.82 - 39.26)
Moisture (% fwt)	6.88 (0.65) (5.17 - 8.47)	7.14 (0.65) (5.79 - 10.60)	-0.26 (0.52) (-3.12 - 1.43)	-1.46, 0.94	0.629	4.27, 9.58 (5.50 - 9.23)
Protein	40.86 (0.39) (39.00 - 42.53)	42.41 (0.39) (40.69 - 43.85)	-1.55 (0.51) (-4.84 - 0.088)	-2.73, -0.37	0.016	35.50, 45.19 (37.06 - 43.42)
Total Fat	15.97 (0.59) (14.00 - 18.56)	15.84 (0.59) (14.40 - 18.39)	0.13 (0.31) (-1.90 - 2.37)	-0.58, 0.84	0.691	12.33, 24.10 (15.47 - 21.34)
Fiber (% dwt)						
Acid Detergent Fiber	13.55 (0.40) (12.45 - 15.57)	12.86 (0.40) (11.62 - 14.57)	0.68 (0.25) (-0.71 - 2.13)	0.18, 1.19	0.009	10.06, 18.04 (12.07 - 17.46)

Table VII-2 (continued). Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Crude Fiber	8.29 (0.26) (6.23 - 9.65)	7.37 (0.26) (6.05 - 8.64)	0.91 (0.26) (-0.34 - 2.67)	0.40, 1.43	<0.001	5.76, 10.76 (6.35 - 11.31)
Neutral Detergent Fiber	15.29 (0.59) (13.11 - 17.83)	14.34 (0.59) (11.81 - 17.99)	0.95 (0.41) (-1.31 - 4.57)	0.11, 1.79	0.028	11.36, 19.38 (11.66 - 19.45)
Amino Acid (% dwt)						
Alanine	1.76 (0.018) (1.66 - 1.83)	1.80 (0.018) (1.69 - 1.90)	-0.037 (0.017) (-0.16 - 0.042)	-0.075, 0.0018	0.059	1.56, 1.91 (1.59 - 1.86)
Arginine	3.30 (0.069) (3.09 - 3.50)	3.58 (0.069) (3.19 - 3.93)	-0.28 (0.078) (-0.83 - 0.0059)	-0.46, -0.10	0.006	2.55, 3.83 (2.88 - 3.74)
Aspartic Acid	4.63 (0.044) (4.44 - 4.80)	4.78 (0.044) (4.46 - 5.01)	-0.15 (0.050) (-0.56 - 0.12)	-0.27, -0.037	0.016	4.04, 5.13 (4.22 - 4.94)
Cystine	0.61 (0.0049) (0.58 - 0.63)	0.59 (0.0049) (0.56 - 0.62)	0.018 (0.0046) (-0.0071 - 0.053)	0.0085, 0.027	<0.001	0.50, 0.68 (0.53 - 0.66)

Table VII-2 (continued). Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Glutamic Acid	7.38 (0.085) (7.05 - 7.73)	7.69 (0.085) (7.12 - 8.14)	-0.31 (0.093) (-1.09 - 0.17)	-0.53, -0.095	0.010	6.28, 8.30 (6.69 - 7.92)
Glycine	1.76 (0.016) (1.67 - 1.83)	1.81 (0.016) (1.70 - 1.89)	-0.048 (0.017) (-0.20 - 0.042)	-0.086, -0.0096	0.020	1.53, 1.92 (1.58 - 1.84)
Histidine	1.06 (0.0095) (1.02 - 1.10)	1.09 (0.0095) (1.02 - 1.14)	-0.033 (0.011) (-0.12 - 0.031)	-0.059, -0.0076	0.017	0.93, 1.16 (0.95 - 1.13)
Isoleucine	1.88 (0.019) (1.75 - 1.97)	1.95 (0.019) (1.79 - 2.04)	-0.070 (0.019) (-0.24 - 0.11)	-0.11, -0.026	0.006	1.65, 2.06 (1.68 - 2.02)
Leucine	3.06 (0.029) (2.93 - 3.19)	3.17 (0.029) (2.96 - 3.32)	-0.11 (0.031) (-0.36 - 0.072)	-0.18, -0.035	0.008	2.72, 3.39 (2.80 - 3.27)
Lysine	2.64 (0.019) (2.53 - 2.71)	2.68 (0.019) (2.54 - 2.77)	-0.041 (0.023) (-0.23 - 0.090)	-0.094, 0.012	0.110	2.33, 2.84 (2.38 - 2.74)

Table VII-2 (continued). Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Methionine	0.58 (0.0053) (0.53 - 0.60)	0.58 (0.0053) (0.53 - 0.60)	0.00012 (0.0062) (-0.039 - 0.071)	-0.013, 0.013	0.985	0.50, 0.64 (0.52 - 0.63)
Phenylalanine	2.06 (0.028) (1.92 - 2.18)	2.13 (0.028) (1.95 - 2.27)	-0.071 (0.028) (-0.27 - 0.048)	-0.13, -0.0067	0.034	1.80, 2.30 (1.85 - 2.21)
Proline	1.99 (0.021) (1.90 - 2.09)	2.05 (0.021) (1.89 - 2.13)	-0.067 (0.022) (-0.17 - 0.065)	-0.12, -0.015	0.017	1.65, 2.26 (1.74 - 2.16)
Serine	2.04 (0.023) (1.92 - 2.12)	2.09 (0.023) (1.95 - 2.21)	-0.048 (0.026) (-0.19 - 0.054)	-0.11, 0.013	0.105	1.78, 2.27 (1.90 - 2.18)
Threonine	1.56 (0.015) (1.48 - 1.62)	1.58 (0.015) (1.51 - 1.64)	-0.023 (0.015) (-0.10 - 0.052)	-0.058, 0.012	0.169	1.40, 1.69 (1.47 - 1.64)
Tryptophan	0.47 (0.0085) (0.44 - 0.53)	0.46 (0.0085) (0.43 - 0.50)	0.0070 (0.0097) (-0.035 - 0.064)	-0.015, 0.029	0.494	0.38, 0.52 (0.39 - 0.50)

Table VII-2 (continued). Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Tyrosine	1.37 (0.018) (1.28 - 1.46)	1.42 (0.018) (1.34 - 1.52)	-0.049 (0.021) (-0.20 - 0.078)	-0.098, -0.00046	0.048	1.24, 1.50 (1.26 - 1.49)
Valine	1.98 (0.020) (1.82 - 2.09)	2.06 (0.020) (1.90 - 2.17)	-0.080 (0.022) (-0.27 - 0.13)	-0.13, -0.030	0.006	1.72, 2.20 (1.73 - 2.13)
Fatty Acid (% Total FA)						
16:0 Palmitic	11.59 (0.16) (11.25 - 12.16)	11.33 (0.16) (10.92 - 12.08)	0.26 (0.060) (-0.15 - 0.62)	0.12, 0.40	0.002	8.44, 12.56 (9.40 - 11.54)
18:0 Stearic	4.06 (0.10) (3.60 - 4.40)	4.04 (0.10) (3.67 - 4.31)	0.028 (0.049) (-0.19 - 0.42)	-0.085, 0.14	0.584	2.90, 5.19 (3.24 - 4.67)
18:1 Oleic	19.20 (0.30) (17.85 - 19.94)	20.91 (0.30) (19.60 - 22.44)	-1.71 (0.19) (-2.71 - -0.90)	-2.15, -1.27	<0.001	15.73, 27.19 (17.88 - 25.31)
18:2 Linoleic	54.40 (0.37) (53.42 - 55.67)	53.59 (0.37) (52.33 - 54.99)	0.81 (0.24) (-0.59 - 1.68)	0.25, 1.37	0.010	48.61, 59.37 (50.95 - 56.68)

Table VII-2 (continued). Statistical Summary of Combined-Site Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
18:3 Linolenic	10.12 (0.27) (8.99 - 10.88)	9.49 (0.27) (8.42 - 10.14)	0.63 (0.072) (0.36 - 1.20)	0.46, 0.80	<0.001	6.01, 12.58 (7.43 - 11.37)
20:0 Arachidic	0.26 (0.0052) (0.23 - 0.27)	0.26 (0.0052) (0.24 - 0.27)	-0.0012 (0.0031) (-0.013 - 0.020)	-0.0082, 0.0059	0.707	0.19, 0.34 (0.20 - 0.30)
20:1 Eicosenoic	0.093 (0.017) (0.069 - 0.16)	0.090 (0.017) (0.068 - 0.17)	0.0029 (0.0042) (-0.010 - 0.050)	-0.0056, 0.011	0.495	0.022, 0.24 (0.065 - 0.17)
22:0 Behenic	0.27 (0.0038) (0.25 - 0.29)	0.28 (0.0038) (0.27 - 0.30)	-0.013 (0.0029) (-0.023 - -0.0024)	-0.020, -0.0066	0.001	0.24, 0.40 (0.28 - 0.36)
Vitamin (mg/100g dwt)						
Vitamin E	1.41 (0.18) (1.08 - 2.17)	1.23 (0.18) (0.89 - 2.11)	0.19 (0.038) (0.018 - 0.42)	0.098, 0.27	0.001	0, 3.49 (0.69 - 2.91)

[1]dwt = dry weight; fwt = fresh weight; FA = fatty acid.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table VII-3. Statistical Summary of Combined-Site Soybean Seed Anti-Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient						
Lectin (H.U./mg dwt)	3.17 (0.76) (0.59 - 10.27)	3.16 (0.76) (0.46 - 10.38)	0.013 (0.67) (-4.27 - 8.13)	-1.54, 1.57	0.984	0, 7.73 (0.68 - 8.34)
Phytic Acid (% dwt)	1.30 (0.071) (1.08 - 1.51)	1.39 (0.071) (1.09 - 1.62)	-0.085 (0.035) (-0.29 - 0.15)	-0.17, -0.0034	0.043	0.77, 1.91 (1.00 - 1.64)
Raffinose (% dwt)	0.43 (0.038) (0.32 - 0.59)	0.47 (0.038) (0.36 - 0.60)	-0.036 (0.018) (-0.24 - 0.069)	-0.072, -0.00077	0.045	0.13, 0.70 (0.26 - 0.59)
Stachyose (% dwt)	3.36 (0.078) (3.07 - 4.02)	3.62 (0.078) (3.07 - 4.15)	-0.26 (0.099) (-1.00 - 0.40)	-0.46, -0.062	0.011	2.30, 4.07 (2.50 - 3.94)
Trypsin Inhibitor (TIU/mg dwt)	32.27 (1.40) (26.09 - 39.27)	30.37 (1.40) (25.22 - 34.22)	1.90 (1.79) (-4.76 - 8.72)	-2.23, 6.04	0.319	22.05, 41.12 (22.81 - 44.56)
Isoflavone (µg/g dwt)						
Daidzein	1494.97 (155.94) (899.83 - 2305.26)	1340.71 (155.94) (762.49 - 1729.91)	154.26 (65.62) (-258.27 - 795.19)	2.95, 305.57	0.046	0, 2271.38 (451.33 - 2033.05)

Table VII-3 (continued). Statistical Summary of Combined-Site Soybean Seed Anti-Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Isoflavone (µg/g dwt)						
Genistein	967.01 (90.36) (594.13 - 1496.78)	886.57 (90.36) (588.17 - 1162.01)	80.44 (41.86) (-185.98 - 513.56)	-4.30, 165.19	0.062	78.36, 1869.48 (533.88 - 1726.03)
Glycitein	108.01 (5.24) (77.67 - 119.09)	95.85 (5.24) (68.68 - 122.09)	12.16 (6.91) (-43.86 - 50.41)	-3.77, 28.09	0.116	31.24, 233.60 (73.61 - 231.75)

[1]dwt = dry weight; H.U. = Hemagglutinating Units; TIU = Trypsin Inhibitor Units.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table VII-4. Statistical Summary of Combined-Site Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	7.29 (0.54) (5.94 - 9.65)	7.39 (0.54) (6.10 - 10.46)	-0.10 (0.27) (-0.89 - 1.56)	-0.71, 0.51	0.712	3.36, 10.84 (5.20 - 9.81)
Carbohydrates	66.48 (1.03) (62.21 - 73.31)	65.66 (1.04) (62.91 - 67.94)	0.83 (0.96) (-3.95 - 6.90)	-1.40, 3.05	0.414	60.69, 73.46 (62.73 - 71.72)
Moisture (% fwt)	75.63 (1.82) (72.40 - 82.80)	75.55 (1.82) (71.60 - 82.70)	0.081 (0.27) (-1.40 - 1.30)	-0.55, 0.71	0.775	62.08, 89.80 (70.40 - 84.10)
Protein	21.52 (0.95) (15.23 - 25.52)	22.32 (0.95) (20.88 - 24.11)	-0.80 (0.80) (-6.26 - 2.75)	-2.67, 1.07	0.350	15.69, 26.63 (18.50 - 25.86)
Total Fat	4.67 (0.66) (2.00 - 7.34)	4.64 (0.66) (2.01 - 6.72)	0.032 (0.26) (-0.68 - 1.96)	-0.57, 0.63	0.904	0, 10.04 (1.57 - 7.99)
Fiber (% dwt)						
Acid Detergent Fiber	30.58 (1.79) (23.30 - 45.11)	27.69 (1.80) (21.79 - 38.15)	2.89 (1.19) (-4.78 - 16.24)	0.45, 5.34	0.021	16.54, 41.80 (20.98 - 39.23)

Table VII-4 (continued). Statistical Summary of Combined-Site Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Neutral Detergent Fiber	29.63 (1.68) (24.21 - 38.51)	30.49 (1.70) (23.66 - 39.42)	-0.86 (1.22) (-8.13 - 11.03)	-3.65, 1.94	0.503	20.28, 44.03 (24.81 - 42.80)

[1]dwt = dry weight; fwt = fresh weight.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

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

Seed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Seed Nutrients		
Proximates (% dwt)		
Ash	4.61 – 6.32[a]; 4.32 – 5.88[b]	3.89 – 6.99
Carbohydrates by calculation	32.75 – 40.98[a]; 29.88 – 43.48[b]	29.6 – 50.2
Moisture (% fwt)	6.24 – 12.10[a]; 5.44 – 11.70[b]	4.7 – 34.4
Protein	34.78 – 43.35[a]; 32.29 – 42.66[b]	33.19 – 45.48
Total Fat	14.40 – 20.91[a]; 15.10 – 23.56[b]; 15.5[c] – 24.7[c]	8.10 – 23.56
Fiber (% dwt)		
Acid Detergent Fiber	9.22 – 26.26[a]; 11.81 – 19.45[b]	7.81 – 18.61
Neutral Detergent Fiber	10.79 – 23.90[a]; 13.32 – 23.57[b]	8.53 – 21.25
Amino Acids (% dwt)		
Alanine	1.62 – 1.89[a]; 1.43 – 1.93[b]	1.51 – 2.10
Arginine	2.57 – 3.34[a]; 2.15 – 3.05[b]	2.29 – 3.40
Aspartic acid	4.16 – 5.02[a]; 4.01 – 5.72[b]	3.81 – 5.12
Cystine/Cysteine	0.52 – 0.69[a]; 0.41 – 0.71[b]	0.37 – 0.81
Glutamic acid	6.52 – 8.19[a]; 5.49 – 8.72[b]	5.84 – 8.20
Glycine	1.59 – 1.90[a]; 1.41 – 1.99[b]	1.46 – 2.00
Histidine	0.96 – 1.13[a]; 0.86 – 1.24[b]	0.88 – 1.18
Isoleucine	1.59 – 2.00[a]; 1.41 – 2.02[b]	1.54 – 2.08
Leucine	2.79 – 3.42[a]; 2.39 – 3.32[b]	2.59 – 3.62
Lysine	2.36 – 2.77[a]; 2.19 – 3.15[b]	2.29 – 2.84
Methionine	0.45 – 0.63[a]; 0.39 – 0.65[b]	0.43 – 0.68
Phenylalanine	1.82 – 2.29[a]; 1.62 – 2.44[b]	1.63 – 2.35
Proline	1.83 – 2.23[a]; 1.63 – 2.25[b]	1.69 – 2.28
Serine	1.95 – 2.42[a]; 1.51 – 2.30[b]	1.11 – 2.48
Threonine	1.44 – 1.71[a]; 1.23 – 1.74[b]	1.14 – 1.86
Tryptophan	0.30 – 0.48[a]; 0.41 – 0.56[b]	0.36 – 0.50
Tyrosine	1.27 – 1.53[a]; 0.74 – 1.31[b]	1.02 – 1.61
Valine	1.68 – 2.11[a]; 1.50 – 2.13[b]	1.60 – 2.20
Fatty Acids (% total FA)		
8:0 Caprylic	not available	0.148 – 0.148
10:0 Capric	0.15 – 0.27[b]	not available
12:0 Lauric	not available	0.082 – 0.132
14:0 Myristic	0.063 – 0.11[b]	0.071 – 0.238
14:1 Myristoleic	not available	0.121 – 0.125
15:0 Pentadecanoic	not available	not available
15:1 Pentadecenoic	not available	not available
16:0 Palmitic	9.80 – 12.63[b]	9.55 – 15.77
16:1 Palmitoleic	0.055 – 0.14[b]	0.086 – 0.194
17:0 Heptadecanoic	0.076 – 0.13[b]	0.085 – 0.146
17:1 Heptadecenoic	0.019 – 0.064[b]	0.073 – 0.087
18:0 Stearic	3.21 – 5.63[b]	2.70 – 5.88
18:1 Oleic	16.69 – 35.16[b]	14.3 – 32.2
18:2 Linoleic	44.17 – 57.72[b]	42.3 – 58.8
18:3 Gamma Linolenic	not available	not available
18:3 Linolenic	4.27 – 9.90[b]	3.00 – 12.52

Table VII-5 (continued). Literature and ILSI Database Ranges for Components in Soybean Seed and Forage

Seed Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Seed Nutrients		
Fatty Acids (% total FA)		
20:0 Arachidic	0.35 – 0.57[b]	0.163 – 0.482
20:1 Eicosenoic	0.13 – 0.30[b]	0.140 – 0.350
20:2 Eicosadienoic	0.016 – 0.071[b]	0.077 – 0.245
20:3 Eicosatrienoic	not available	not available
20:4 Arachidonic	not available	not available
22:0 Behenic	0.35 – 0.59[b]	0.277 – 0.595
22:1 Erucic	not available	not available
Vitamins (mg/100g dwt)		
Vitamin E	1.29 – 4.80[a]; 1.12 – 8.08[b]	0.19 – 6.17
Seed Anti-Nutrients		
Lectin (H.U./mg fwt)	0.45 – 10.87[a]; 0.090 – 11.18[b]	0.09 – 8.46
Trypsin Inhibitor (TIU/mg dwt)	20.79 – 59.03[a]; 18.14 – 42.51[b]	19.59 – 118.68
Phytic Acid (% dwt)	0.41 – 1.92[a]; 0.81 – 2.66[b]	0.63 – 1.96
Raffinose (% dwt)	0.26 – 0.84[a]; 0.43 – 1.85[b]	0.21 – 0.66
Stachyose (% dwt)	1.53 – 3.04[a]; 1.97 – 6.65[b]	1.21 – 3.50
Isoflavones	**(µg/g dwt)**	**(mg/kg dwt)**
Daidzein	224.03 – 1571.91[a]; 198.95 – 1458.24[b]	60.0 – 2453.5
Genistein	338.24 – 1488.89[a]; 148.06 – 1095.57[b]	144.3 – 2837.2
Glycitein	52.72 – 298.57[a]; 32.42 – 255.94[b]	15.3 – 310.4

Forage Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Forage Nutrients		
Proximate (% dwt)		
Ash	5.28 – 9.24[a]; 4.77 – 8.54[b]	6.72 – 10.78
Carbohydrates by calculation	62.25 – 72.30[a]; 60.61 – 77.26[b]	59.8 – 74.7
Moisture (% fwt)	68.50 – 78.40[a]; 62.76 – 80.20[b]	73.5 – 81.6
Protein	16.48 – 24.29[a]; 12.68 – 23.29[b]	14.38 – 24.71
Total Fat	2.65[4] – 9.87[a]; 2.96 – 7.88[b]	1.302 – 5.132
Fiber (% dwt)		
Acid Detergent Fiber	23.86 – 50.89[a]; 25.49 – 47.33[b]	not available
Neutral Detergent Fiber	19.61 – 43.70[a]; 30.96 – 54.55[b]	not available

[1] fwt = fresh weight; dwt = dry weight; H.U. = hemagglutinating unit; TIU = trypsin inhibitor unit.
[2] Literature range references: [a](Lundry et al., 2008); [b](Berman et al., 2009); [c](OECD, 2001).
[4] ILSI Crop Composition Database (2006).

VII.B. Compositional Assessment Conclusion

Analyses of nutrient and anti-nutrient levels in MON 87708 and the near isogenic conventional soybean control A3525 were conducted to assess compositional equivalence. The tissues analyzed included seed and forage harvested from plants grown at five field sites in the U.S. during the 2008 field season. The composition analysis, conducted in accordance with OECD guidelines, also included measurement of nutrients and anti-nutrients in the commercial reference varieties concurrently grown with MON 87708 to provide data on natural variability of each compositional component. All soybean plants including MON 87708, the conventional control, and the commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition, MON 87708 plots were treated at the V2-V3 growth stage with dicamba herbicide at the maximum in-crop label rate (0.5 lb a.e./acre).

For MON 87708 the combined-site analysis of both seed and forage showed no statistically significant differences between MON 87708 and conventional control for 21 (42.0%) of the 50 mean value comparisons. Of the statistically significant differences observed, one was from the forage analysis, and 28 were from the seed analysis. Nutrient component differences in seed included mean values for ash, carbohydrates by calculation, protein and 12 amino acids, five fatty acids, ADF, NDF, crude fiber, and vitamin E. In the combined-site analysis, all nutrient component differences in seed between MON 87708 and the conventional control were of small relative magnitude with respect to the conventional control and, whether increased or decreased, ranged from 1.51% to 12.37% for the three proximates, amino acids, fatty acids, and fibers, and 15.13% for vitamin E. Two of the nutrient components in the combined-site analysis (decreased levels of 18:1 oleic acid and increased levels of 18:3 linolenic acid) were also observed to be statistically different at all five individual sites, and one nutrient component (vitamin E) was observed to be increased at four of the five individual sites as in the combined-site analysis. The other combined-site differences occurred at fewer or none of the individual sites. Anti-nutrient component differences in seed were observed in mean values for phytic acid, raffinose, stachyose, and daidzein. In the combined-site analysis, all anti-nutrient component differences in seed between MON 87708 and the conventional control were of small relative magnitude, with respect to the conventional control, and ranged from a 6.14% decrease (phytic acid) to an 11.51% increase (daidzein). None of the anti-nutrient components were observed to be statistically different at more than two of the five individual sites. The only nutrient component difference in forage for the combined-site analysis was observed in ADF and its relative magnitude of difference, with respect to the conventional control, was 10.45%. No differences between MON 87708 and the conventional control ADF mean values were observed at any of the five individual sites. Mean values of MON 87708 components with statistically significant differences to the conventional control were all within the 99% tolerance interval established from the commercial reference varieties grown concurrently and at the same field sites, as well as ranges in the scientific literature and the ILSI Crop Composition Database.

In summary, a comprehensive evaluation of key nutrients and anti-nutrients in seed and key nutrients in forage supports the conclusion that soybean seed and forage produced

from MON 87708 are compositionally equivalent to that of conventional soybean and that neither the dicamba tolerance trait in MON 87708, nor the dicamba herbicide treatment, applied according to maximum in-crop label rates (including the associated dicamba residue levels) have a meaningful impact on the composition and therefore on the food and feed safety or the nutritional quality of MON 87708 compared to conventional soybean.

VIII. USE OF ANTIBIOTIC RESISTANCE MARKER GENES

The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 5, paragraphs 55 through 58.

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

No genes that encode resistance to an antibiotic marker were inserted into the soybean genome during the development of MON 87708. The backbone of the PV-GMHT4355 plasmid vector contained the *aadA* antibiotic resistant marker gene. Molecular characterization data presented in Section V demonstrate the absence of the *aadA* antibiotic resistant marker gene in MON 87708.

IX. SUMMARY OF FOOD AND 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.

IX.A. Donor Organism

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.

IX.B. Genetic Insert

As described in more detail in Section IV, MON 87708 was produced by *Agrobacterium tumefaciens*-mediated transformation of soybean with PV-GMHT4355, which is a binary vector containing two T-DNAs (Figure IV-1). T-DNA I contains the *dmo* coding sequence under the regulation of the *PC1SV* promoter, *TEV* leader, the *RbcS* targeting sequence, and the *E9* 3′ non-translated region. In addition, T-DNA I contains Left and Right Border regions. T-DNA II contains the *cp4 epsps* expression cassette. During plant transformation, both T-DNAs were inserted into the soybean genome, with the *cp4 epsps* expression cassette functioning as a selectable marker. Subsequently, conventional self-pollinated breeding methods and segregation were used to isolate those plants that contain the *dmo* expression cassette (T-DNA I) and do not contain the *cp4 epsps* expression cassette (T-DNA II), resulting in the production of marker-free MON 87708.

Molecular analyses demonstrated that MON 87708 contains a single copy of the inserted T-DNA I at a single integration locus. No T –DNA II or backbone sequences from the PV-GHMT4355 were detected in the genome of MON 87708. Data confirmed the organization and sequence of the inert and the stability of the insert over several generations.

IX.C. Safety of MON 87708 DMO

A history of safe use has been established for MON 87708 DMO (Section VI.C.). MON 87708 DMO lacks structural similarity to known allergens (Section VI.F.1.3.) or toxins (Section VI.F.2.1.) known to have adverse effects on mammals. MON 87708 DMO is present at very low levels in MON 87708 seed and will constitute a

very small portion of the total protein present in food and feed derived from MON 87708 (Section VI.F.1.2.). MON 87708 DMO is readily digestible in simulated gastric and simulated intestinal fluids (Section VI.F.1.4.), is heat labile (Section VI.F.1.5.), and shows no oral toxicity in mice (Section VI.F.2.3.). In addition, large MOEs have been demonstrated for consumption of MON 87708 DMO derived from MON 87708 for the general population and for non-nursing infants, the most highly exposed sub-population (Section VI.F.3.1.).

IX.D. Compositional Characteristics of MON 87708

Detailed compositional comparisons were presented (Section VII.A.) to assess whether levels of nutrients and anti-nutrients in seed and forage derived from MON 87708 are comparable to levels in the near isogenic conventional soybean control A3525 and several commercially available reference soybean varieties for which there is an established history of safe consumption. The analysis included proximates (ash, carbohydrates, moisture, protein, and fat), fiber, amino acids, fatty acids, vitamin E, and anti-nutrients (raffinose, stachyose, lectin, phytic acid, trypsin inhibitors, and isoflavones) in seed; and proximate (ash, carbohydrates, moisture, protein, and fat) and fiber in forage.

Compositional and nutritional comparisons were conducted using a combined-site analysis to determine statistically significant differences (5% level of significance) between MON 87708 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, the reproducibility of differences across individual sites, and whether the mean component value was 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 field trial and 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 87708. In addition, the levels of assessed components in MON 87708 were compositionally equivalent to the conventional control and within the range of variability of commercial soybean grown concurrently. These results support the overall conclusion that MON 87708 seed and forage are compositionally equivalent to conventional soybean in accordance with OECD guidelines.

IX.E. Summary of Food and Feed Safety Assessment of MON 87708

Collectively, these data and the history of safe use of soybean as a common source of processed human foods and animal feeds (Section II) support a conclusion of "no concerns" for every criterion specified in the flowcharts outlined in the FDA's Food Policy document (Figure IX-1). MON 87708 is not materially different in composition, safety, or nutrition from conventional soybean, other than the introduction of the dicamba-tolerance trait. Sales or consumption of soybean seed or processed products derived from MON 87708 would be fully consistent with the FDA's Food Policy, the

Federal Food, Drug and Cosmetic Act, and current practice for the development and introduction of new soybean varieties and biotechnology traits.

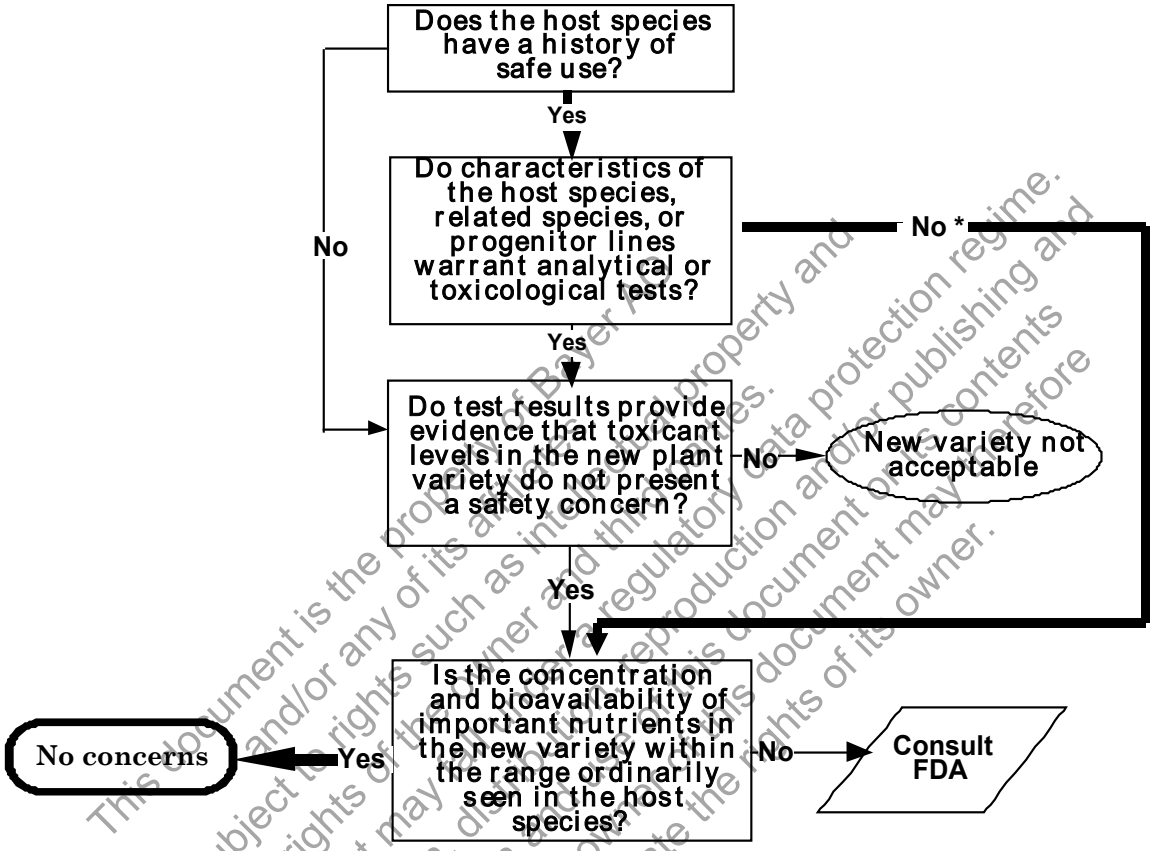


Figure IX-1. Safety Assessment of New Varieties: The Host Plant

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APPENDICES

Appendix A: Materials and Methods Used for Molecular Analyses of MON 87708

A.1. Materials

The genomic DNA used in molecular analyses was isolated from leaf tissue harvested from MON 87708 and the near isogenic conventional soybean control A3525 (seed lot: GLP-0707-18882-S, and GLP-0707-18884-S, respectively). Additional DNA extracted from leaf tissue of various MON 87708 generations was used in generational stability analyses. The conventional control has a similar genetic background as MON 87708. Plasmid vector PV-GMHT4355 (Figure IV-1) was used as a positive hybridization control in Southern blot analyses. Probe templates generated from PV-GMHT4355 were used as additional positive hybridization controls. As additional reference standards, the 1 kb DNA extension ladder and λ DNA/*Hind* III segments from Invitrogen (Carlsbad, CA) were used for size estimations on Southern blots and agarose gels. The GeneRuler™ 1 kb Plus DNA ladder from Fermentas (Hanover, MD) was used for size estimations on agarose gels for polymerase chain reaction (PCR) analyses.

A.2. Characterization of the Materials

The identity of the leaf material from MON 87708 and the conventional control was verified by event specific PCR analysis to confirm the presence or absence of the *dmo* expression cassette. The stability of the genomic DNA was confirmed in each Southern blot analysis by observation of the digested DNA sample on an ethidium bromide-stained agarose gel, and/or interpretable signals on Southern blots, and/or produced specific PCR products.

A.3. DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA from MON 87708 and the conventional control was isolated from leaf tissue. The leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. DNA was extracted 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 minutes with intermittent mixing. Twenty milliliters of chloroform was added to the samples and mixed by hand for 2-3 minutes, then centrifuged at 10,300 × g for 8-10 minutes. 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 × gravity for 5 minutes to pellet the DNA. DNA pellets were air dried, then resuspended in 300 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). All extracted DNA was stored in a 4 C refrigerator or a -20 C freezer.

A.4. Quantification of Genomic DNA

Extracted genomic DNA was quantified using a Hoefer DyNA Quant 200 Fluorometer. Molecular size marker IX (Roche, Indianapolis, Indiana) was used as the calibration standard.

A.5. Restriction Enzyme Digestion of Genomic DNA

Approximately 10 µg of genomic DNA extracted from MON 87708 and the conventional control was digested with appropriate combinations of restriction enzymes *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I (New England Biolabs, Ipswich, Massachusetts). All digests were conducted in 1 × NEBuffer 4 (New England Biolabs) plus 1 × BSA (New England Biolabs) at 37 C in a total volume of ~500 µl with ~50 units of each 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 combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I and the appropriate positive hybridization control(s) were added to these digests.

A.6. Agarose Gel Electrophoresis

Digested genomic DNA was resolved on ~0.8% (w/v) agarose gels. Individual digests of MON 87708 and the 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 the detection of small molecular weight DNA. For the insert stability analysis, individual digests of genomic DNA extracted from leaf tissue across multiple generations were loaded on the agarose gel in a single short-run format. The positive hybridization controls were only run in the short-run format.

A.7. DNA Probe Preparation for Southern Blot Analyses

Probe templates were prepared by PCR amplification from plasmid vector PV GMHT4355. Approximately 25 ng of each probe template were radiolabeled with ^{32}P-deoxycytidine triphosphate (dCTP) (6000 Ci/mmol) or ^{32}P-deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using the RadPrime DNA Labeling System (Invitrogen). Probe locations relative to the genetic elements in plasmid vector PV-GMHT4355 are depicted in Figure IV-1.

A.8. Southern Blot Analyses of Genomic DNA

Digested genomic DNA isolated from MON 87708 and the conventional control was evaluated using Southern blot analyses. The plasmid vector PV-GMHT4355 DNA digested with the enzyme combination *Aat* II/*Nde* I was added to the conventional control genomic DNA previously digested with the enzyme combination *Bsp1286* I/*Pvu* II or *Hpa* I/*Kpn* I to serve as a positive hybridization control. When multiple probes were hybridized simultaneously to one Southern blot, the appropriate probe templates generated from PV-GMHT4355 were mixed with previously digested conventional control genomic DNA to serve as additional positive hybridization controls. The

digested DNA was then separated by agarose gel electrophoresis and transferred onto a nylon membrane. Southern blots were hybridized and washed at 55, 60, or 65°C, depending on the melting temperature (Tm) of the probes. 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 in conjunction with one or two Kodak Biomax MS intensifying screen(s) in a 80°C freezer.

Table A-1. Hybridization Conditions of Utilized Probes

Probe	DNA Probe	Element Sequence Spanned by DNA Probe	Probe labeled with dNTP (^{32}P)	Hybridization Temperature (°C)
1	Backbone Probe	Backbone sequence	dCTP	60
2	Backbone Probe	Backbone sequence	dCTP	60
3	Backbone Probe	Backbone sequence	dCTP	60
4	T-DNA II Probe	T-*E9*, and CS-*cp4 epsps* (portion)	dATP	55
5	T-DNA II Probe	CS-*cp4 epsps* (portion), and TS-*CTP2* (portion)	dCTP	60
6	T-DNA II Probe	TS-*CTP2* (portion), L-*DnaK*, P-*FMV*	dATP	55
7	Backbone Probe	Backbone sequence	dCTP	60
8	T-DNA I Probe	B-Right Border, P-*PC1SV*, L-*TEV*, TS-*RbcS*	dATP	60
9	T-DNA I Probe	TS-*RbcS* (portion), CS-*dmo*, T-*E9* (portion)	dCTP	65
10	T-DNA I Probe	T-*E9*, and B-Left Border	dATP	55

A.9. DNA Sequence Analyses of the Insert

Overlapping PCR products that span the insert and adjacent 5′ and 3′ flanking DNA sequences in MON 87708 (Figure A-1) were generated. These products were sequenced to determine the nucleotide sequence of the insert in MON 87708 as well as the nucleotide sequence of the DNA flanking the 5′ and 3′ ends of the insert.

The PCR analyses were conducted using 50 ng of genomic DNA template in a 25 µl reaction volume containing a final concentration of 1 M betaine, 1 mM MgSO4, 0.8 µM of each primer, 0.2 mM of each dNTP, and 0.5 units of KOD Hot Start DNA polymerase (Novagen, Madison, Wisconsin). The amplification of Product A (Figure A-1) was performed under the following cycling conditions: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 45 seconds, 60.2°C for 45 seconds, 72°C for 5 minutes; one cycle at 72°C for 10 minutes. The amplification of Product B (Figure A-1) was performed under

the following cycling conditions: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 45 seconds, 60.8°C for 45 seconds, 72°C for 5 minutes; one cycle at 72°C for 10 minutes.

Following PCR amplification, exonuclease I (Exo; US Biochemicals, Cleveland, OH)/ shrimp alkaline phosphatase (SAP; US Biochemicals) purification of the PCR products used for sequencing was performed in a 21 µl reaction volume containing 15 µl of the PCR product and a final concentration of 0.1 units/µl of Exo and 0.1 units/µl of SAP. The reaction was incubated at 37ºC for 15 minutes, followed by 80ºC for an additional 15 minutes.

Prior to sequencing, aliquots of untreated and Exo/SAP treated PCR product were separated on 0.8% (w/v) agarose E-gels (Invitrogen) and visualized by ethidium bromide staining to verify that the products were of the expected size. The PCR products were sequenced using multiple primers including primers used for PCR amplification and primers designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry (ABI, Foster City, California).

A.10. PCR and DNA Sequence Analysis to Examine the MON 87708 Insertion Site

To examine the insertion site of conventional soybean and MON 87708, PCR analysis was performed on genomic DNA from both MON 87708 and the conventional control (Figure B 2). The primers used in this analysis were designed from the genomic DNA sequences flanking the insert in MON 87708. One primer designed from the genomic DNA sequence flanking the 5′ end of the insert was paired with a second primer located in the genomic DNA sequence flanking the 3′ end of the insert.

The PCR analysis was conducted using approximately 50 ng of genomic DNA template in a 25 µl reaction volume containing a final concentration of 1 M betaine, 1 mM MgSO4, 0.8 µM of each primer, 0.2 mM of each dNTP, and 0.5 units of KOD Hot Start DNA polymerase (Novagen). The amplification of the product was performed under the following cycling conditions: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 45 seconds, 60.2°C for 45 seconds, 72°C for 5 minutes; one cycle at 72°C for 10 minutes.

Following PCR amplification, Exo/SAP purification of the PCR products used for sequencing was performed in a 21 µl reaction volume containing 15 µl of the PCR product and a final concentration of 0.1 units/µl of Exo and 0.1 units/µl of SAP (U.S. Biochemicals). The reaction was incubated at 37ºC for 15 minutes, followed by 80ºC for an additional 15 minutes.

Prior to sequencing, aliquots of untreated and Exo/SAP treated PCR product were separated on 0.8 % (w/v) agarose E-gels (Invitrogen) and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. The PCR products were sequenced using multiple primers, including primers used for PCR amplification and primers designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye terminator chemistry.

Appendix B: Materials and Methods for Characterization of MON 87708 DMO Produced in MON 87708

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 87708. The wild-type DMO was first isolated and characterized from *Stenotrophomonas maltophilia* (Herman et al., 2005). The MON 87708 DMO protein present in MON 87708 is identical to the wild-type DMO except for an additional alanine at position two added for cloning purposes and a cysteine instead of tryptophan at position 112 (Figure B-1). The MON 87708 DMO+27 protein also present in MON 87708 is identical to the MON 87708 DMO protein except for the additional 27 amino acids at the N-terminus and the presence on the N-terminal methionine. As previously stated, this document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. Additionally, 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 some 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 (Figure B-2, Panel A).

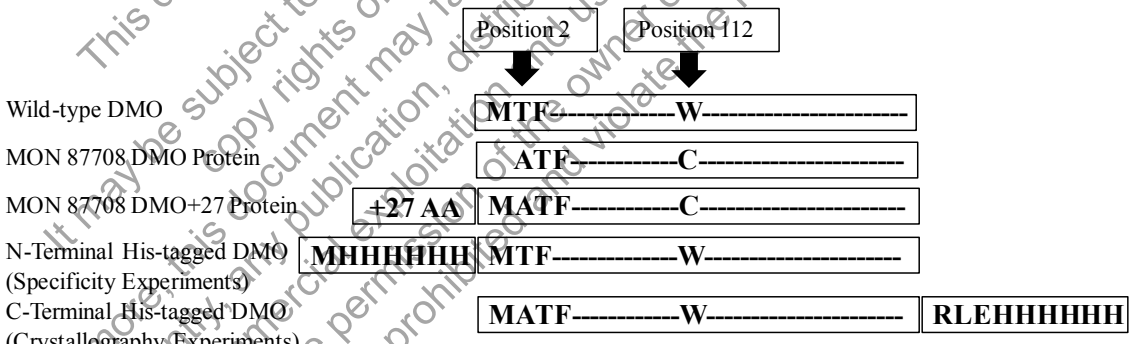

Figure B-1. Forms of DMO Protein and Their Relation to the Wild-Type DMO Protein

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). MON 87708 DMO was purified from soybean seed of MON 87708 and contained two forms of the protein; the MON 87708 DMO protein and the MON 87708 DMO+27 protein. The purified MON 87708 DMO used in specificity studies was active (Section B.3.). The N-terminal histidine-tagged DMO was produced in *E. coli* and was used for *in vitro* specificity studies. The C-terminal histidine-tagged DMO was produced in *E. coli* and was used for crystallography studies.

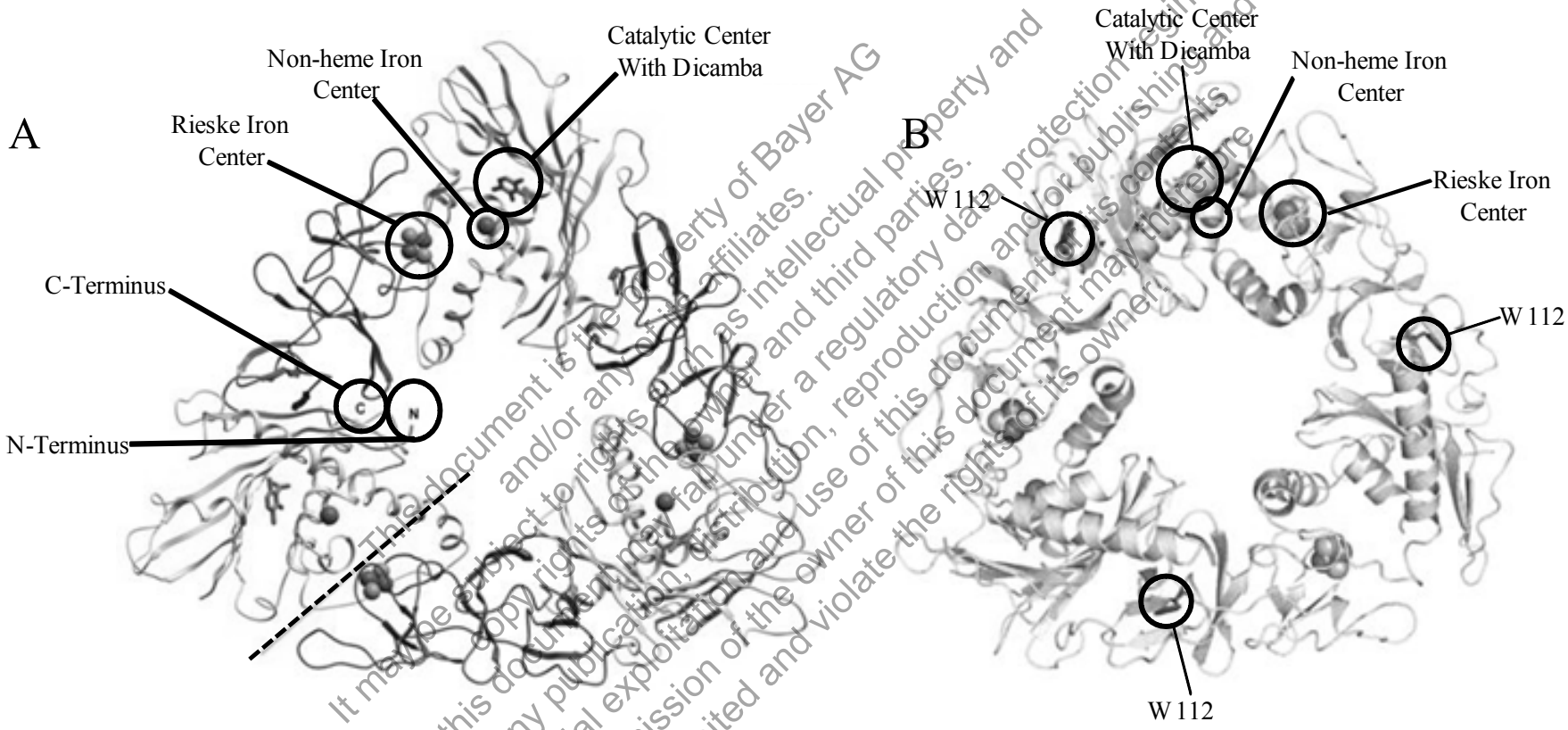

Figure B-2. Crystal Structures of C-Terminal Histidine Tagged DMO.
Structures depicting the crystal structure of the C-terminal histidine-tagged DMO as a trimer, which is the active form of DMO (D'Ordine et al., 2009). Panel A depicts various structural elements including the Rieske Iron cluster, the non-heme iron, and the catalytic sites containing dicamba. The dashed segmented line represents the interface where electron transfer takes place between the subunits at the adjoining non-heme iron site and the Rieske center (D'Ordine et al., 2009). Panel B depicts the localization of the tryptophan amino acid (W112) at position 112, which in MON 87708 DMO is a cysteine.

B.2. Substrate Specificity of MON 87708 DMO

B.2.1. Materials

Two DMO proteins were used in the endogenous substrate specificity *in vitro* experiments. Most of the testing was done with the N-terminal histidine-tagged DMO (Figure B-1) and a sub-set of these compounds were tested with MON 87708 DMO. The compounds tested and standards used in the *in vitro* experiments are listed in Table B-1.

Table B-1. Compounds Used in Specificity *In Vitro* Experiments

Manufacturer/ Retailer	Compound	Common Name	Lot/Product Number
Compounds Tested as Substrates:			
Aldrich	2-methoxybenzoic acid	*o*-anisic acid	A0230443
Chem Service	3,6-dichloro-2-methoxybenzoic acid	dicamba	341-9143
Fluka	3,5-dimethoxy-4-hydroxybenzoic acid	syringic acid	86230
Fluka	4-hydroxy-3-methoxybenzoic acid	vanillic acid	94770
Fluka	3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid	ferulic acid	46278
Sigma	3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid	sinapic acid	D7927-1G
Compound Used as Standard:			
Monsanto	3,6-dichlorosalicylic acid	DCSA	GLP-0603-16959-T

B.2.2. *In Vitro* Specificity Experiments Enzymatic Reaction Mixture Method

The reaction of the N-terminal histidine-tagged DMO and MON 87708 DMO with different compounds evaluated as potential substrates was carried out using similar reaction conditions to those described in the characterization portion of this appendix (Appendix B.3.9.). The compounds tested (Table B-1) were combined with the N-terminal histidine-tagged DMO at 200 and/or 12 µM. In addition, MON 87708 DMO was combined with dicamba or *o*-anisic acid at 200 and/or 12 µM. The concentrations tested ensured adequate reaction conditions in terms of the substrate for the detection of product formation or disappearance of substrate.

B.2.3. *In Vitro* Experiments Liquid Chromatography Separation Method

The reaction mixture was separated by Ultra Performance Liquid Chromatography (UPLC®) (Waters Corp., Milford, MA) using an ACQUITY UPLC BEH C18 Column containing 1.7 µm Bridged Ethyl Hybrid (BEH) particles and an ACQUITY BEH C18 VanGuard Pre-column. The column was heated to 40°C. The tested substrates and

potential oxidative products were monitored by ACQUITY UPLC photodiode array (PDA) with wavelength range from 200 nm to 320 nm with 1.2 nm resolution. The chromatography was performed at 0.25 ml/min and directed to the mass spectrometer following the separation. Both mobile phase A (water) and solvent B (acetonitrile) contained 0.1% v/v formic acid. Gradients used were substrate specific:

- The gradient for dicamba was run from 40 to 50% solvent B in 3 minutes, 50 to 100% solvent B in 0.1 minutes and then kept at 100% solvent B for 1 minute before returning to 40% solvent B in 0.1 minutes.

- The gradient for ferulic acid, *o*-anisic acid, sinapic acid, syringic acid, and vanillic acid were run from 0 to 100% solvent B in 4 minutes and then held at 100% solvent B for 1 minute before returning to 0% solvent B in 0.1 minutes.

A 5 µl injection of the reaction mixture was used for UPLC analysis where the disappearance of the potential substrate was monitored, and a 50 µl injection of the reaction mixture was used for UPLC analysis where formation of the potential oxidative product was monitored.

B.2.4. *In Vitro* Experiments Mass Spectrometry Detection Method

Elution from the UPLC column flowed directly to a Waters Micro Q-TOF mass spectrometer. The parameters used for the mass determination of all analytes were: negative mode, capillary voltage of 2800 V, sample cone voltage of 26 V, extraction cone of 1.5 V, source temperature of 150°C, and the desolvation temperature was 390°C. The desolvation gas flow was 500 L/hour and scan time was 0.76 seconds and inter scan delay was 0.1 sec. The m/z range used was specific to each compound and product. The m/z range for dicamba and DCSA was from 160 to 225 from 0 to 4 min. The m/z at 175, which is the fragment ion of dicamba, was used as a detection method for dicamba. This fragment ion of dicamba gave better sensitivity, than the parent ion. The m/z at 205 was used to detect DCSA. The m/z range for all other acids is from 120 to 250 within 4 minutes.

B.2.5. Results of *In Vitro* Experiments with Endogenous Soybean Compounds

The reaction of dicamba with N-terminal histidine-tagged DMO has been well characterized utilizing an *in vitro* enzymatic assay that monitors the formation of DCSA by LC-UV as well as LC-MS, which allows the detection of the product with high sensitivity. The substrate (dicamba) and oxidative product (DCSA) can be detected by UV absorbance and LC-MS after separation by UPLC. The results demonstrate that when using dicamba as a substrate, the formation of DCSA is clearly observed in the presence of the N-terminal histidine-tagged DMO (Figure B-3). Furthermore, these results demonstrate the validity of the *in vitro* enzymatic assay to determine the specificity of MON 87708 DMO and N-terminal histidine-tagged DMO.

Compounds structurally similar to dicamba and present in soybean (Table B-1) were used as potential substrates to determine if these compounds could be metabolized by MON 87708 DMO and the N-terminal histidine-tagged DMO. The compounds tested

were syringic acid, *o*-anisic acid, vanillic acid, ferulic acid, and sinapic acid. Mass spectrometric scans were taken from 120 m/z to 250 m/z to cover the range of all potential oxidation products formed by DMO. Observations were also focused on the m/z of the predicted oxidative products of: dicamba m/z 205; ferulic acid m/z 179; *o*-anisic acid m/z 137; sinapic acid m/z 209; syringic acid m/z 183; and vanillic acid m/z 153. Standard reaction conditions of dicamba with DMO were used as a positive control. Analysis of LC-MS data demonstrated that there are no additional peaks formed when reactions of each compound incubated with and without the N-terminal histidine-tagged DMO are compared (Figure B-4). No peaks were observed at the respective masses for the predicted oxidative products of each compound incubated with the N-terminal histidine-tagged DMO, indicating these compounds are not metabolized. The same reaction conditions were used with *o*-anisic acid and MON 87708 DMO. Similarly, no formation of potential oxidative product or disappearance of *o*-anisic acid was observed (Figure B-5). These results further confirm the identical function of both forms of the protein used in the specificity experiments, and confirm the specificity of DMO for dicamba as a substrate.

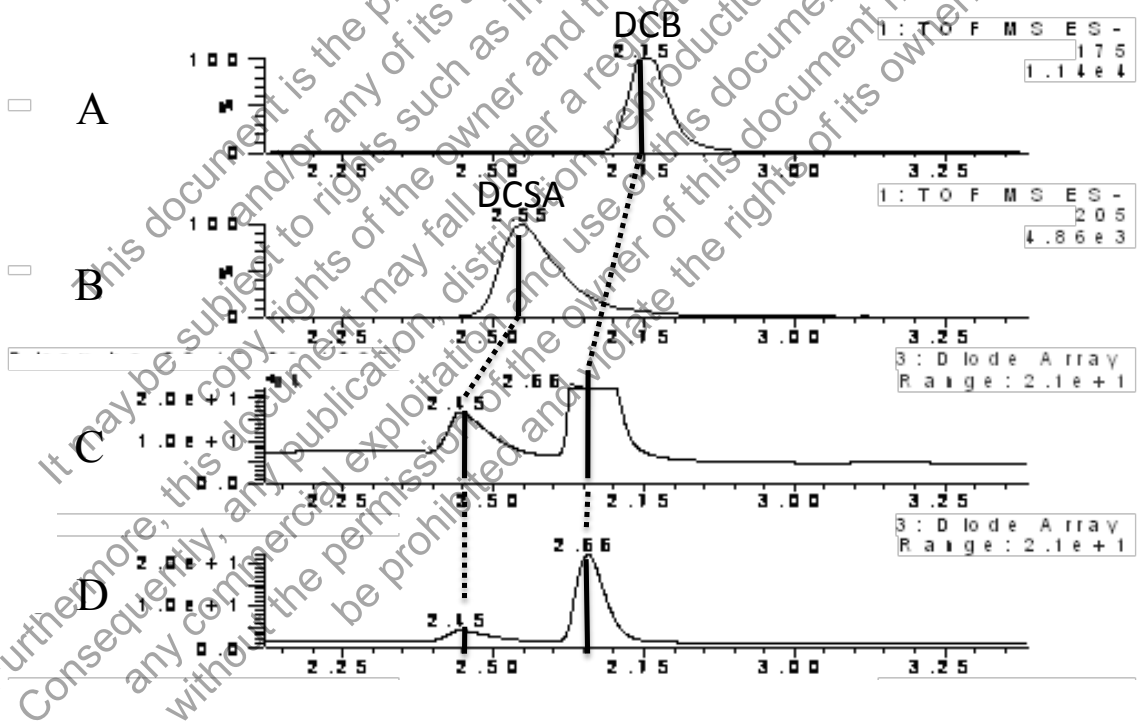

Figure B-3. UPLC Separation of Dicamba (DCB) and DCSA
Dicamba (DCB) and DCSA were separated by UPLC and detected by mass spectrometry (A and B, respectively) and UV absorbance (C and D, respectively).

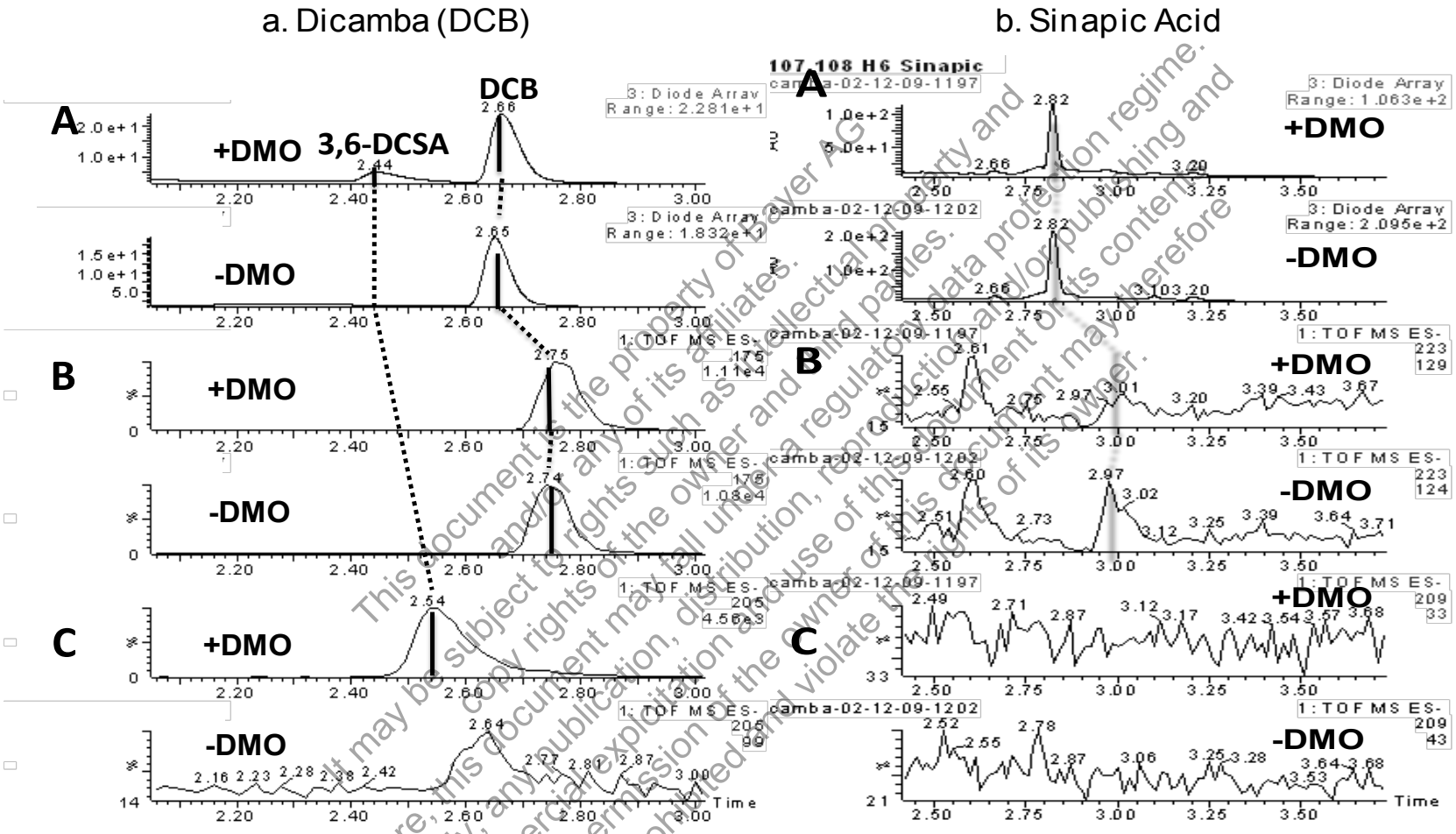

Figure B-4. DMO Conversion of Endogenous Substrates

Endogenous substrates, as well as dicamba, were incubated with the N-terminal histidine-tagged DMO, and the formation of predicted oxidative products and the disappearance of each substrate was monitored by LC-UV (top two chromatograms, A) and LC-MS (bottom chromatograms B and C). Dicamba (a) was used as a positive control. Each endogenous compound, sinapic acid(b), ferulic acid (c), *o*-anisic acid (d), syringic acid (e), and vanillic acid (f), was included in a reaction mixture made with (+DMO, upper) and without (-DMO, lower) DMO. The dotted line indicates the migration of the substrates (and DCSA in the case of dicamba) in each chromatogram as a result of the UV and MS detectors being run in series. Of the four LC-MS chromatograms; chromatograms B represents the mass scan for the intact compound, while chromatograms C represents the scan at the m/z of the most probable oxidative product.

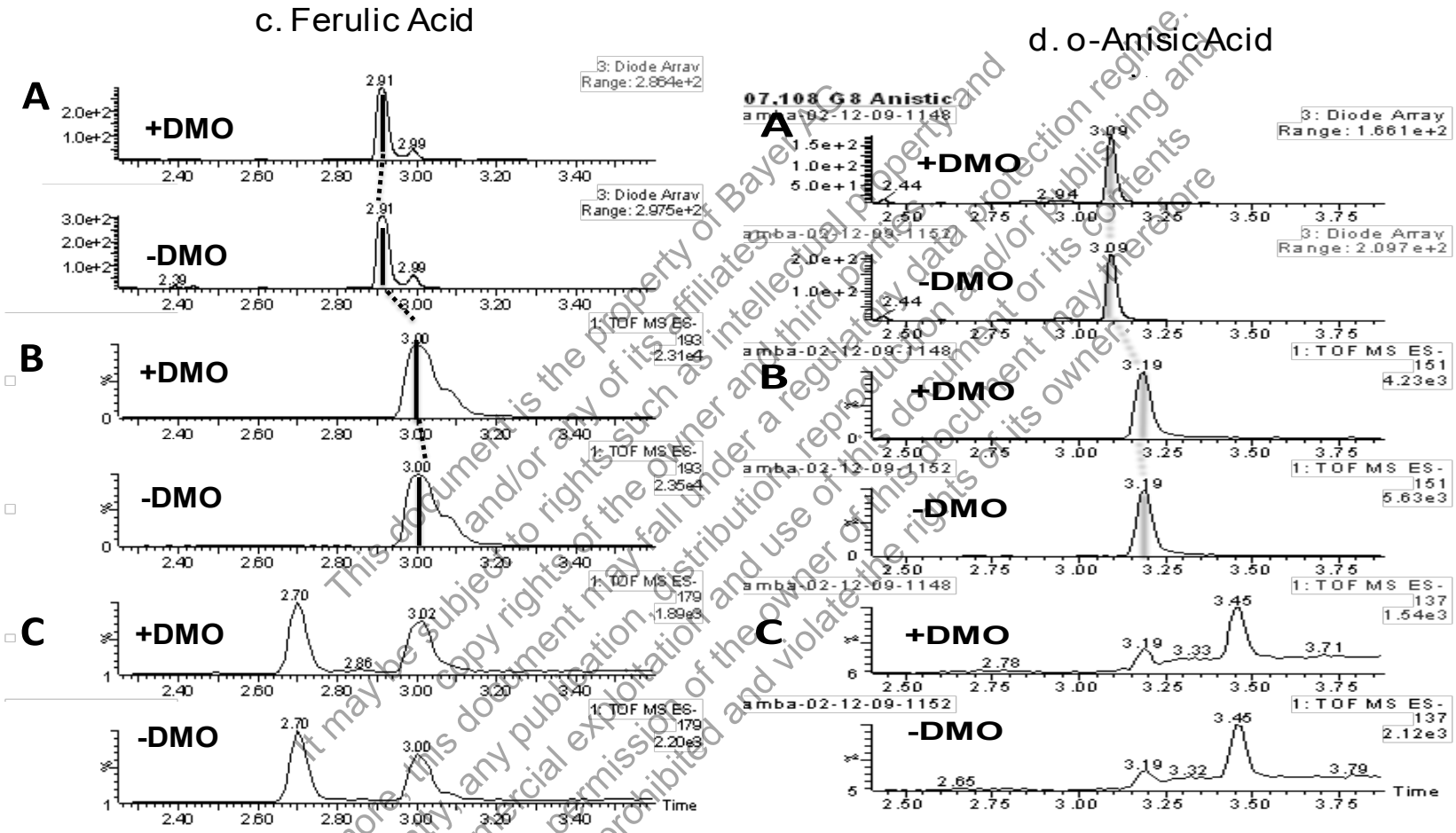

Figure B-4 (continued). DMO Conversion of Endogenous Substrates

Endogenous substrates, as well as dicamba, were incubated with the N-terminal histidine-tagged DMO, and the formation of predicted oxidative products and the disappearance of each substrate was monitored by LC-UV (top two chromatograms, A) and LC-MS (bottom chromatograms B and C). Dicamba (a) was used as a positive control. Each endogenous compound, sinapic acid(b), ferulic acid (c), *o*-anisic acid (d), syringic acid (e), and vanillic acid (f), was included in a reaction mixture made with (+DMO, upper) and without (-DMO, lower) DMO. The dotted line indicates the migration of the substrates (and DCSA in the case of dicamba) in each chromatogram as a result of the UV and MS detectors being run in series. Of the four LC-MS chromatograms; chromatograms B represents the mass scan for the intact compound, while chromatograms C represents the scan at the m/z of the most probable oxidative product.

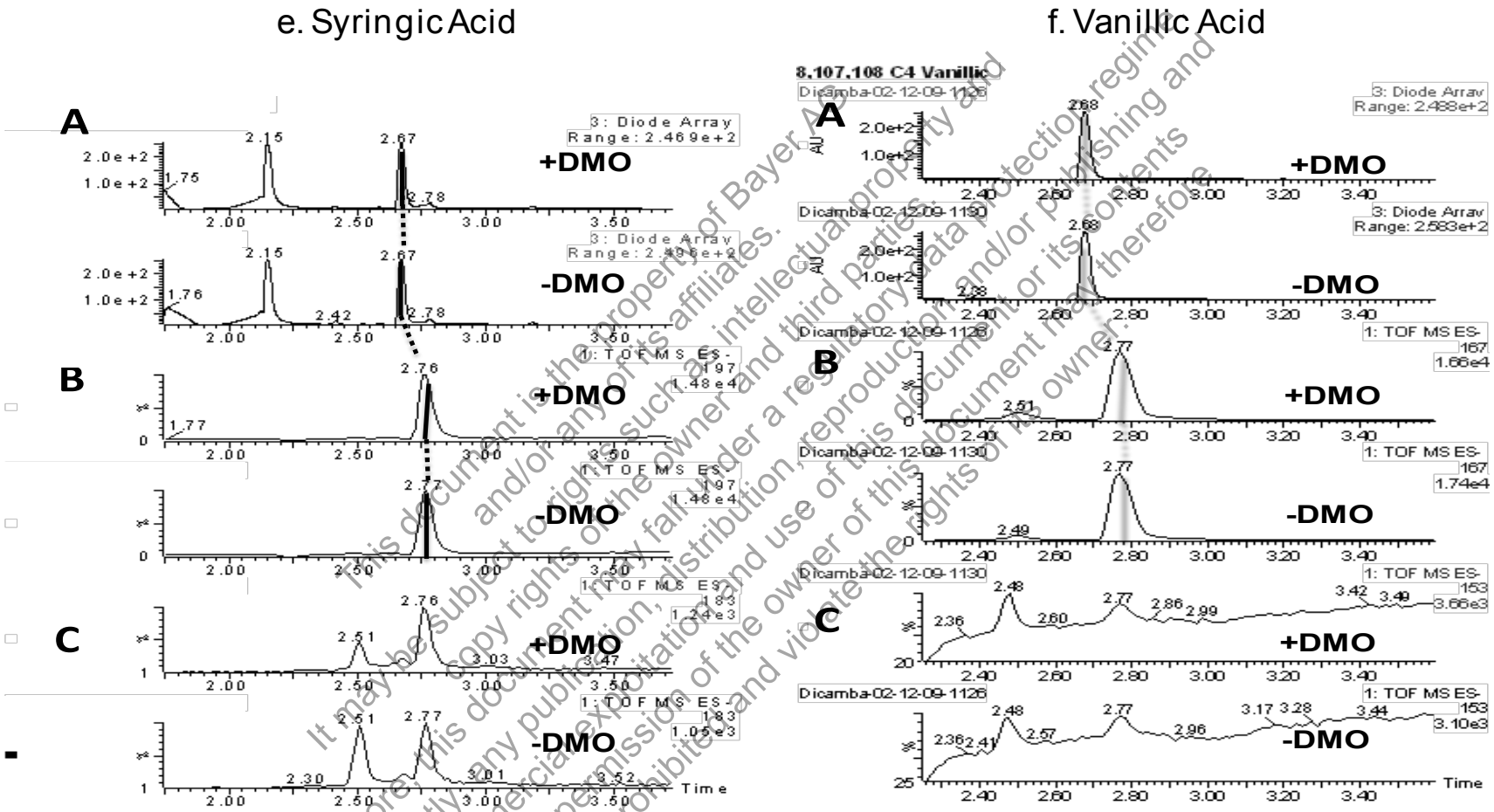

Figure B-4 (continued). DMO Conversion of Endogenous Substrates

Endogenous substrates, as well as dicamba, were incubated with the N-terminal histidine-tagged DMO, and the formation of predicted oxidative products and the disappearance of each substrate was monitored by LC-UV (top two chromatograms, A) and LC-MS (bottom chromatograms B and C). Dicamba (a) was used as a positive control. Each endogenous compound, sinapic acid(b), ferulic acid (c), *o*-anisic acid (d), syringic acid (e), and vanillic acid (f), was included in a reaction mixture made with (+DMO, upper) and without (-DMO, lower) DMO. The dotted line indicates the migration of the substrates (and DCSA in the case of dicamba) in each chromatogram as a result of the UV and MS detectors being run in series. Of the four LC-MS chromatograms; chromatograms B represents the mass scan for the intact compound, while chromatograms C represents the scan at the m/z of the most probable oxidative product.

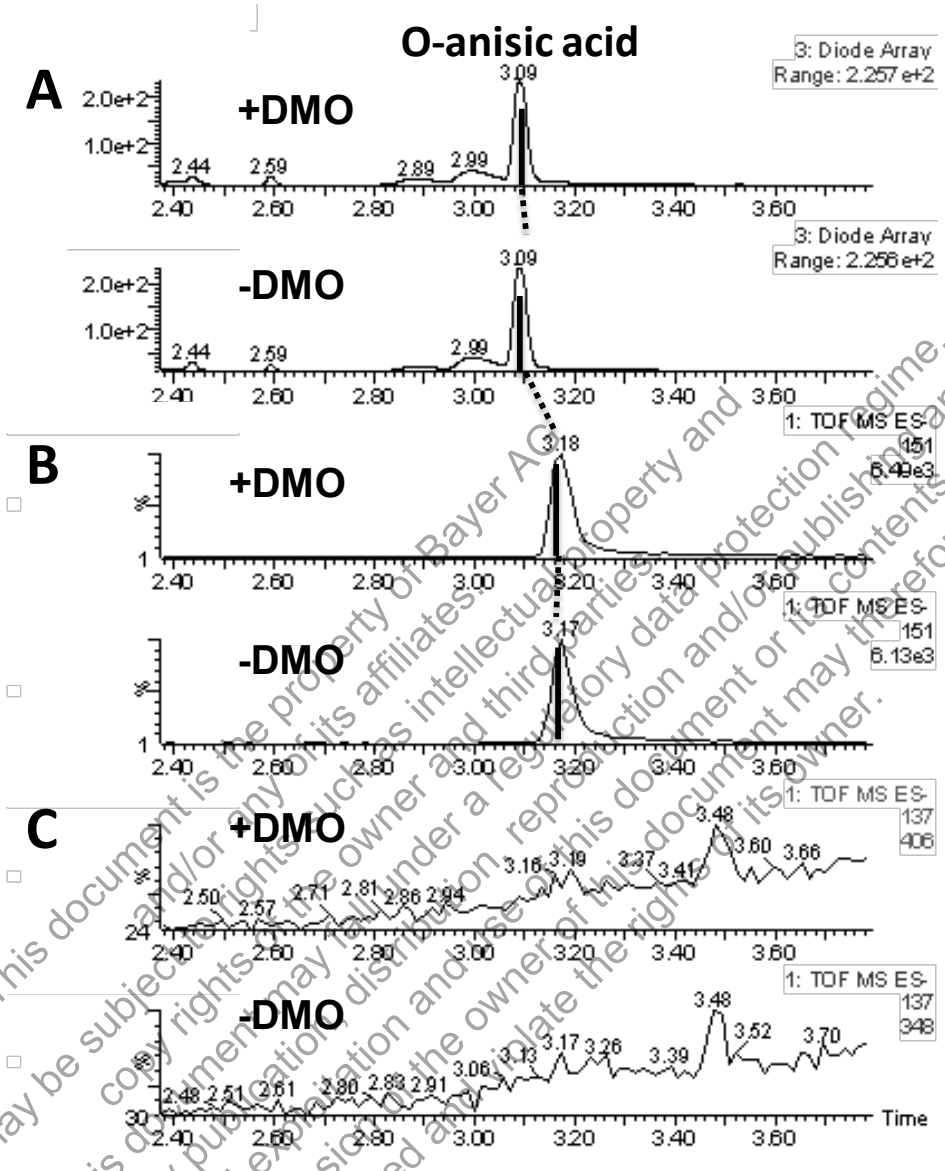

Figure B-5. MON 87708 DMO Conversion of *o*-Anisic Acid

200 µM *o*-anisic acid was incubated with MON 87708 DMO and the formation of products and disappearance of substrate was monitored by LC-UV (top two chromatograms, A) and LC-MS (bottom chromatograms, B and C). For each experiment the reaction mixture was made with (+DMO) and without (-DMO) MON 87708 DMO. The dotted line indicates the migration of the *o*-anisic acid in each chromatogram. The chromatograms in C represent a scan at an m/z of 137, the potential mass of a demethylated form of *o*-anisic acid. These chromatograms are on a smaller scale to detect any possible products and the peaks observed are within the variability of the baseline.

B.3. Biochemical Characterization of MON 87708 DMO

B.3.1. Materials

MON 87708 DMO (lot 11261646) was purified from defatted soybean flour as described in Appendix Section B.3.3. As described in Section VI, processing of the MON 87708 DMO precursor protein results in two forms of DMO, MON 87708 DMO protein and MON 87708 DMO+27 protein. This document refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein, and all forms of the trimer, collectively, as MON 87708 DMO. The identity of the MON 87708 harvested seed, processed to make the defatted soybean flour, was confirmed by event-specific polymerase chain reaction (PCR); a copy of the verification of identity is archived in the Monsanto Regulatory archives with the records documenting protein isolation. The purified MON 87708 DMO was stored in a -80 ºC freezer in a buffer solution containing 50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT) and 5% glycerol. The records describing the purification of the MON 87708 DMO are archived under lot 11261646.

B.3.2. Description of Assay Controls

Protein molecular weight standards (SeeBlue® Plus2 Pre-stained, Invitrogen,) were used to calibrate SDS-PAGE gels and verify protein transfer to polyvinylidene difluoride (PVDF) membranes. Broad range SDS PAGE molecular weight standards (Bio-Rad, Hercules, CA) were used to determine the apparent molecular weight of MON 87708 DMO. 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 and intact mass analysis. Transferrin provided with the kit (GE Healthcare, Piscataway, NJ) was used as a positive control for glycosylation analysis.

B.3.3. MON 87708 DMO Purification

MON 87708 DMO was purified from defatted flour processed from harvested seed of MON 87708. MON 87708 DMO was purified using a combination of extraction, filtration and diafiltration, and various chromatographic separations. A brief description of the purification process is below.

Defatting of seed from MON 87708 was completed at Pilot Plant Corporation in Saskatoon, Canada. The seed was cracked, dehulled, and ground to meal in the presence of dry ice. The meal was then solvent extracted, dried, and shipped to Monsanto and stored in a -20°C cold room.

Aliquots of the defatted flour were used as starting material in the purification process. Approximately 7.5 kg of defatted MON 87708 flour were extracted with 75 liters (L) of extraction buffer [25 mM potassium phosphate, pH 7.2, 10 mM MgCl2, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µM E-64, and 0.1 µM bestatin]. The extraction was conducted at room temperature (RT) for 2 hours using a Lightnin® mixer with slow stirring (Graham Transmissions Inc, Menomonee

Falls, WI). The resulting slurry was filtered using an Ertel Alsop filter press (Kingston, NY) with Die 42 micro media filter pads and a Cuno filter (Hagedorn and Gannon Co., Inc) after the addition of 7.5 kg of diatomaceous earth [5.6 kg fine hy-flo (Celite Corporation, Lompoc, CA) and 1.9 kg Celite 560 coarse (Sigma-Aldrich, St Louis, MO)]. The pads on the press were pre-coated with 1.8 kg of fine hyflo prior to the filtration of the extract. After washing the press with an additional volume of extraction buffer, the filtrate was collected (final volume: 150 L).

The filtrate was then concentrated at RT to 75 L using a hollow fiber cartridge with a 30,000 kDa molecular weight cutoff (MWCO) (GE Healthcare) to remove small molecules. Solid KCl was added to a final concentration of 0.15 M. The concentrated filtrate was diafiltered with four exchanges of 25 L each of a phenyl sepharose equilibration buffer (25 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 0.15 M KCl).

The first chromatographic step was performed at RT. A 30 L phenyl sepharose (GE Healthcare) column equilibrated with phenyl sepharose equilibration buffer was charged with the diafiltered extract and then washed with three column volumes (CV) of the phenyl sepharose equilibration buffer. A single CV of elution buffer (50 mM triethanolamine, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 100 μM dicamba) was loaded onto the column, the flow stopped and the column incubated for 1 hour. The released proteins were eluted with an additional CV of elution buffer and stored at 4°C.

Solid potassium phosphate was added to the phenyl column elution to a final concentration of 25 mM and the pH adjusted to 8.0, followed by the addition of 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, and 0.1 μM bestatin. A 3 L ceramic hydroxyapatite column (CHT) (Bio-Rad) was packed at 4°C and equilibrated in a buffer containing 25 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 100 μM dicamba. Half of the adjusted phenyl elution was charged on the CHT column. The column was washed with two CV of the CHT equilibration buffer. The bound proteins were then eluted with 400 mM potassium phosphate, pH 8.0. The flow-through containing the MON 87708 DMO, detected by immunoblot analysis, was collected. The eluted fractions without the MON 87708 DMO were discarded and the column was re-equilibrated. The second half of the phenyl elution was processed with the CHT column in the same manner as the first half. The flow-through collected from each CHT column run was combined into a single pool.

Before charging onto the next column, fresh, solid DTT and protease inhibitors were added to the CHT column flow-through pool. The flow-through pool from the CHT step was then charged on a 5 L DEAE macroprep (Bio-Rad) column at 4°C and equilibrated in a buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 μM E-64, 0.1 μM bestatin, and 100 μM dicamba. The DEAE column was then washed with five CV of the DEAE equilibration buffer followed by five CV of the equilibration buffer plus 100 mM NaCl. The bound MON 87708 DMO was eluted with a 20 CV linear NaCl gradient from 100 mM to 350 mM in the equilibration buffer.

The fractions collected throughout the gradient were analyzed by immunoblot and those fractions containing the MON 87708 DMO were pooled.

To concentrate the DEAE macroprep pool, it was first diluted with the DEAE equilibration buffer (to reduce the conductivity) and then charged onto a 1 L DEAE macroprep column. After charging, the column was washed with three CV of equilibration buffer and then eluted with minimal volume of the equilibration buffer plus 1 M NaCl. This concentrated the DEAE macroprep pool from 16 to 1.6 L.

The concentrated DEAE macroprep pool was mixed with 1 L of concanavalin A (Con A) sepharose 4B (Sigma-Aldrich) that was previously equilibrated with the DEAE equilibration buffer with fresh, solid DTT and protease inhibitors added. The purification step was run in batch mode at RT and was intended to remove contaminants that bind to Con A, while not binding MON 87708 DMO. The concentrated DEAE macroprep pool was stirred Con A resin for 1 hour, the resin was filtered out using a Büchner funnel and Whatman® filter paper (GE Healthcare). The resin was washed with 3 L of equilibration buffer. All filtrates containing MON 87708 DMO were combined.

The Con A filtrate pool was concentrated on ice for approximately 4 hours using a tangential flow membrane (Sartorius-Stedim, Goettingen, Germany) with a 100 kDa MWCO. After a 10× concentration step, the retentate containing MON 87708 DMO was diafiltered with 10 volume exchanges of DEAE macroprep equilibration buffer containing 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 µM E-64, and 0.1 µM bestatin.

The concentrated and diafiltered Con A pool was further purified on CHT at RT, this time in a binding mode where MON 87708 DMO was bound to the resin. This is achieved in the complete absence of phosphate where MON 87708 DMO binds to the CHT column and is then eluted. A 1 L CHT column was packed and equilibrated with the DEAE macroprep equilibration buffer with fresh DTT and protease inhibitors. The column was washed with three CV of equilibration buffer. The protein was eluted with a linear phosphate gradient using an elution buffer (400 mM potassium phosphate, pH 8.0, 1 mM DTT, 1 mM benzamidine-HCl, 1 mM PMSF, 1 µM E-64, 0.1 µM bestatin, and 100 µM dicamba) increasing from 0% to 50% over 10 CV. The fractions were collected and analyzed by SDS PAGE. Those containing at least 80% pure MON 87708 DMO as estimated by gel densitometry were pooled.

This entire purification procedure was repeated with two additional batches of 7.5 kg of defatted flour from MON 87708. After analysis, all final CHT pools were combined into a single final pool that was concentrated on ice for approximately 2 hours to 370 ml with a tangential flow membrane with a 30 kDa MWCO. The concentrated pool was dialyzed against enzyme storage buffer (50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 5% glycerol, and 1 mM DTT). Four liters of storage buffer were used and exchanged twice over two days and the dialysis was conducted at 4°C. The dialysate was aliquoted, assigned APS lot 11261646 and stored at -80°C.

B.3.4. Molecular Weight and Purity Estimation Using SDS-PAGE Method

SDS-PAGE analysis was performed to determine the molecular weight and purity of MON 87708 DMO.

An aliquot of MON 87708 DMO was mixed with 5 × loading buffer (LB) to a final total protein concentration of 0.09 µg/µl and heated at 99°C for three minutes. A molecular weight marker (Broad Range MW Marker, Bio-Rad) was diluted to a final total protein concentration of 0.9 µg/µl. MON 87708 DMO was loaded in duplicate at 0.5, 1.0, and 1.5 µg of total protein per lane onto a pre-cast Tris glycine 4-20% polyacrylamide gradient 10 well gel (Invitrogen). The molecular weight markers were loaded in parallel at 4.5 µg protein per lane. Electrophoresis was performed at a constant 125 V for 90 minutes. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) acetic acid for 30 minutes, stained for 16 hours with Brilliant Blue G Colloidal stain (Sigma-Aldrich), destained 30 seconds with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and finally destained with 25% (v/v) methanol for 6 hours. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One® software (version 4.4.0). Molecular weight markers were used to estimate the apparent molecular weight of each observed band. All visible bands within each lane were quantified using Quantity One software. Apparent molecular weights were obtained for the MON 87708 DMO protein and the MON 87708 DMO+27 protein, which were separated in the denaturing SDS-PAGE, while the purity for MON 87708 DMO was calculated based on the addition of the average purity of both the MON 87708 DMO protein and the MON 87708 DMO+27 protein. The results were reported as an average of all six samples loaded onto the gel containing MON 87708 DMO.

B.3.5. Immunoblot Analysis Method

Immunoblot analysis was performed to confirm the identity of MON 87708 DMO.

An aliquot of MON 87708 DMO was diluted with water and mixed with 5 × LB [312 mM Tris-HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) SDS, 0.025% (w/v) bromophenol blue, and 50% (v/v) glycerol, pH 6.8], heated at 99°C for 3 minutes, and applied on a pre-cast Tris glycine 4-20% polyacrylamide gradient 10 well gel (Invitrogen). Three amounts (20, 30, and 40 ng) of MON 87708 DMO were loaded in duplicate on the gel, where due to the denaturing conditions would be separated into the MON 87708 DMO protein and the MON 87708 DMO+27 protein. Electrophoresis was performed at a constant 125 V for 90 minutes. Pre-stained molecular weight markers (SeeBlue® Plus2 Pre-stained, Invitrogen) were loaded in parallel to verify electrotransfer of the proteins to the membrane and estimate the size of the immunoreactive bands observed. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen) was performed for 90 minutes at a constant 25 V.

For immunodetection, the membrane was blocked for 1 hour with 10% (w/v) Non-Fat Dried Milk (NFDM) in 1× Phosphate Buffered Saline containing 0.05% (v/v) Tween-20 (PBST). The membrane was then probed with a 1:3,000 dilution of goat anti-DMO

antibody, which is specific for MON 87708 DMO, in 5% (w/v) NFDM in PBST for one hour. Excess antibody was removed using three 10 minutes washes with PBST. Finally, the membrane was probed with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Thermo, Rockford, IL) at a dilution of 1:10,000 in 5% (w/v) NFDM in PBST for 1 hour. Excess HRP-conjugate was removed using three 10 minutes washes with PBST. All incubations were performed at RT. Immunoreactive bands were visualized using the ECL (Enhanced Chemiluminescence) detection system (GE Healthcare) and exposed to Amersham Hyperfilm (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan). Three exposures (20, 30, and 60 seconds) were taken and the 20 second exposure was scanned using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0).

B.3.6. N-Terminal Sequencing Method

N-terminal sequencing using automated Edman degradation chemistry (Hunkapiller et al., 1983) was used to confirm the identity of MON 87708 DMO.

Ninety microliters of MON 87708 DMO were mixed with 22.5 µl of 5× LB, heated at 99 C for 3 minutes and loaded in four lanes (10 µl/lane) onto a pre-cast Tris-glycine 4-20% polyacrylamide gradient 10 well gel (Invitrogen). Electrophoresis was carried out at a constant voltage of 150 V for 80 minutes. Proteins in the gel were electrotransferred to a PVDF (Invitrogen) membrane for 90 minutes in a buffer containing 10 mM CAPS, pH 11 and 10% methanol at a constant voltage of 25 V. Pre-stained molecular weight markers (SeeBlue Plus2 Pre-stained, Invitrogen) were loaded in parallel to verify the electrotransfer of protein to the membrane and estimate the size of the stained bands observed. The blot was stained with Ponceau S (Sigma-Aldrich).

Following electrotransfer and staining, the bands corresponding to the MON 87708 DMO protein and the MON 87708 DMO+27 protein were excised based on apparent molecular weight from the blot and N-terminal sequence analyses were performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller et al., 1983). An Applied Biosystems 494 Procise™ Protein Sequencing System with 140C Microgradient HPLC pump, ABI 785A Programmable Absorbance Detector and Procise™ Control Software (version 2.1) were used. Chromatographic data were collected using Atlas™ 2003 software (Thermo Fisher Scientific Inc, Waltham, MA). A PTH (Phenylthiohydantoin) - amino acid standard mixture (Applied Biosystems) was used as the calibration standard in the chromatographic analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein, 10 pmol β lactoglobulin (Applied Biosystems), was analyzed before and after the analysis to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

B.3.7. Tryptic Mapping Method

MALDI-TOF mass spectrometry was used to confirm the identity of MON 87708 DMO. The proteins were first separated by SDS-PAGE prior to trypsinization.

An aliquot (89.5 µl) of MON 87708 DMO was mixed with 22.5 µl of 5× LB, heated at 99°C for 3 minutes and loaded in four lanes (three lanes each loaded with 4.2 µg and one lane with 3.1 µg of total protein) onto a pre-cast Tris-glycine 4-20% polyacrylamide gradient 10 well gel (Invitrogen). Pre-stained MW markers (SeeBlue Plus2 Pre-stained, Invitrogen) were loaded in parallel to estimate the size of the stained bands observed. Electrophoresis was carried out at a constant 150 V for 80 minutes, where due to the denaturing conditions MON 87708 DMO would be separated into the MON 87708 DMO protein and the MON 87708 DMO+27 protein. Following electrophoresis, the gel was stained with Brilliant Blue G Colloidal (Sigma-Aldrich). The bands corresponding to the MON 87708 DMO protein or the MON 87708 DMO+27 protein were excised from four lanes of the gel, destained, reduced, and alkylated. Each gel band was destained for 30 minutes by incubation in 100 µl of destain solution (40% methanol, 50% water, and 10% glacial acetic acid) in a microfuge tube. This step was repeated twice for 60 minutes each, removing all visible Brilliant Blue G Colloidal stain. Following destaining, the gel bands were incubated in 100 µl per band of 100 mM ammonium bicarbonate buffer for 16 hours at RT. The protein was reduced in 100 µl of 10 mM DTT solution for two hours at 37°C. After removing the reducing solution, the protein in the gel was alkylated by incubating in 100 µl of 20 mM iodoacetic acid. The alkylation reaction was allowed to proceed at RT for 20 minutes in the dark. The gel containing the protein band was incubated in 200 µl of 25 mM ammonium bicarbonate buffer for 15 minutes at RT. This step was repeated two additional times for 15 minutes each; then the gel band was dried using a Savant Speed Vac concentrator (Holbrook, NY). Each gel band was rehydrated with 20 µl of 0.02 µg/µl trypsin in 25 mM ammonium bicarbonate and 10% acetonitrile, and the incubated for approximately one hour at RT. Following incubation, the excess solution was removed and the gel/trypsin reaction mixture was incubated overnight at 37°C in 40 µl of 25 mM ammonium bicarbonate and 10% acetonitrile. The following day, the sample was sonicated for 5 minutes, and the supernatant transferred to a new tube and dried using a Speed Vac concentrator (Extract 1). The gel band(s) was resuspended in 30 µl of a solution consisting of 60% acetonitrile, 0.1% trifluoroacetic acid (TFA), and 0.1% octyl-β-D-glucopyranoside, and then sonicated for 5 minutes. After transfer of the supernatant to a new microcentrifuge tube, this step was repeated once and the combined supernatants were dried using the Speed Vac concentrator (Extract 2). Extracts 1 and 2 were separately dissolved in 20 µl 0.1% TFA and then dried using a Speed Vac concentrator. Finally, Extract 1 was dissolved in 5 µl of 50% acetonitrile/0.1% TFA, while Extract 2 was dissolved in 10 µl of the same solution. To maximize the solubilization, each sample was sonicated for 5 minutes.

Mass calibration of the mass spectrometer was performed using an external peptide mixture (Sequazyme™ Peptide Mass Standards Kit, Calibration Mixture 2, Applied Biosystems). The samples Extract 1 and Extract 2 (0.3 µl) were co-crystallized with 0.75 µl each of the following matrix solutions: dihydroxybenzoic acid (DHB), α-cyano-4-hydroxy cinnamic acid (α-cyano), and 3,5 dimethoxy-4 hydroxycinnamic acid (sinapinic acid) at separate locations on the analysis plate. The samples in DHB matrix were analyzed in the 300 to 5,000 Dalton (Da) range. The samples in α-cyano matrix were analyzed in the 500 to 5,000 Da range. The samples in sinapinic acid matrix were analyzed in the 500 to 7,000 Da range. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993), except above 3,000 Da, where

mass-averaged values were used. GPMAW32® software (Lighthouse data, Denmark) was used to generate a theoretical trypsin digestion of the deduced MON 87708 DMO protein and the MON 87708 DMO+27 protein amino acid sequences. Masses were calculated for each theoretical peptide and compared to the raw experimental mass data. Below 1000 Da, experimental masses (MH+) were assigned to peaks when two or more isotopically resolved peaks were observed. Above 1000 Da, experimental masses (MH+) were assigned to peaks when three or more isotopically resolved peaks were observed. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger mass signal. Known autocatalytic segments from trypsin digestion were identified in the raw data. The list of experimental masses was compared to the theoretical list from the GPMAW software. Those experimental masses within 1 Da of a theoretical mass were matched. All matching masses were tallied and a coverage map was generated. The tryptic mass map coverage was considered acceptable if ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide segments to the expected masses for the segments.

B.3.8. Glycosylation Analysis Method

Glycosylation analysis was used to determine whether MON 87708 DMO was post-translationally modified with covalently bound carbohydrate moieties.

An aliquot of MON 87708 DMO and the positive control, transferrin (GE Healthcare) were each diluted with water and mixed with 5 × LB. These samples were heated at 101.0°C for three minutes, cooled, and loaded on a Tris-glycine 4-20% polyacrylamide gradient 10 well mini-gel (Invitrogen). Three amounts of transferrin (50, 100, and 200 ng) and two amounts (100 and 200 ng) of the purity corrected MON 87708 DMO was loaded in the gel. SeeBlue® Plus2 Pre-stained protein molecular weight markers (Invitrogen) were loaded to verify electrotransfer of the proteins to the membrane. Electrophoresis was performed at a constant 150 V for 87 minutes. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen) was performed for 60 minutes at a constant 25 V, followed by 30 minutes at 30 V.

Carbohydrate detection was performed directly on the PVDF membrane using the GE Healthcare Glycosylation Detection Module (Cat. No. RPN 2190). The manufacturer's protocol was followed and all the reagents except phosphate buffered saline (PBS) were provided with the kit. All steps were performed at RT. Following electrotransfer to PVDF membrane, the blot was incubated in 30 ml of PBS for 10 minutes, followed by incubation with 20 ml of 10 mM $NaIO_4$ for 20 minutes in the dark. The membrane was rinsed twice with PBS and washed three times with 20 ml PBS for 10 minutes each. The membrane was incubated with 20 ml solution consisting of 0.125 mM biotin-hydrazide, 100 mM acetate, pH 5.5 for 60 minutes followed by two PBS rinses and three 10 minute washes with PBS. The membrane was blocked for 60 minutes using 5% blocking reagent in PBS followed by two PBS rinses and three 10 minute washes with PBS. The membrane was incubated with strepavidin-HRP at a 1:6000 dilution for 30 minutes. After two PBS rinses and three 10 minute washes with PBS, the membrane was developed with ECL detection reagents by mixing 1 ml of Reagent 1 and 1 ml of

Reagent 2. After one minute incubation, the excess detection solution was removed by blotting with paper towels and the blot was exposed to Hyperfilm ECL (GE Healthcare). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan). Three exposures (30 seconds, 1 and 2 minutes) were performed. The image was captured using a Bio-Rad GS-800 densitometer with the supplied Quantity One® software (version 4.4.0).

B.3.9. Specific Activity Assay Method

The specific activity of MON 87708 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). The standard assays were conducted in 200 µl solutions consisting of 25 mM potassium phosphate, pH 7.2, 3.4 µg ferredoxin, 3.4 µg reductase, 0.5 mM FeSO4, 10 mM MgCl2, 0.7 mM NADH, 0.3 mM dicamba, 2 µl (42.48 U/ml) of formaldehyde dehydrogenase and either 2 µg MON 87708 DMO or 1 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 minutes. Reactions 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 minutes, followed by a step to 10% solvent A for 1 minute and then re-equilibration at 90% solvent A for 10 minutes 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 87708 DMO added to the reaction mixture and expressed as nmol of DCSA produced per minute per mg of MON 87708 DMO (nmol/min/mg).

References for Appendix B

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Appendix C: Materials and Methods for the Analysis of MON 87708 DMO Levels in MON 87708

C.1. Materials

Over-season leaf, root, forage, and seed tissue samples from MON 87708 and the near isogenic conventional soybean control A3525 were harvested from five field sites in the U.S. during 2008 from plants grown from starting seed lot 10001256 and 10001257, respectively. An *E. coli*-produced DMO protein (lot 11247247) identical in sequence to the MON 87708 DMO protein was used as the analytical reference standard.

C.2. Characterization of the Materials

The identities of MON 87708 and conventional control were confirmed by verifying the chain of custody documentation prior to analysis. To further confirm the identities of MON 87708 and conventional control, event specific polymerase chain reaction (PCR) analyses were conducted on the seed tissue samples from each site to confirm the presence or absence of the *dmo* expression cassette. The PCR analyses and the resulting verification of identities were archived under the starting seed lot numbers.

C.3. Field Design and Tissue Collection

Field trials were initiated during the 2008 planting season to generate MON 87708 and conventional control samples at the following locations in the U.S.: Jefferson County, Iowa (IARL); Stark County, Illinois (ILWY); Clinton County, Illinois (ILCY); Parke County, Indiana (INRC); and Berks County, Pennsylvania (PAHM). The field sites were representative of soybean producing regions suitable for soybean commercial production. At each site, three replicated plots containing MON 87708, as well as the conventional control, were planted using a randomized complete block field design. Over-season leaf (OSL), root, forage, and seed samples were collected from each replicated plot at each field site (except for the conventional control from Berks County, Pennsylvania where only two replicates were collected).

The OSL tissue samples were collected from the youngest set of fully expanded trifoliate leaves at the following growth stages: OSL-1 at V3-V4; OSL-2 at V5-V8; OSL-3 at R2 V12; and OSL-4 at R5 V16. The root and forage tissue samples were collected at approximately the R6 growth stage. Seed tissue samples were collected at the R8 growth stage

C.4. Tissue Processing and Protein Extraction

MON 87708 DMO was extracted from the seed tissue samples at a tissue to buffer ratio of 1:100 with a Tris-borate buffer [0.1 M Tris, 0.1 M Na_2B4O7 • $10H_2O$, 0.005 M $MgCl_2$, 0.05% (v/v) Tween-20 at pH 7.8]. MON 87708 DMO was extracted from OSL, forage, and root tissues samples at a tissue to buffer ratio of 1:100, 1:50, and 1:50, respectively, using phosphate buffered saline (PBS) with Tween 20 and 0.5% (w/v) bovine serum albumin (BSA) [(1 × PBST with 0.5% (w/v) BSA)]. Extractions were done using 8 1/4″ chrome-steel beads, and shaking two times for 3.5 minutes 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 aliquotted and stored frozen in a -80°C freezer until enzyme-linked immunosorbent assay (ELISA) analysis.

C.5. DMO Antibodies

Goat polyclonal anti-DMO antibodies 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, pH 7.4. The purified anti-DMO antibodies were coupled with biotin (Pierce, Rockford, IL) according to the manufacturer's instructions and assigned lot G-84413. The detection reagent was NeutrAvidin (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP). The goat polyclonal anti-DMO antibodies that react with MON 87708 DMO, which refers to both the MON 87708 DMO protein, the MON 87708 DMO+27 protein and all forms of the trimer, were used as capture antibodies for the DMO ELISA method.

C.6. DMO ELISA Method

Goat polyclonal anti-DMO antibodies were diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) to a final concentration of 5.0 µg/ml and then immobilized onto 96 well microtiter plates followed by incubation in a 4°C refrigerator for≥8 hours. Plates were washed with 1×PBS containing 0.05% (v/v) Tween-20 (1×PBST). The plates were blocked using 10% Casein in Tris-buffered saline (TBS) blocking buffer (Pierce, Rockford, IL) at 200 µl per well for 1 hour at room temperature. The blocking buffer was aspirated and DMO reference standard or tissue sample extract was added at 100 µl per well and incubated for 1 hour at 37°C. Prior to the addition of a biotinylated detection antibody, NeutrAvidin-HRP and 3,3',5,5'-tetramethyl-benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD) reagents, plates were washed with 1×PBST. The captured MON 87708 DMO was detected by the addition of 100 µl per well of biotinylated goat anti-DMO antibodies and NeutrAvidin-HRP. Plates were developed by adding 100 µl per well of TMB. The enzymatic reaction was terminated by the addition of 100 µl per well of 3 M H_3PO_4. Quantification of MON 87708 DMO was accomplished by interpolation on a DMO reference standard curve that ranged from 0.313-20 ng/ml.

C.7. Moisture Analysis

Tissue moisture content was determined using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO). A homogeneous site and tissue-specific pool (TSSP) was prepared using MON 87708 and conventional control samples from each tissue type grown at each site. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$\mathrm{DWCF} = 1 - \frac{(\text{Mean \% TSSP Moisture})}{(100)}$$

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

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

The protein levels that were reported on a fwt basis to be less than or equal to the limit of detection (LOD) or less than the limit of quantification (LOQ) were not reported on a dwt basis.

C.8. Data Analyses

All MON 87708 DMO 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 reported on a µg/g fwt basis for data that were greater than or equal to the LOQ. For MON 87708 DMO, this conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The MON 87708 DMO values in µg/g fwt were converted to µg/g dwt by applying the DWCF. Microsoft Excel 2007 (Version 12.0.6514.5000 SP2 Microsoft, Redmond, WA) was used to calculate MON 87708 DMO levels in tissue samples. The sample mean, standard deviations, and ranges were also calculated using Microsoft Excel 2007.

Appendix D: Western Blot Analysis of MON 87708 DMO in Leaf Across Multiple Generations

D.1. Materials

Leaf tissues harvested from five generations of MON 87708 plants grown in the greenhouse were analyzed to determine MON 87708 DMO presence across multiple generations. The R_3 generation and the control substance tissues were collected in 2007 from a greenhouse production. Generations R_2, R_4, R_5, and R_6 were collected in 2008 from a greenhouse production.

D.1.1. MON 87708 Materials

Leaf tissues harvested from five generations of MON 87708 were evaluated.

Table D-1. MON 87708 Starting Seed Planted in 2007 and 2008 Greenhouse Productions

Generation	Lot Number
R_2	10002130
R_3	GLP-0707-18882-S
R_4	10002129
R_5	10001256
R_6	10001821

D.1.2. Control Material

The control substance was a near isogenic conventional soybean variety A3525. The leaf tissue of the control substance was evaluated.

Table D-2. Control Starting Seed Planted in 2007 Greenhouse Production

Generation	Lot Number
N/A	GLP-0707-18884-S

D.1.3. Characterization of MON 87708 and Control Materials

The identities of the MON 87708 and conventional control samples were characterized prior to their use. The identity of the MON 87708 R_3 sample and the conventional control were confirmed by event specific polymerase chain reaction (PCR) analysis of the starting seed and harvested seed and archived under the lot numbers.

The identity of the MON 87708 R_2, R_4, R_5, and R_6 samples was confirmed by event-specific PCR of the harvested leaf tissue prior to use.

D.1.4. Reference Standard

The *E. coli*–produced DMO (lot 10002215) was used as the analytical reference standard. This DMO is identical to the MON 87708 DMO protein.

D.2. Methods

Leaf tissues from five generations of MON 87708 were analyzed by western blot to demonstrate the presence of MON 87708 DMO in R_2, R_3, R_4, R_5, and R_6 generations. The conventional control was also analyzed by western blot to confirm the expected absence of MON 87708 DMO. The presence or absence of MON 87708 DMO was determined using a goat anti-DMO polyclonal antibody (lot G-830119). The *E. coli*-produced DMO reference standard was used as a reference for molecular weight comparison and confirmation of the specificity of the antibody detection reagent.

D.2.1. MON 87708 Tissue Processing

The leaf tissues were processed by grinding with liquid nitrogen in a mortar and pestle. Processed tissue samples were stored in a -80°C freezer until shipped on dry ice to the analytical facility.

D.2.2. Extraction

MON 87708 DMO was extracted from processed leaf tissue samples in a Tris borate buffer at a tissue-to-buffer ratio (w/v) of 1:100. Each extract was prepared by homogenization of the tissue using a Harbil mixer (Eight beads; two, 3.5 minute cycles). After extraction, insoluble material was removed from the leaf tissue extracts by serum filter (Fisher Scientific, Pittsburg, PA). The filtered extracts were transferred into labeled tubes and the aliquots stored in a -80°C freezer until analysis.

D.2.3. SDS-PAGE

Extracts were analyzed by SDS-PAGE on a 4-20% Tris-Glycine gradient gel (Invitrogen). Electrophoresis was conducted at 150 V for approximately 90 minutes in 1× Novex Tris-Glycine-SDS running buffer (Invitrogen). Prior to gel loading, the extracts were diluted initially 1:2.5 (v/v) in Bio-Rad 1×Laemmli Buffer and then further diluted 1:2 (v/v) in Bio-Rad 2×Laemmli Buffer, then heated in a 75°C metal block for five minutes. Sample extracts were loaded on the gel with the *E. coli*–produced DMO reference standard and the Precision Plus molecular weight marker (Bio-Rad) to demonstrate the transfer of proteins to the membrane and to approximate the molecular weight of the MON 87708 DMO protein and the MON 87708 DMO+27 protein.

D.2.4. Western Blot Analysis (Immunoblotting)

Proteins separated by SDS-PAGE were electrophoretically transferred to a 0.45 µm Invitrolon PVDF membrane (Invitrogen) using 1× Novex Tris-Glycine transfer buffer (Invitrogen) containing 20% methanol. Non-specific sites were blocked using 5% (w/v) non-fat dry milk (NFDM, Bio-Rad) in Phosphate-Buffered Saline with 0.05% (v/v) Tween-20 (PBST). The membrane was probed for the presence of the MON 87708 DMO protein and the MON 87708 DMO+27 protein using a 1:1000 dilution of purified goat anti-DMO antibody (lot G-830119) in 1×PBST with 5% (w/v) NFDM. The membrane was rinsed briefly, then washed three times for ten minutes each in PBST to remove unbound antibody. Bound antibody was then probed with a 1:3000 dilution of anti-goat IgG antibody conjugated to horseradish peroxidase (HRP; Pierce, Rockford, IL). The membrane was rinsed briefly and then washed three times for ten minutes each in PBST. The SuperSignal West Pico Chemiluminescent Substrate (Pierce) was added to the membrane according to the manufacturer's instructions. The membrane was exposed to Hyperfilm ECL (Amersham) to record an image of the immunoreactive bands.

Appendix E: Bioinformatics Evaluation of MON 87708

E.1. Databases Assembly

The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from FARRP (2010) and was used as provided. The AD_2010 database contains 1,471 sequences.

GenBank protein database, release 175.0 was downloaded from the National Center of Biotechnology Information (NCBI) and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2010 database and contains 17,815,538 sequences.

The toxin database is a subset of sequences derived from the PRT_2010 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_2010 database and contains 8,448 sequences.

E.2. Bioinformatics Evaluation of MON 87708 DMO

E.2.1. Sequence Database Searches

FASTA analyses using the AD_2010, PRT_2010 and TOX_2010 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_2010, TOX_2010, and PRT_2010) 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 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 significant homology. The expectation threshold (E-score) limit was set to one. 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 87708 DMO+27 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_2010). 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 (Hileman et al., 2002; Goodman 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. 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_2010 database were inspected to determine whether they exceeded the CODEX threshold of 35% or greater identity in 80 or greater amino acids.

E.3. Bioinformatics Evaluation of the Transfer-DNA Insert in MON 87708

E.3.1. 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 or SeqBuilder 8.0.2 (13). The resultant amino acid sequences were used to search the AD_2010, PRT_2010, and TOX_2010 databases.

E.3.2. Sequence Database Searches

FASTA analyses using the AD_2010, PRT_2010, and TOX_2010 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_2010, TOX_2010, and PRT_2010) 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 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 less than 1×10^{-5} or smaller 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).

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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_2010). 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 (Hileman et al., 2002; Goodman 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, 1996).

E.3.3. 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_2010 database were inspected to determine whether they exceeded the CODEX threshold of 35% or greater identity in 80 or greater amino acids.

E.4. Bioinformatics Evaluation of DNA Sequences Flanking the 5′ and 3′ Junctions of Inserted DNA in MON 87708: Assessment of Putative Polypeptides

E.4.1. Translation of Putative Polypeptides

DNA sequence spanning the 5′ and 3′ junctions of the MON 87708 insertion site was analyzed for translational stop codons (TGA, TAG, TAA). All six reading frames originating or terminating within the MON 87708 insertion and originating or terminating within the intervening sequence were translated using the standard genetic code from stop codon to stop codon using DNAStar, version 8.0.2 (13), 412. A total of twenty sequences of eight amino acids or greater that spanned the junction(s) were analyzed.

E.4.2. Sequence Database Searches

FASTA analyses using the AD_2010, TOX_2010, and PRT_2010 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 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_2010, TOX_2010, and PRT_2010) 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 *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 will need to have an *E*-score of 1×10^{-5} or smaller to be considered to have significant homology. The expectation threshold (*E*-score) limit was set to one. 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

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contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

E.4.3. Significance of the Alignment

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Appendix F: Materials and Methods Used in Assessing Stability of MON 87708 DMO in Gastrointestinal Fluids

F.1. Materials

MON 87708 DMO (lot 11261646) purified from soybean seed of MON 87708 was used to assess its stability in simulated gastrointestinal fluids. The MON 87708 DMO was stored in a -80ºC freezer in a buffer solution containing 50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT) and 5% glycerol. MON 87708 DMO has a purity of 81% and a concentration of 0.18 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).

Simulated intestinal fluid (SIF) contained a mixture of proteolytic enzymes known as pancreatin in a buffer adjusted to pH of ~7.5. SIF was prepared according to the method described in The United States Pharmacopoeia (USP, 1995). The pancreatin used for the preparation of SIF was obtained from Sigma Company (catalog number P 1500, St. Louis, MO)

F.2. Digestion of MON 87708 in Simulated Gastric Fluid Method

Digestions were initiated by addition of MON 87708 DMO to tubes containing SGF, where 10 units of pepsin activity were used per 1 µg of total protein. Digestions were incubated at 37 ± 2°C in separate tubes for various durations, and the reactions were quenched by addition of a sodium carbonate solution to each tube. Zero incubation time points (T = 0) were quenched by addition of sodium carbonate solution to SGF prior to addition of the MON 87708 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 MON 87708 DMO in the system without pepsin. These controls were incubated for 0 and 60 minutes and were designated with the letter "P"(SGF P). Additionally, experimental controls to characterize the system without MON 87708 DMO were also included. These experimental controls were prepared by substituting MON 87708 DMO storage buffer for MON 87708 DMO, and were designated with the letter "N"(SGF N).

All quenched specimens, were heated to 75-100ºC for 5-10 minutes, frozen on dry ice, and stored in a –80ºC freezer until analyzed. The digestibility of MON 87708 DMO in SGF was assessed using SDS-PAGE gel followed by Brilliant Blue G Colloidal dye (Sigma P/N B-2025) staining or western blotting, where due to the denaturing conditions MON 87708 DMO would be separated into the MON 87708 DMO protein and the MON 87708 DMO+27 protein. Limits of detection (LOD) were determined for the gel staining and western blot methods.

F.3. Digestion of MON 87708 DMO in Simulated Intestinal Fluid Method

Digestions were initiated by addition of MON 87708 DMO to tubes containing SIF, where 55.3 µg of pancreatin were used per 1 µg of total protein. Digestions were incubated at 37 ± 2°C in separate tubes for various durations, and the reactions were quenched by addition of 5×LB, heated to 75-100ºC for 5-10 minutes, and frozen on dry ice. Zero incubation time points (T = 0) were quenched by addition of 5×LB, heated to 75-100ºC for 5-10 minutes to SIF prior to addition of MON 87708 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 MON 87708 DMO in the system without pancreatin. These controls were incubated for 0 and 60 minutes and were designated with the letter "P" (SIF P). Additionally, experimental controls to characterize the system without MON 87708 DMO were also included. These experimental controls were prepared by substituting MON 87708 DMO storage buffer for MON 87708 DMO, and were designated with the letter "N" (SIF N).

All quenched specimens, were heated to 75-100ºC for 5-10 minutes, frozen on dry ice, and stored in a -80ºC freezer until analyzed. The digestibility of purified MON 87708 DMO in SIF was assessed using western blotting, where due to the denaturing conditions MON 87708 DMO would be separated into the MON 87708 DMO protein and the MON 87708 DMO+27 protein. Limits of detection (LOD) were determined for the western blot methods.

References for Appendix F

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Appendix G: Heat Stability of MON 87708 DMO

G.1. Materials

MON 87708 DMO (lot 11271156) purified from soybean seed of MON 87708 was used to assess its stability after heat treatment. The MON 87708 DMO was stored in a -80ºC freezer in a buffer solution containing 50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT) and 5 % glycerol. MON 87708 DMO has a purity of 81 % and a concentration of 0.18 mg/ml.

G.2. Description of Assay Controls

Bio-Rad Broad Range molecular weight standards (Bio-Rad, Hercules, CA) were used to visually estimate the molecular weight (MW) of the protein bands on SDS-PAGE.

G.3. Methods

G.3.1. MON 87708 DMO Dilution and Heat Treatment

Aliquots of 100 µl of MON 87708 DMO were transferred to eleven tubes. Five tubes were incubated for 15 minutes and five tubes were incubated for 30 minutes at temperatures of 25, 37, 55, 75, or 95°C. The eleventh tube (*i.e.*, the non-heated control sample) was maintained on wet ice throughout the heat treatment incubation period. All heat-treated samples were returned immediately to wet ice following heat treatment. The samples were diluted, frozen, or assayed as quickly as practical.

G.3.2. MON 87708 DMO Functional Assay

The functional activity of MON 87708 DMO in the heated and non-heated samples was determined according to the method used in Appendix B.3.9. Samples were assayed in triplicate. The percentage of remaining activity was calculated based on the unheated sample (100%).

G.3.3 SDS-PAGE Analysis

For the ten heat-treated samples plus the control treatment sample that were diluted, 40 µl of each heat-treated sample and the non-heated control sample was transferred to a clean tube and mixed with 10 µl of 5×SDS-PAGE loading buffer to a total protein concentration of 0.14 mg/ml. All samples were heated at 95 ± 5°C for 3-5 minutes and quickly frozen by placement on dry ice and stored in a -80°C prior to SDS-PAGE analysis. The samples prepared for SDS-PAGE analysis were then thawed, heated at 95 ± 5°C for 3-5 minutes, and then twenty microliters of each sample, each at ~0.14 mg total protein/ml in 1×loading buffer, were loaded per lane. Twenty microliters of MON 87708 DMO at 0.14 mg/ml total protein (100% equivalent to the heat-treated sample concentration) were loaded on the same gel. Twenty microliters of MON 87708 DMO 0.014 mg/ml total protein (10% equivalent to the heat-treated sample concentration) were also loaded on the same gel. BioRad Broad Range molecular

markers (Hercules, CA) were loaded on two lanes on the same gel at 40.5 µg total protein per lane.

Proteins were separated by SDS-PAGE using a pre-cast 10 well Tris-glycine 4-20 % polyacrylamide gradient gels and Tris-glycine-SDS running buffer. Electrophoresis was performed at 125 V for 90 minutes. Following electrophoresis, the gel was stained with Colloidal Brilliant Blue G (Sigma, St. Louis, MO).

After staining, the presence of the MON 87708 DMO protein and the MON 87708 DMO+27 protein of each heat-treated sample was estimated qualitatively from the scanned image by visual inspection. The intensity of the major protein bands at ~39.8 and ~42.0 kDa in the lanes loaded with the heat-treated sample was compared visually to the same bands in the lanes loaded with the control treatment sample, 100 % reference protein equivalent, and 10% reference protein equivalent to determine presence.

Appendix H: Quantitative ELISA Assessment of Human IgE Binding to MON 87708, Conventional Control, and Commercial Reference Soybean Using Sera from Soybean-Allergic Subjects

H.1. Materials

Soybean seeds of MON 87708, near isogenic conventional soybean control A3525, and commercial soybean reference varieties were coarsely ground and stored in a -20ºC freezer until analysis.

H.1.1. MON 87708 Material

The test substance was soybean seed from MON 87708 (lot 10001256; Study Sample ID 22).

H.1.2. Conventional Control Material

The control substance was soybean seed from near-isogenic conventional soybean variety A3525 that has a similar genetic background to MON 87708 (lot 10001257; Study Sample ID 23).

H.1.3. Commercial Reference Varieties Materials

The commercial reference varieties were 17 commercial-available soybean varieties.

Table H-1. Identification of Commercial Reference Varieties Materials

Study Sample Identification	Soybean Variety	Lot Number	Type
1	A4922	10001425	Reference
2	A5427	10001395	Reference
3	Beck	10001424	Reference
4	Dwight	10001434	Reference
5	Hutcheson	10001432	Reference
6	M-SOY 8411	10001430	Reference
7	Pioneer 93B15	10001304	Reference
8	Stewart 3454	10001435	Reference
9	Stine ST2788	10001133	Reference
10	EXP125	10001433	Reference
11	Opal	10001431	Reference
12	A2553	10001295	Reference
13	A1900	10001299	Reference
14	A2442	10001297	Reference
15	A2824	10001294	Reference
16	AJB2501KOC	10001503	Reference
17	A241QT-211	10001504	Reference

H.1.4. ELISA Assay Internal Reference

The PEI internal reference substance is conventional seed from yellow soybean "Hensel – GMO-free", W. Schoenenberger GmbH & Co. KG, Magstadt, Germany.

H.1.5. Characterization of Materials

The identity of the seed from MON 87708 and the conventional control were confirmed by event specific polymerase chain reaction (PCR). Chain of custody (COC) records denoting the assigned lot number served as the identification for the commercial reference varieties.

H.1.6. Sera

Sera for this experiment contained soybean-specific IgE antibody. The sera were collected from soybean-allergic subjects prior to this experiment by the principal investigator in conjunction with approved clinical partners. The study subjects had been diagnosed as soybean allergic on the basis of:

1) a documented case history of anaphylactic reactions to soybean

2) a positive Double-Blind Placebo Controlled Food Challenge (DBPCFC) to soybean.

A total of 20 sera from soybean-allergic subjects met the criteria for inclusion in the study. Sera from five non-allergic individuals were also collected from either the clinical partners or obtained from the commercial supplier, PlasmaLab. Sera from soybean-allergic subjects were coded numerically as shown in Table H-2. The level of total soybean-specific IgE was measured for screening purposes using Capsulated Hydrolic Carrier Polymer-FluoroEnzyme Immunoassay (CAP-FEIA; Phadia, Uppsala, Sweden) as shown in Table H.2.

Table H-2. Characteristics of Sera from Soybean-Allergic Subjects

Serum ID	CAP-FEIA (kU/l)[1]	IgE ELISA Result	DBPCFC with Soybean
KB 1	0.68	Positive	Positive
KB 2	2.64	Positive	Positive
ME 1	25.0	Positive	Positive
ME 2	<100	Positive	Positive
ME 3	38.7	Positive	Positive
MS 01	0.09	Negative[2]	Positive
MS 02	0.17	Negative[2]	Positive
MS 03	0.23	Negative[2]	Positive
MS 04	1.05	Negative[2]	Positive
MS 05	1.73	Positive	Positive
MS 06	4.62	Positive	Positive
MS 07	4.70	Positive	Positive
MS 08	7.12	Positive	Positive
MS 09	2.76	Positive	Positive
MS 10	0.28	Negative[2]	Positive
MS 11	12.7	Positive	Positive
MS 12	0.06	Negative[2]	Positive
MS 13	2.10	Positive	Positive
MS 14	1.87	Positive	Positive
MS 15	0.02	Negative[2]	Positive

[1]CAP-FEIA values were obtained for total soybean-specific IgE.
[2]These sera had three or more IgE binding values against the 17 conventional references below the ELISA limit of quantitation (LOQ).

H.2. Analytical Methods

H.2.1. Grinding of Soybean Seed

Seeds from the MON 87708, conventional control, and commercial reference varieties were roughly ground and stored in a -20ºC freezer until it was re-ground to a fine powder prior to analysis. The fine powder was stored in a -80ºC freezer until extraction. After thawing, the fine powder was maintained on wet ice prior to extraction.

H.2.2. Preparation of Soybean Extracts

Aqueous protein extracts were prepared according to the following methodology. Finely ground, raw, full-fat soybean were extracted by shaking in 1×PBST (1 g tissue /10 ml PBST) at 4-8ºC for 4-5 hours. Two independent extracts were prepared for each sample and pooled together, clarified by centrifugation at ~13, 000 × g and then passed through a 0.22 µm cellulose acetate filter. The variability (% CV) in the total protein content between independent extractions using this method was demonstrated to be <10%. The clarified extracts were divided into 10 equal volumes of 75 µl and stored at -80°C until used. Once thawed, the extracts were maintained on wet ice and used within 6 hours.

The total protein concentrations in the clarified extracts were determined using a commercially available ready-to-use Bradford reagent according to the manufacturer's instructions. Each pooled soybean extract, diluted with coating buffer to a total protein concentration of 10 µg/ml, was tested in triplicate wells and was added to a 96 well plate at 100 µl/well.

H.2.3. ELISA Method

The PEI laboratory developed and performed the human IgE immunoassays for this study. A validated ELISA method was used for testing the amount of soybean-specific IgE in sera from soybean allergic and non-allergic subjects to extracts from test, control, and reference substances. The method is as follows: Assay plates were coated with soybean extracts at a concentration of 10 µg/ml and all other immunoreagents were added at 100 µl/well. Coated plates were incubated overnight and then washed four times with 300 µl wash buffer/well. This overnight incubation and all subsequent incubations were completed at room temperature. Following washing, 100 µl 2% BSA in PBS/well was added and plates were incubated for 60-65 minutes. The plates were again washed four times with 300 µl wash buffer/well. Standards, positive and negative control sera, incubation buffer for the non-specific reagent binding control and study specific sera were added to the plate at 100 µl/well and the plates were incubated for two hours. Following incubation, the plates were washed four times with 300 µl wash buffer/well. After washing, 100 µl/well of horseradish peroxidase labeled anti-human IgE antibody was added, and plates were incubated for 60-65 minutes. Plates were then washed four times with 300 µl wash buffer/well. To develop the plate, 100 µl/well TMB/Peroxide solution in citrate buffer was added to each well and incubated for 10-11 minutes. Plates were read bi-chromatically as described in Section H.2.6. after 100 µl stop solution was added to each well.

H.2.4. ELISA Plate Design

Each 96-well microtiter plate contained a standard curve, an internal reference soybean extract (Hensel), and human serum PEI 46 (PEI is the designation given to all sera collected at PEI) containing soybean-specific IgE, which served as a positive control for inter-assay precision. Each plate also contained MON 87708, conventional control, and commercial soybean reference varieties sample extracts. Controls utilized for data reduction included non-specific reagent binding (NSB) and IgE binding from the non-allergic (NA) serum pool. A mixture of equal volumes of five sera from non-allergic subjects were used to create the non-allergic serum pool (PEI designations; NA1, NA2, NA5, C, and E).

H.2.5. ELISA Standard Curve

Soybean-specific IgE binding was quantified by use of a soybean-specific IgE standard curve and expressed as ng/ml of serum. The standard curve was created by loading serial dilutions of human serum PEI 163 that contains a known amount of soybean-specific IgE into wells coated with internal reference soybean extract. Concentration of soybean-specific IgE in serum PEI 163 was 36 kU/l as measured by CAP-FEIA.

Conversion of IgE concentration expressed as U/ml into ng/ml was done according to the conversion ratio: 2.4 ng/ml IgE = 1 U/ml. Standard curves were generated with serial 4-fold dilutions of human serum PEI 163 in an incubation buffer and then loading the following concentrations of soybean-specific IgE: 21.6, 5.4, 1.35, 0.34, 0.084, and 0.021 ng/ml.

H.2.6. Quantifying IgE Binding and Data Reduction

Plates were read bi-chromatically at 450 nm with a 630 nm reference wavelength. Optical density (OD) values recorded at 630 nm were subtracted from OD values recorded at 450 nm for each well to produce reduced OD values using Softmax Pro® software (Molecular Devices; version 5.2 Rev C). Mean values of triplicate ODs from each sample were calculated. To calculate a limit of detection (LOD) for the standard curve (LOD1), the mean OD values for non-specific binding reagent control (NSB1) added to the wells coated with internal non-transgenic reference soybean extract were subtracted from the OD values obtained for the non-allergic serum pool added to the wells coated with internal non-transgenic reference soybean extract (designated as NA1). For NA1 the standard deviation (SD) of the calculated mean OD values was determined. The LOD1 was calculated as follows: LOD1 = [Mean OD (NA1) + 3 × SD (NA1)] – Mean OD (NSB1). The obtained OD values were interpolated versus the standard curve and expressed as ng/ml of IgE. For each test, control, and reference substance extract, a specific LOD was calculated (LOD2). Mean OD values for non-specific binding reagent control added to the wells coated with tested soybean extracts (designated as NSB2) were subtracted from the mean OD values obtained for non-allergic serum pool added to the wells coated with tested soybean extracts (designated as NA2). For NA2, the SD of the calculated mean OD values was determined. The LOD2 was calculated as follows: LOD2 = [OD (NA2) + 3 × SD (NA2)] - OD (NSB2). The obtained OD values were interpolated versus the standard curve and expressed as ng/ml of IgE.

All data on each ELISA plate were normalized for non-specific binding reagent control and for IgE binding to the non-allergic serum pool.

H.2.7. ELISA Acceptance Criteria

The following criteria were applied to ELISA performance and used to determine if the assay was generating acceptable data:

a) Standard curve: maximum OD value (ODmax) is≥ 1.5 absorbance units. The LOD1 is ≤ 0.2 ng/ml (at 1:10 dilution).

b) Positive control serum PEI 46 quantified at 3.31 ng/ml soybean-specific IgE with a CV for inter-assay precision of less than 25 % (range 2.48 – 4.14 ng/ml).

c) The minimum LOQ must be greater than LOD1 and LOD2.

d) The soybean-specific serum IgE levels determined for the soybean sample extracts were considered “positive” if the calculated IgE concentrations were larger than LOD1

and LOD2, and if the %CV for each triplicate was ≤ 25 %. Sera not meeting these criteria were considered to be "negative" for the ELISA assay.

H.2.8. Statistical Analysis of ELISA Results

Data evaluation was based on the IgE concentrations in each serum calculated for each extract. Values that failed to satisfy the ELISA acceptance criteria were treated as missing values for the purpose of the statistical analysis.

The proposed statistical model for the analysis was a randomized complete block design model with serum as the block and MON 87708, conventional control, and commercial reference varieties as the treatments. The test for non-additivity was done using Tukey's one degree of freedom test for non-additivity (Snedecor and Cochran, 1980). The test was conducted using a SAS® macro developed by Oliver Schabenberger, SAS Institute (1997). The non-additivity test p-value < 0.0001 rejected the additivity assumption and thus a randomized complete block design could not be used to analyze the data and consequently an alternate analysis was done.

The alternate statistical analysis consisted of calculating a 99% tolerance interval with 95% confidence for individual sera using the IgE binding values obtained for the commercial soybean reference varieties soybean extracts. MON 87708 and conventional control IgE binding values were then compared to the tolerance interval (Figure VI-19).

H.2.9. Control of Bias in Test Design and Statistical Analyses

Inclusion of a standard curve, positive and negative controls, and a control for inter-assay precision on each ELISA plate, in addition to the tested soybean extracts, served as a control of bias in this study.

H.2.10. ELISA Results

The results of the quantitative ELISA assays are summarized in Table H-3. Sera from 13 soybean-allergic subjects yielded positive IgE values for all of the soybean extracts and were included in the statistical analysis. Sera MS01, MS02, MS03, MS04, MS10, MS12, and MS15 had IgE binding values below the LOQ for at least three reference extracts (Table H-4). Therefore, IgE binding values from these sera were considered an incomplete data set and were excluded from the statistical analysis. One IgE value was deemed as a statistical outlier for serum ME03, this included a value against a commercial soybean reference variety (soybean extract #3). This serum had substantially more variability than the other sera even after removal of this outlier. None of the results for MON 87708, conventional control, and commercial soybean reference varieties showed IgE binding to sera from non-allergic subjects; therefore, these data were not submitted for statistical analysis.

To compare IgE binding values for each of the 13 positive sera, the ELISA values generated for the MON 87708, conventional control, and commercial soybean reference varieties were subjected to a statistical data evaluation. The IgE binding values obtained for the 17 commercial reference varieties soybean extracts were used to calculate a 99%

tolerance interval for each subject's serum. The 99% tolerance interval represents the range of IgE binding for each subject's serum to the commercial soybean reference varieties soybean extracts. The tolerance interval describes the value range that includes 99% of the IgE binding values and has a statistically predicted 95% confidence level. The IgE binding values obtained for extracts prepared from MON 87708 and control were compared to the tolerance interval derived for each serum. All of the IgE binding values observed for MON 87708 and control were within the commercial reference varieties tolerance limits for each subject's serum (Figure VI-18). None of the soybean varieties showed IgE binding to sera from non-allergic subjects.

Table H-3. Soybean-Specific IgE Bound to Protein Extracts Prepared from MON 87708, Conventional Control, and Commercial Reference Materials for Soybean Allergic Sera

	Serum									
	KB 1	KB 2	ME 1	ME 2	ME 3	MS05	MS06	LOQ	LOD1	LOD2
Extract								ng/ml at 1:10 dilution		
1	0.677	3.686	57.419	200.754	304.025	0.720	7.364	0.035	0.034	0.028
2	0.675	3.272	60.357	205.103	332.570	0.551	7.372	0.042	0.041	0.038
3	0.552	2.589	58.930	154.827	158.722	0.477	5.999	0.028	0.027	0.023
4	0.684	1.720	60.012	167.993	186.808	0.696	6.276	0.034	0.033	0.030
5	0.610	2.933	60.000	166.297	198.268	0.626	6.838	0.035	0.034	0.022
6	0.820	3.666	71.013	194.110	221.817	0.806	6.473	0.068	0.034	0.067
7	0.909	4.172	72.189	196.566	227.815	0.849	6.030	0.042	0.038	0.041
8	0.766	3.369	66.549	187.269	234.202	0.665	6.244	0.032	0.031	0.025
9	0.687	1.508	71.386	156.588	234.571	0.677	6.704	0.046	0.045	0.030
10	0.839	3.486	90.108	231.531	353.648	0.752	7.519	0.049	0.048	0.044
11	0.862	1.828	73.225	166.311	257.072	0.840	6.643	0.040	0.039	0.034
12	0.840	3.123	62.901	217.216	264.275	0.905	5.658	0.043	0.042	0.041
13	1.044	4.009	74.894	176.490	352.677	0.914	6.865	0.051	0.044	0.050
14	0.693	4.326	62.627	199.664	279.766	0.791	6.074	0.050	0.049	0.040
15	0.829	3.580	66.639	217.984	321.875	0.790	5.809	0.053	0.052	0.040
16	0.796	4.180	74.802	205.931	272.795	0.826	6.865	0.045	0.044	0.035
17	0.631	3.311	75.616	241.355	264.880	0.627	6.027	0.061	0.060	0.032
22	0.898	4.687	76.520	248.056	326.365	0.860	6.980	0.039	0.038	0.033
23	0.707	4.605	69.853	230.358	280.647	0.747	6.698	0.039	0.038	0.031

Table H-3 (continued). Soybean-Specific IgE Bound to Protein Extracts Prepared from MON 87708, Conventional Control, and Commercial Reference Varieties for Soybean Allergic Sera

	Serum								
	MS07	**MS08**	**MS09**	**MS11**	**MS13**	**MS14**	**LOQ**	**LOD1**	**LOD2**
Extract							ng/ml at 1:10 dilution		
1	4.440	6.594	12.515	12.774	1.628	1.211	0.035	0.034	0.028
2	2.133	7.019	15.624	17.410	1.158	1.124	0.042	0.041	0.038
3	3.292	6.336	11.983	11.589	1.362	1.008	0.028	0.027	0.023
4	5.673	6.530	13.982	10.261	1.074	1.409	0.034	0.033	0.030
5	3.953	6.321	13.037	13.260	1.425	1.041	0.035	0.034	0.022
6	6.279	7.432	14.021	14.077	1.715	1.335	0.068	0.034	0.067
7	5.488	7.426	13.560	11.301	1.860	1.552	0.042	0.038	0.041
8	6.032	7.058	13.985	11.825	1.547	1.255	0.032	0.031	0.025
9	2.345	7.097	18.217	9.757	1.624	1.184	0.046	0.045	0.030
10	5.206	7.253	14.293	12.427	1.683	1.315	0.049	0.048	0.044
11	3.682	7.510	14.286	8.547	1.722	1.374	0.040	0.039	0.034
12	3.566	6.881	14.770	9.666	1.734	1.314	0.043	0.042	0.041
13	5.002	7.977	16.996	11.662	1.990	1.573	0.051	0.044	0.050
14	4.965	7.150	14.481	12.643	1.688	1.094	0.050	0.049	0.040
15	4.987	6.978	15.204	11.328	1.687	1.215	0.053	0.052	0.040
16	5.075	7.134	13.996	13.113	1.697	1.318	0.045	0.044	0.035
17	4.407	6.273	14.639	10.668	1.479	1.069	0.061	0.060	0.032
22	5.032	8.095	16.411	13.290	1.943	1.360	0.039	0.038	0.033
23	3.900	7.308	14.819	13.873	1.675	1.138	0.039	0.038	0.031

Table H-4. Soybean-Specific IgE Bound to Protein Extracts Prepared from MON 87708, Conventional Control, and Commercial Reference Materials for Soybean Allergic Sera that were Considered Negative[1]

	Serum									
	MS01	**MS02**	**MS03**	**MS04**	**MS10**	**MS12**	**MS15**	**LOQ**	**LOD1**	**LOD2**
Extract	ng/ml at 1:10 dilution							ng/ml at 1:10 dilution		
1	0.027	0.013	0.033	0.042	0.026	0.011	0.014	0.035	0.034	0.028
2	0.032	0.023	0.038	0.043	0.038	0.015	0.028	0.042	0.041	0.038
3	0.021	0.011	0.024	0.032	0.023	0.004	0.010	0.028	0.027	0.023
4	0.027	0.011	0.028	0.047	0.039	0.010	0.013	0.034	0.033	0.030
5	0.020	0.007	0.025	0.035	0.026	0.000	0.008	0.035	0.034	0.022
6	0.032	0.014	0.036	0.054	0.037	0.006	0.014	0.068	0.034	0.067
7	0.033	0.014	0.042	0.066	0.040	0.007	0.020	0.042	0.038	0.041
8	0.025	0.008	0.033	0.046	0.033	n/a	0.014	0.032	0.031	0.025
9	0.030	0.016	0.037	0.043	0.040	0.008	0.012	0.046	0.045	0.030
10	0.033	0.021	0.042	0.055	0.036	0.013	0.019	0.049	0.048	0.044
11	0.028	0.022	0.039	0.056	0.037	0.012	0.016	0.040	0.039	0.034
12	0.036	0.019	0.043	0.065	0.049	0.011	0.017	0.043	0.042	0.041
13	0.047	0.028	0.050	0.071	0.059	0.017	0.030	0.051	0.044	0.050
14	0.028	0.015	0.037	0.051	0.039	0.005	0.017	0.050	0.049	0.040
15	0.037	0.020	0.043	0.064	0.047	0.012	0.018	0.053	0.052	0.040
16	0.027	0.015	0.038	0.060	0.045	0.007	0.017	0.045	0.044	0.035
17	0.015	0.001	0.021	0.040	0.031	n/a	0.009	0.061	0.060	0.032
22	0.033	0.018	0.041	0.054	0.042	0.009	0.023	0.039	0.038	0.033
23	0.030	0.014	0.037	0.051	0.033	0.007	0.014	0.039	0.038	0.031

n/a – Value not reportable due to reduced OD value < 0.00.

[1]Below LOQ for 3 or more reference soybean extracts**.**

References for Appendix H

Snedecor, G.W. and W.G. Cochran. 1980. One-way classifications; Analysis of variance. Pages 214-237 in Statistical Methods. Seventh Edition. Iowa State University Press, Ames, Iowa.

Appendix I: Materials, Methods, and Individual Site Results for Compositional Analysis of MON 87708 Soybean Seed and Forage

Compositional comparisons between MON 87708 and the near isogenic conventional soybean control A3525 were performed using the principles and analytes outlined in the OECD consensus documents for soybean composition (OECD, 2001). These principles are accepted globally and have been employed previously in assessments of soybean products derived through biotechnology. The compositional assessment was conducted on seed and forage samples harvested from a single growing season conducted in the U.S. during 2008 under typical agronomic practices.

The materials and methods for compositional analysis, as well as the individual-site results (Tables I-4 to I-18), are provided below.

I.1. Materials

Forage and harvested seed from MON 87708, a near isogenic conventional soybean control A3525 that has similar genetic background to that of MON 87708, and commercial reference varieties were compositionally assessed. The commercial reference varieties are listed in Table I-1.

Table I-1. Commercial Reference Varieties

Material Name	Seed Lot#	Field Site Code
CST3461	10000890	IARL
Wilken 3316	10001505	IARL
Midland 363	10001570	IARL
Stine 3300-0	10001312	IARL
Croplan HT3596STS	10001450	ILWY
FS 3591	10001448	ILWY
Garst 3585N	10000883	ILWY
Pioneer 93M52	10001311	ILWY
Stine 3608-0	10001392	ILCY
Quality Plus 365C	10001608	ILCY
Crows C37003N	10001508	ILCY
NK S38-T8	10001509	ILCY
Lewis 372	10001475	INRC
Pioneer 93M50	10000888	INRC
Dekalb DKB34-51	10000889	INRC
Stewart SB3454	10000887	INRC
Dekalb DKB31-51	10001285	PAHM
NK 32Z3	10001607	PAHM
Hoegemeyer 333	10001590	PAHM
Pioneer 93B15	10001304	PAHM

I.2. Characterization of the Materials

The identities of the forage and seed samples from MON 87708, the conventional control, and the commercial reference varieties were verified by the Study Director prior to the study by confirming the chain-of-custody documentation supplied with the forage and seed harvested from the field sites. The seed of MON 87708, the conventional control, and the commercial reference varieties were characterized by event-specific polymerase chain reaction (PCR) analysis to confirm the presence or absence of the *dmo* expression cassette.

I.3. Field Production of the Samples

Harvested seed and forage of MON 87708, the conventional control, and the commercial reference varieties were collected from five replicated sites in the U.S. during the 2008 growing season. These sites are Jefferson County, Iowa (IARL); Stark County, Illinois (ILWY); Clinton County, Illinois (ILCY); Parke County, Indiana (INRC); and Berks County, Pennsylvania (PAHM). Starting seeds were planted in a randomized complete block design with three plots for each of MON 87708, the conventional control, and the commercial reference varieties. The production was conducted under normal agronomic field conditions. All soybean plants including MON 87708, the conventional control, and commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition, MON 87708 plots were treated at the V2-V3 growth stage with dicamba herbicide at the target label rate (0.5 lb/Acre a.e.). Seed and forage samples were harvested from all plots and shipped on dry ice (forage) or at ambient temperature (harvested seed) to Monsanto Company, St. Louis, MO. A subsample for compositional analysis was obtained for each tissue sample collected. 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, WI) for analysis.

I.4. Summary of Analytical Methods

Nutrients assessed in this study included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), fiber, amino acids (18 components), fatty acids (FA, C8-C22), and vitamin E (α-tochopherol) in seed, and proximates (ash, carbohydrates by calculation, moisture, protein, and fat) and fiber in forage. Anti-nutrients assessed in seed included raffinose, stachyose, lectin, phytic acid, trypsin inhibitors, and isoflavones (daidzein, genistein, and glycitein).

All compositional analyses were performed at Covance Laboratories, Inc. (Madison, WI). Methods for analysis were based on internationally-recognized procedures and literature publications. Brief descriptions of the methods utilized for the analyses are described below.

I.5. Analytical Method Summaries and Reference Standards

I.5.1. Acid Detergent Fiber

The sample was placed in a fritted vessel and washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. An acetone wash removed the fats and pigments. The lignocellulose fraction was collected on the frit and determined gravimetrically (Goering and Van Soest, 1970). The limit of quantitation (LOQ) for this analysis was 0.100%.

I.5.2. Amino Acid Composition

The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct acid hydrolysis with hydrochloric acid. Once hydrolyzed, the individual amino acids were then quantitated using an automated amino acid analyzer (AOAC-International, 2005a). The LOQ for this analysis was 0.100 mg/g.

Reference Standards:

- ThermoScientific K18, 2.5 µmol/mL per constituent except cystine (1.25 µmol/mL), Lot Number JK126327
- Sigma, L-Tryptophan, 100%, Lot Number 076K0075
- Sigma/BioChemika, L-Cysteic Acid Monohydrate, 99.5% (used as 100%), Lot Number 1305674
- Sigma, L-Methionine Sulfone, 100%, Lot Number 047K1321

I.5.3. Ash

The sample was placed in an electric furnace at 550 °C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash (AOAC-International, 2005b). The LOQ for this analysis was 0.100%.

I.5.4. Carbohydrates

The total carbohydrate level was calculated by difference using the fwt-derived data and the following equation (USDA, 1973):

% carbohydrates = 100 % - (% protein + % fat + % moisture + % ash)

The LOQ for this analysis was 0.100%.

I.5.5. 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-International, 2005c). The limit of quantitation for this study was 0.100%.

I.5.6. Fat by Acid Hydrolysis

The sample was hydrolyzed with hydrochloric acid at an elevated temperature. The fat was extracted with ether and hexane. The extract was evaporated on a steambath, re-dissolved in hexane and filtered through a sodium sulfate column. The hexane extract was then evaporated again on a steambath under nitrogen, dried, and weighed (AOAC-International, 2005d). The LOQ for this analysis was 0.100%.

I.5.7. 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-International, 2005e). The LOQ for this analysis was 0.100%.

I.5.8. Fatty Acids

The lipid was extracted and saponified with 0.5 N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride in methanol. The resulting methyl esters 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 (AOAC-International, 2005f; AOCS, 1997a; AOCS, 2001). The limit of quantitation was 0.0200%.

Reference Standards:

- Nu Chek Prep GLC Reference Standard Hazleton No. 1, *, Lot Number AU18-S
- Nu Chek Prep GLC Reference Standard Hazleton No. 2, *, Lot Number M13-O
- Nu Chek Prep GLC Reference Standard Hazleton No. 3, *, Lot Number MA18-S
- Nu Chek Prep GLC Reference Standard Hazleton No. 4, *, Lot Number JA16-T
- Nu Chek Prep Methyl Gamma Linolenate, used as 100%, Lot Number U-63M-JY12-R
- Nu Chek Prep Methyl Tridecanoate, used as 100%, Lot Number N-13M-JA16-T

* Overall purity of the sum of the mixture of components is used as 100%.

I.5.9. Isoflavones

The sample was extracted using a solution of hydrochloric acid and reagent alcohol heated on steam baths or hot plates. The extract was brought to volume, diluted, and centrifuged. An aliquot of the supernatant was placed onto a C18 solid-phase extraction column. Unwanted components of the matrix were rinsed off with 20% methanol and then the isoflavones were eluted with 80% methanol. The sample was analyzed on a

high-performance liquid chromatography system with ultraviolet detection and was compared to an external standard curve of known standards for quantitation (Pettersson and Kiessling, 1984; Seo and Morr, 1984). The LOQ for each component was 10.0 ppm (µg/g).

Reference Standards:

- Chromadex, Daidzein, 96.5%, Lot Number 04007-120
- Chromadex, Glycitein, 96.3%, Lot Number 07344-571
- Indofine, Genistein, ≥99% (100% used in calculations), Lot Number 0309074

I.5.10. Lectin

The sample was suspended in phosphate buffered saline (PBS), shaken, and filtered. An aliquot of the resulting extract was serially diluted in 10 cuvettes containing PBS. A 10% hematocrit of lyophilized rabbit blood in PBS was added to each dilution. After 2.5 hours, the absorbance of each dilution of the sample and lectin control was measured on a spectrophotometer at 620 nm, using PBS to zero the instrument. One hemagglutinating unit (H.U.) was defined as the level that caused 50% of the standard cell suspension to sediment in 2.5 hours (Klurfeld and Kritchevsky, 1987; Liener, 1955). The LOQ for this analysis was 0.10 H.U./mg.

Reference Standard:

- Sigma-Aldrich, Red Blood Cells, Rabbit, Product #R1629, Lot Number 105K6042

I.5.11. 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-International, 2005g). The LOQ for this analysis was 0.100%.

I.5.12. Neutral Detergent Fiber, Enzyme Method

The sample was placed in a fritted vessel and washed with a neutral boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. An acetone wash removed the fats and pigments. Hemicellulose, cellulose, and lignin fractions were collected on the frit and determined gravimetrically (AACC, 1998; Goering and Van Soest, 1970). The LOQ for this analysis was 0.100%.

I.5.13. Phytic Acid

The sample was extracted using 0.5 M HCl with ultrasonication. Purification and concentration were accomplished on a silica-based anion-exchange column. The sample was analyzed on a polymer high-performance liquid chromatography column PRP-1, 5 µm (150 × 4.1 mm) with a refractive index detector (Lehrfeld, 1989; Lehrfeld, 1994). The LOQ for this analysis was 0.100%.

Reference Standard:

- Aldrich, Phytic Acid, Dodecasodium Salt Hydrate, 98%, Lot Number 068K0755

I.5.14. Protein

Nitrogenous compounds in the sample were reduced in the presence of boiling sulfuric acid and a mercury catalyst mixture to form ammonia. 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-International, 2005h; Bradstreet, 1965; Kalthoff and Sandell, 1948). The LOQ for this analysis was 0.100%.

I.5.15. Raffinose and Stachyose

The sample was extracted with deionized water and the extract treated with a hydroxylamine hydrochloride solution in pyridine, containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector (Brobst, 1972; Mason and Slover, 1971). The LOQ for this analysis was 0.0500%.

Reference Standards:

- Sigma, D-(+)-Raffinose Pentahydrate, 95.5% after correction for degree of hydration, Lot Number 037K1059
- Sigma, Stachyose, 97.1% after correction for degree of hydration, Lot Number 078K3802

I.5.16. Trypsin Inhibitor

The sample was ground and defatted with petroleum ether. A sample of matrix was extracted with 0.01 N sodium hydroxide. Varying aliquots of the sample suspension were exposed to a known amount of trypsin and benzoy1-DL-arginine~p~nitroanilide hydrochloride. The sample was allowed to react for 10 minutes at 37°C. After 10 minutes, the reaction was halted by the addition of acetic acid. The solution was centrifuged and then the absorbance was determined at 410 nm. Trypsin inhibitor activity was determined by photometrically measuring the inhibition of trypsin's reaction with benzoyl-DL-arginine~p~nitroanilide hydrochloride (AOCS, 1997b). The LOQ for this analysis was 1.00 Trypsin Inhibitor Units (TIU)/mg.

I.5.17. 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 quantitated by high-performance liquid chromatography using a silica column (Cort et al., 1983; McMurray et al., 1980; Speek et al., 1985). The LOQ for this analysis was 0.500 mg/100g.

Reference Standard:

- USP, Alpha Tocopherol, 100%, Lot Number M

I.5. Data Processing and Statistical Analysis

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

The following formulas were used for re-expression of soybean composition data for statistical analysis (Table I-2):

Table I-2. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)	To	Formula[1]
Proximates (excluding Moisture), Fiber, Phytic Acid, Raffinose, Stachyose	% fwt	% dwt	X/d
Isoflavones	µg/g fwt	µg/g dwt	X/d
Lectin	H.U./fwt	H.U./dwt	X/d
Trypsin Inhibitor	TIU/mg fwt	TIU/mg dwt	X/d
Vitamin E	mg/100g fwt	mg/100g dwt	X/d
Amino Acids (AA)	mg/g fwt	% dwt	X/(10d)
Fatty Acids (FA)	% fwt	% Total FA	$(100)X_j/\Sigma X$, for each FA_j where ΣX is over all the FA

[1]'X' is the individual sample value; 'd' is the fraction of the sample that is dry matter.

In order to complete a statistical analysis for a compositional analyte, at least 50% of the values for an analyte had to be greater than the assay LOQ. The following 14 analytes with more than 50% of observations below the assay LOQ were excluded from statistical analysis: 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 16:1 palmitoleic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma-linolenic acid, 20:3 eicosatrienoic acid, 20:2 eicosadienoic 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. The following analyte was assigned a value (Table I-3):

Table I-3. Component with Observations Below the Assay Limit of Quantitation Not Excluded from Statistical Analysis

		Obs. Below LOQ		Total		Value
Component	Units	N	(%)	N	LOQ	Assigned
Seed Fatty Acid						
20:1 Eicosenoic	% fwt	45	42.9	105	0.020	0.010

The data were assessed for potential outliers using a studentized PRESS residuals calculation. A PRESS residual is the difference between any value and its predicted value from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ± 3. Extreme data points that are also outside of the ± 6 studentized PRESS residual range are considered for exclusion, as outliers, from the final analyses. No results had PRESS residual values outside of the ± 6 range.

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

(1) $Y_{ij} = U + T_i + B_j + e_{ij},$

where Y_{ij} = unique individual observation, U = overall mean, T_i = substance effect, B_j = random block effect, and e_{ij} = residual error.

Combined-site analyses used model *(2)*.

(2) $Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk},$

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.

A range of observed values from the reference varieties was determined for each analytical component. Additionally, data from the reference varieties were used to develop tolerance intervals. A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured.

For each compositional component, 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of commercial reference varieties. 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 I-4. Statistical Summary of Site IARL Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	5.30 (0.070) (5.20 - 5.37)	5.29 (0.070) (5.19 - 5.47)	0.0063 (0.098) (-0.27 - 0.17)	-0.27, 0.28	0.952	4.74, 6.01 (4.93 - 5.88)
Carbohydrates	38.55 (0.56) (38.03 - 39.11)	37.97 (0.56) (37.17 - 38.45)	0.57 (0.55) (-0.27 - 1.33)	-0.96, 2.11	0.358	32.07, 40.08 (33.82 - 39.26)
Moisture (% fwt)	7.13 (0.28) (6.92 - 7.27)	6.07 (0.28) (5.84 - 6.36)	1.06 (0.39) (0.56 - 1.43)	-0.033, 2.15	0.054	4.27, 9.58 (5.50 - 9.23)
Protein	40.92 (0.27) (40.40 - 41.41)	41.09 (0.27) (40.69 - 41.74)	-0.18 (0.16) (-0.33 - 0.088)	-0.63, 0.28	0.342	35.50, 45.19 (37.06 - 43.42)
Total Fat	15.22 (0.47) (14.77 - 15.62)	15.61 (0.47) (15.38 - 15.82)	-0.40 (0.66) (-1.05 - -0.017)	-2.23, 1.43	0.579	12.33, 24.10 (15.47 - 21.34)
Fiber (% dwt)						
Acid Detergent Fiber	13.03 (0.38) (12.68 - 13.58)	12.60 (0.38) (11.92 - 13.17)	0.43 (0.54) (-0.34 - 1.66)	-1.08, 1.94	0.472	10.06, 18.04 (12.07 - 17.46)

Table I-4 (continued). Statistical Summary of Site IARL Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Crude Fiber	7.71 (0.33) (7.26 - 8.48)	7.41 (0.33) (7.17 - 7.60)	0.30 (0.46) (-0.34 - 1.03)	-0.98, 1.58	0.545	5.76, 10.76 (6.35 - 11.31)
Neutral Detergent Fiber	14.25 (0.89) (13.11 - 16.38)	13.27 (0.89) (11.81 - 14.42)	0.98 (1.25) (-1.31 - 4.57)	-2.51, 4.46	0.479	11.36, 19.38 (11.66 - 19.45)
Amino Acid (% dwt)						
Alanine	1.72 (0.026) (1.66 - 1.76)	1.74 (0.026) (1.69 - 1.77)	-0.027 (0.036) (-0.11 - 0.042)	-0.13, 0.074	0.502	1.56, 1.91 (1.59 - 1.86)
Arginine	3.28 (0.055) (3.26 - 3.30)	3.45 (0.055) (3.27 - 3.56)	-0.17 (0.061) (-0.26 - -0.013)	-0.33, 0.0040	0.053	2.55, 3.83 (2.88 - 3.74)
Aspartic Acid	4.55 (0.060) (4.44 - 4.63)	4.63 (0.060) (4.46 - 4.74)	-0.076 (0.084) (-0.29 - 0.12)	-0.31, 0.16	0.416	4.04, 5.13 (4.22 - 4.94)
Cystine	0.61 (0.0094) (0.60 - 0.62)	0.59 (0.0094) (0.56 - 0.62)	0.018 (0.013) (-0.0056 - 0.053)	-0.019, 0.054	0.257	0.50, 0.68 (0.53 - 0.66)

Table I-4 (continued). Statistical Summary of Site IARL Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Glutamic Acid	7.24 (0.10) (7.05 - 7.38)	7.41 (0.10) (7.12 - 7.58)	-0.16 (0.15) (-0.53 - 0.17)	-0.57, 0.25	0.332	6.28, 8.30 (6.69 - 7.92)
Glycine	1.72 (0.024) (1.67 - 1.76)	1.76 (0.024) (1.70 - 1.79)	-0.037 (0.034) (-0.12 - 0.042)	-0.13, 0.057	0.331	1.53, 1.92 (1.58 - 1.84)
Histidine	1.04 (0.014) (1.02 - 1.06)	1.06 (0.014) (1.02 - 1.08)	-0.018 (0.019) (-0.066 - 0.031)	-0.071, 0.036	0.408	0.93, 1.16 (0.95 - 1.13)
Isoleucine	1.84 (0.038) (1.75 - 1.90)	1.88 (0.038) (1.79 - 1.94)	-0.036 (0.054) (-0.16 - 0.11)	-0.19, 0.11	0.540	1.65, 2.06 (1.68 - 2.02)
Leucine	3.01 (0.041) (2.93 - 3.07)	3.07 (0.041) (2.96 - 3.14)	-0.055 (0.058) (-0.21 - 0.072)	-0.22, 0.11	0.401	2.72, 3.39 (2.80 - 3.27)
Lysine	2.60 (0.030) (2.53 - 2.64)	2.62 (0.030) (2.54 - 2.66)	-0.017 (0.042) (-0.12 - 0.090)	-0.13, 0.10	0.710	2.33, 2.84 (2.38 - 2.74)

Table I-4 (continued). Statistical Summary of Site IARL Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Methionine	0.58 (0.013) (0.56 - 0.60)	0.56 (0.013) (0.53 - 0.60)	0.016 (0.019) (-0.022 - 0.071)	-0.037, 0.068	0.452	0.50, 0.64 (0.52 - 0.63)
Phenylalanine	1.98 (0.036) (1.92 - 2.04)	2.03 (0.036) (1.95 - 2.09)	-0.052 (0.050) (-0.17 - 0.023)	-0.19, 0.087	0.357	1.80, 2.30 (1.85 - 2.21)
Proline	1.92 (0.035) (1.90 - 1.96)	1.98 (0.035) (1.89 - 2.07)	-0.063 (0.050) (-0.17 - 0.065)	-0.20, 0.076	0.274	1.65, 2.26 (1.74 - 2.16)
Serine	1.98 (0.044) (1.92 - 2.03)	2.00 (0.044) (1.95 - 2.08)	-0.024 (0.036) (-0.087 - 0.047)	-0.12, 0.076	0.545	1.78, 2.27 (1.90 - 2.18)
Threonine	1.53 (0.023) (1.48 - 1.56)	1.54 (0.023) (1.51 - 1.58)	-0.011 (0.023) (-0.051 - 0.052)	-0.075, 0.053	0.653	1.40, 1.69 (1.47 - 1.64)
Tryptophan	0.45 (0.0092) (0.45 - 0.46)	0.45 (0.0092) (0.44 - 0.47)	0.00085 (0.013) (-0.0079 - 0.0069)	-0.035, 0.037	0.950	0.38, 0.52 (0.39 - 0.50)

Table I-4 (continued). Statistical Summary of Site IARL Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Tyrosine	1.34 (0.013) (1.32 - 1.37)	1.38 (0.013) (1.37 - 1.42)	-0.042 (0.016) (-0.079 - -0.00044)	-0.087, 0.0035	0.062	1.24, 1.50 (1.26 - 1.49)
Valine	1.95 (0.046) (1.82 - 2.03)	1.99 (0.046) (1.90 - 2.05)	-0.042 (0.065) (-0.20 - 0.13)	-0.22, 0.14	0.552	1.72, 2.20 (1.73 - 2.13)
Fatty Acid (% Total FA)						
16:0 Palmitic	11.49 (0.051) (11.44 - 11.54)	11.00 (0.051) (10.92 - 11.08)	0.49 (0.062) (0.39 - 0.62)	0.32, 0.66	0.001	8.44, 12.56 (9.40 - 11.54)
18:0 Stearic	4.06 (0.067) (3.99 - 4.19)	4.00 (0.067) (3.99 - 4.01)	0.059 (0.095) (-0.016 - 0.20)	-0.21, 0.32	0.568	2.90, 5.19 (3.24 - 4.67)
18:1 Oleic	19.38 (0.20) (19.07 - 19.73)	21.67 (0.20) (21.48 - 21.78)	-2.29 (0.28) (-2.71 - -1.75)	-3.07, -1.52	0.001	15.73, 27.19 (17.88 - 25.31)
18:2 Linoleic	53.85 (0.33) (53.42 - 54.07)	52.70 (0.33) (52.66 - 52.73)	1.16 (0.46) (0.68 - 1.41)	-0.13, 2.44	0.066	48.61, 59.37 (50.95 - 56.68)

Table I-4 (continued). Statistical Summary of Site IARL Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
18:3 Linolenic	10.64 (0.13) (10.58 - 10.74)	10.04 (0.13) (10.00 - 10.12)	0.60 (0.19) (0.46 - 0.74)	0.074, 1.12	0.033	6.01, 12.58 (7.43 - 11.37)
20:0 Arachidic	0.25 (0.0038) (0.25 - 0.26)	0.25 (0.0038) (0.25 - 0.26)	0.0016 (0.0054) (-0.0021 - 0.0080)	-0.013, 0.017	0.777	0.19, 0.34 (0.20 - 0.30)
20:1 Eicosenoic	0.073 (0.0020) (0.071 - 0.075)	0.070 (0.0020) (0.069 - 0.071)	0.0030 (0.0028) (0.0011 - 0.0062)	-0.0048, 0.011	0.348	0.022, 0.24 (0.065 - 0.17)
22:0 Behenic	0.26 (0.0035) (0.25 - 0.27)	0.28 (0.0035) (0.27 - 0.28)	-0.015 (0.0042) (-0.023 - -0.0038)	-0.027, -0.0036	0.022	0.24, 0.40 (0.28 - 0.36)
Vitamin (mg/100g dwt)						
Vitamin E	1.15 (0.058) (1.10 - 1.22)	0.94 (0.058) (0.89 - 0.97)	0.21 (0.066) (0.18 - 0.24)	0.027, 0.39	0.033	0, 3.49 (0.69 - 2.91)

[1]dwt = dry weight; fwt = fresh weight; FA = fatty acid.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-5. Statistical Summary of Site IARL Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dwt)						
Lectin (H.U./mg dwt)	2.04 (0.64) (0.80 - 3.44)	1.53 (0.64) (0.46 - 2.70)	0.52 (0.90) (-0.81 - 2.98)	-1.98, 3.02	0.595	0, 7.73 (0.68 - 8.34)
Phytic Acid	1.36 (0.032) (1.33 - 1.38)	1.53 (0.032) (1.47 - 1.62)	-0.17 (0.045) (-0.29 - -0.10)	-0.30, -0.048	0.018	0.77, 1.91 (1.00 - 1.64)
Raffinose	0.38 (0.022) (0.34 - 0.42)	0.43 (0.022) (0.40 - 0.45)	-0.051 (0.020) (-0.10 - -0.018)	-0.11, 0.0034	0.059	0.13, 0.70 (0.26 - 0.59)
Stachyose	3.61 (0.20) (3.15 - 4.02)	3.89 (0.20) (3.76 - 4.15)	-0.29 (0.28) (-1.00 - 0.26)	-1.06, 0.49	0.365	2.30, 4.07 (2.50 - 3.94)
Trypsin Inhibitor (TIU/mg dwt)	33.59 (2.63) (29.66 - 37.60)	29.67 (2.63) (25.64 - 32.71)	3.92 (3.19) (-3.05 - 7.87)	-4.94, 12.79	0.286	22.05, 41.12 (22.81 - 44.56)
Isoflavone (µg/g dwt)						
Daidzein	1489.23 (145.92) (1175.46 - 1654.49)	1447.97 (145.92) (1404.40 - 1505.77)	41.27 (206.36) (-258.27 - 233.35)	-531.67, 614.20	0.851	0, 2271.38 (451.33 - 2033.05)

Table I-5 (continued). Statistical Summary of Site IARL Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Isoflavone (µg/g dwt)						
Genistein	1050.87 (83.29) (1019.09 - 1070.05)	954.99 (83.29) (900.10 - 984.62)	95.88 (117.79) (38.84 - 163.37)	-231.16, 422.92	0.461	78.36, 1869.48 (533.88 - 1726.03)
Glycitein	108.39 (5.35) (97.06 - 117.44)	106.40 (5.35) (97.49 - 111.71)	1.98 (2.24) (-0.44 - 5.73)	-4.23, 8.20	0.425	31.24, 233.60 (73.61 - 231.75)

[1]dwt= dry weight; H.U. = Hemagglutinating Units; TIU = Trypsin Inhibitor Units.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-6. Statistical Summary of Site IARL Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	9.44 (0.62) (9.14 - 9.65)	8.87 (0.62) (7.58 - 10.46)	0.57 (0.52) (-0.81 - 1.56)	-0.88, 2.03	0.334	3.36, 10.84 (5.20 - 9.81)
Carbohydrates	63.02 (0.80) (62.21 - 63.79)	65.57 (0.80) (63.58 - 67.74)	-2.55 (1.13) (-3.95 - -1.37)	-5.69, 0.60	0.087	60.69, 73.46 (62.73 - 71.72)
Moisture (% fwt)	82.60 (0.33) (82.40 - 82.80)	81.97 (0.33) (81.40 - 82.70)	0.63 (0.44) (0.10 - 1.20)	-0.59, 1.86	0.223	62.08, 89.80 (70.40 - 84.10)
Protein	25.21 (0.54) (24.71 - 25.52)	23.00 (0.54) (22.15 - 24.07)	2.21 (0.76) (1.33 - 2.75)	0.098, 4.33	0.043	15.69, 26.63 (18.50 - 25.86)
Total Fat	2.30 (0.32) (2.00 - 2.59)	2.52 (0.32) (2.01 - 3.27)	-0.22 (0.46) (-0.68 - 0.042)	-1.49, 1.06	0.662	0, 10.04 (1.57 - 7.99)
Fiber (% dwt)						
Acid Detergent Fiber	40.04 (2.83) (32.90 - 45.11)	32.62 (2.83) (28.87 - 38.15)	7.42 (3.30) (2.07 - 16.24)	-1.75, 16.59	0.088	16.54, 41.80 (20.98 - 39.23)

Table I-6 (continued). Statistical Summary of Site IARL Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Neutral Detergent Fiber	35.16 (2.66) (30.00 - 38.51)	35.01 (2.66) (27.47 - 39.42)	0.15 (3.76) (-8.13 - 11.03)	-10.30, 10.60	0.969	20.28, 44.03 (24.81 - 42.80)

[1]dwt = dry weight; fwt = fresh weight.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-7. Statistical Summary of Site ILCY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	5.10 (0.080) (5.07 - 5.13)	5.05 (0.080) (4.88 - 5.22)	0.050 (0.089) (-0.14 - 0.23)	-0.20, 0.30	0.602	4.74, 6.01 (4.93 - 5.88)
Carbohydrates	36.35 (0.68) (35.65 - 36.91)	35.77 (0.68) (34.11 - 37.16)	0.58 (0.97) (-0.38 - 2.37)	-2.11, 3.26	0.583	32.07, 40.08 (33.82 - 39.26)
Moisture (% fwt)	5.73 (0.20) (5.17 - 6.25)	6.44 (0.20) (6.20 - 6.63)	-0.71 (0.28) (-1.46 - -0.24)	-1.49, 0.062	0.062	4.27, 9.58 (5.50 - 9.23)
Protein	40.17 (0.40) (39.44 - 40.96)	41.72 (0.40) (40.81 - 42.67)	-1.55 (0.55) (-2.56 - -0.73)	-3.08, -0.024	0.047	35.50, 45.19 (37.06 - 43.42)
Total Fat	18.39 (0.45) (18.25 - 18.56)	17.49 (0.45) (16.81 - 18.39)	0.89 (0.63) (-0.047 - 1.74)	-0.87, 2.65	0.231	12.33, 24.10 (15.47 - 21.34)
Fiber (% dwt)						
Acid Detergent Fiber	15.13 (0.45) (14.86 - 15.57)	14.04 (0.45) (13.47 - 14.57)	1.10 (0.61) (0.41 - 2.10)	-0.59, 2.78	0.144	10.06, 18.04 (12.07 - 17.46)

Table I-7 (continued). Statistical Summary of Site ILCY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Crude Fiber	9.13 (0.29) (8.84 - 9.39)	8.38 (0.29) (8.20 - 8.64)	0.76 (0.41) (0.55 - 0.97)	-0.38, 1.90	0.139	5.76, 10.76 (6.35 - 11.31)
Neutral Detergent Fiber	16.62 (0.63) (16.34 - 16.77)	16.85 (0.63) (15.19 - 17.99)	-0.23 (0.73) (-1.23 - 1.56)	-2.27, 1.81	0.766	11.36, 19.38 (11.66 - 19.45)
Amino Acid (% dwt)						
Alanine	1.80 (0.014) (1.77 - 1.82)	1.81 (0.014) (1.78 - 1.84)	-0.0030 (0.020) (-0.028 - 0.034)	-0.060, 0.054	0.891	1.56, 1.91 (1.59 - 1.86)
Arginine	3.22 (0.053) (3.14 - 3.28)	3.30 (0.053) (3.19 - 3.43)	-0.081 (0.049) (-0.20 - 0.0059)	-0.22, 0.056	0.174	2.55, 3.83 (2.88 - 3.74)
Aspartic Acid	4.67 (0.037) (4.59 - 4.75)	4.76 (0.037) (4.73 - 4.82)	-0.089 (0.034) (-0.15 - -0.043)	-0.18, 0.0051	0.058	4.04, 5.13 (4.22 - 4.94)
Cystine	0.60 (0.0078) (0.58 - 0.62)	0.59 (0.0078) (0.58 - 0.60)	0.010 (0.011) (-0.0071 - 0.034)	-0.021, 0.041	0.416	0.50, 0.68 (0.53 - 0.66)

Table I-7 (continued). Statistical Summary of Site ILCY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Glutamic Acid	7.43 (0.073) (7.27 - 7.54)	7.61 (0.073) (7.52 - 7.76)	-0.18 (0.056) (-0.25 - -0.067)	-0.34, -0.024	0.032	6.28, 8.30 (6.69 - 7.92)
Glycine	1.79 (0.013) (1.75 - 1.81)	1.81 (0.013) (1.79 - 1.83)	-0.021 (0.014) (-0.049 - 0.0022)	-0.059, 0.018	0.213	1.53, 1.92 (1.58 - 1.84)
Histidine	1.06 (0.0071) (1.04 - 1.07)	1.08 (0.0071) (1.07 - 1.09)	-0.020 (0.0055) (-0.030 - -0.0050)	-0.035, -0.0046	0.022	0.93, 1.16 (0.95 - 1.13)
Isoleucine	1.89 (0.013) (1.87 - 1.93)	1.97 (0.013) (1.97 - 1.97)	-0.078 (0.017) (-0.10 - -0.037)	-0.13, -0.031	0.010	1.65, 2.06 (1.68 - 2.02)
Leucine	3.09 (0.022) (3.04 - 3.14)	3.17 (0.022) (3.14 - 3.19)	-0.077 (0.012) (-0.10 - -0.051)	-0.11, -0.044	0.002	2.72, 3.39 (2.80 - 3.27)
Lysine	2.66 (0.017) (2.62 - 2.69)	2.67 (0.017) (2.65 - 2.69)	-0.013 (0.013) (-0.041 - 0.0088)	-0.048, 0.022	0.366	2.33, 2.84 (2.38 - 2.74)

Table I-7 (continued). Statistical Summary of Site ILCY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Methionine	0.55 (0.012) (0.53 - 0.59)	0.57 (0.012) (0.56 - 0.58)	-0.017 (0.017) (-0.039 - 0.024)	-0.064, 0.030	0.370	0.50, 0.64 (0.52 - 0.63)
Phenylalanine	2.11 (0.022) (2.08 - 2.14)	2.13 (0.022) (2.09 - 2.19)	-0.016 (0.018) (-0.048 - 0.011)	-0.067, 0.035	0.429	1.80, 2.30 (1.85 - 2.21)
Proline	2.02 (0.019) (1.98 - 2.04)	2.06 (0.019) (2.04 - 2.09)	-0.044 (0.019) (-0.063 - -0.016)	-0.096, 0.0082	0.079	1.65, 2.26 (1.74 - 2.16)
Serine	2.10 (0.027) (2.06 - 2.12)	2.08 (0.027) (2.01 - 2.15)	0.023 (0.025) (-0.027 - 0.054)	-0.046, 0.093	0.404	1.78, 2.27 (1.90 - 2.18)
Threonine	1.59 (0.015) (1.55 - 1.62)	1.59 (0.015) (1.58 - 1.59)	0.0058 (0.019) (-0.035 - 0.046)	-0.046, 0.058	0.772	1.40, 1.69 (1.47 - 1.64)
Tryptophan	0.51 (0.0090) (0.49 - 0.53)	0.48 (0.0090) (0.47 - 0.50)	0.030 (0.0084) (0.015 - 0.045)	0.0064, 0.053	0.024	0.38, 0.52 (0.39 - 0.50)

Table I-7 (continued). Statistical Summary of Site ILCY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Tyrosine	1.44 (0.033) (1.43 - 1.46)	1.43 (0.033) (1.39 - 1.51)	0.014 (0.045) (-0.079 - 0.078)	-0.11, 0.14	0.773	1.24, 1.50 (1.26 - 1.49)
Valine	1.96 (0.015) (1.94 - 2.01)	2.05 (0.015) (2.05 - 2.06)	-0.090 (0.021) (-0.12 - -0.048)	-0.15, -0.031	0.013	1.72, 2.20 (1.73 - 2.13)
Fatty Acid (% Total FA)						
16:0 Palmitic	12.05 (0.073) (11.95 - 12.16)	11.95 (0.073) (11.73 - 12.08)	0.096 (0.089) (-0.082 - 0.29)	-0.15, 0.34	0.340	8.44, 12.56 (9.40 - 11.54)
18:0 Stearic	3.91 (0.044) (3.88 - 3.93)	3.93 (0.044) (3.86 - 4.02)	-0.025 (0.062) (-0.086 - 0.018)	-0.20, 0.15	0.705	2.90, 5.19 (3.24 - 4.67)
18:1 Oleic	19.74 (0.29) (19.44 - 19.94)	21.57 (0.29) (21.07 - 22.44)	-1.83 (0.41) (-2.51 - -1.21)	-2.96, -0.69	0.011	15.73, 27.19 (17.88 - 25.31)
18:2 Linoleic	54.54 (0.25) (54.45 - 54.70)	53.26 (0.25) (52.77 - 53.74)	1.28 (0.35) (0.73 - 1.68)	0.31, 2.26	0.021	48.61, 59.37 (50.95 - 56.68)

Table I-7 (continued). Statistical Summary of Site ILCY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
18:3 Linolenic	9.07 (0.074) (8.99 - 9.16)	8.58 (0.074) (8.42 - 8.71)	0.50 (0.099) (0.36 - 0.56)	0.22, 0.77	0.007	6.01, 12.58 (7.43 - 11.37)
20:0 Arachidic	0.26 (0.0035) (0.26 - 0.26)	0.26 (0.0035) (0.26 - 0.27)	-0.0048 (0.0050) (-0.012 - 0.00087)	-0.019, 0.0091	0.393	0.19, 0.34 (0.20 - 0.30)
20:1 Eicosenoic	0.16 (0.0016) (0.16 - 0.16)	0.16 (0.0016) (0.16 - 0.17)	-0.0064 (0.0023) (-0.010 - -0.0034)	-0.013, 0.00005	0.051	0.022, 0.24 (0.065 - 0.17)
22:0 Behenic	0.28 (0.0030) (0.27 - 0.28)	0.29 (0.0030) (0.28 - 0.30)	-0.011 (0.0042) (-0.019 - -0.0066)	-0.023, 0.00033	0.054	0.24, 0.40 (0.28 - 0.36)
Vitamin (mg/100g dwt)						
Vitamin E	2.13 (0.077) (2.10 - 2.17)	1.86 (0.077) (1.71 - 2.11)	0.27 (0.089) (0.059 - 0.42)	0.022, 0.52	0.038	0, 3.49 (0.69 - 2.91)

[1]dwt = dry weight; fwt = fresh weight; FA = fatty acid.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-8. Statistical Summary of Site ILCY Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dwt)						
Lectin (H.U./mg dwt)	6.89 (1.81) (4.28 - 10.27)	5.54 (1.81) (2.14 - 10.38)	1.34 (2.55) (-4.27 - 8.13)	-5.75, 8.43	0.626	0, 7.73 (0.68 - 8.34)
Phytic Acid	1.12 (0.032) (1.08 - 1.20)	1.16 (0.032) (1.10 - 1.22)	-0.037 (0.045) (-0.14 - 0.10)	-0.16, 0.088	0.456	0.77, 1.91 (1.00 - 1.64)
Raffinose	0.58 (0.013) (0.57 - 0.59)	0.57 (0.013) (0.54 - 0.60)	0.011 (0.018) (-0.027 - 0.046)	-0.039, 0.061	0.575	0.13, 0.70 (0.26 - 0.59)
Stachyose	3.33 (0.10) (3.25 - 3.46)	3.64 (0.10) (3.43 - 3.83)	-0.31 (0.14) (-0.58 - 0.023)	-0.70, 0.085	0.095	2.30, 4.07 (2.50 - 3.94)
Trypsin Inhibitor (TIU/mg dwt)	31.75 (1.10) (30.61 - 33.32)	32.78 (1.10) (31.06 - 34.22)	-1.02 (1.56) (-2.43 - 0.26)	-5.35, 3.30	0.546	22.05, 41.12 (22.81 - 44.56)
Isoflavone (µg/g dwt)						
Daidzein	925.54 (72.66) (899.83 - 974.38)	922.21 (72.66) (762.49 - 1098.08)	3.32 (102.76) (-198.25 - 139.91)	-281.99, 288.63	0.975	0, 2271.38 (451.33 - 2033.05)

Table I-8 (continued). Statistical Summary of Site ILCY Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Isoflavone (µg/g dwt)						
Genistein	655.78 (45.60) (594.13 - 712.01)	653.27 (45.60) (588.17 - 770.79)	2.51 (64.49) (-58.78 - 60.35)	-176.54, 181.56	0.970	78.36, 1869.48 (533.88 - 1726.03)
Glycitein	98.02 (8.11) (77.67 - 112.00)	113.29 (8.11) (96.25 - 122.09)	-15.27 (11.47) (-43.86 - 15.75)	-47.13, 16.59	0.254	31.24, 233.60 (73.61 - 231.75)

[1]dwt = dry weight; H.U. = Hemagglutinating Units; TIU = Trypsin Inhibitor Units.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-9. Statistical Summary of Site ILCY Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	6.45 (0.22) (6.12 - 6.70)	6.27 (0.22) (6.10 - 6.49)	0.18 (0.32) (-0.37 - 0.48)	-0.70, 1.05	0.601	3.36, 10.84 (5.20 - 9.81)
Carbohydrates	65.26 (0.61) (64.42 - 65.78)	66.38 (0.61) (65.53 - 67.94)	-1.12 (0.87) (-3.52 - 0.11)	-3.53, 1.29	0.265	60.69, 73.46 (62.73 - 71.72)
Moisture (% fwt)	73.13 (0.25) (72.40 - 73.70)	73.53 (0.25) (73.20 - 73.80)	-0.40 (0.35) (-1.20 - 0.50)	-1.37, 0.57	0.316	62.08, 89.80 (70.40 - 84.10)
Protein	22.20 (0.51) (21.25 - 23.11)	21.64 (0.51) (20.88 - 23.03)	0.56 (0.68) (-0.78 - 2.23)	-1.33, 2.46	0.454	15.69, 26.63 (18.50 - 25.86)
Total Fat	6.11 (0.38) (5.62 - 6.88)	5.77 (0.38) (5.15 - 6.72)	0.34 (0.52) (0.17 - 0.47)	-1.09, 1.77	0.549	0, 10.04 (1.57 - 7.99)
Fiber (% dwt)						
Acid Detergent Fiber	30.24 (1.09) (28.75 - 32.14)	28.46 (1.09) (27.05 - 30.90)	1.78 (1.54) (-2.15 - 5.09)	-2.49, 6.05	0.310	16.54, 41.80 (20.98 - 39.23)

Table I-9 (continued). Statistical Summary of Site ILCY Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Neutral Detergent Fiber	27.72 (1.65) (27.34 - 27.98)	28.06 (1.65) (23.66 - 32.73)	-0.35 (2.33) (-4.90 - 3.68)	-6.82, 6.13	0.889	20.28, 44.03 (24.81 - 42.80)

[1]dwt = dry weight; fwt = fresh weight.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-10. Statistical Summary of Site ILWY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	5.43 (0.084) (5.24 - 5.69)	5.23 (0.084) (5.13 - 5.29)	0.20 (0.084) (0.097 - 0.40)	-0.031, 0.44	0.073	4.74, 6.01 (4.93 - 5.88)
Carbohydrates	38.84 (0.44) (38.13 - 39.21)	38.29 (0.44) (38.19 - 38.42)	0.54 (0.45) (-0.064 - 0.96)	-0.71, 1.79	0.292	32.07, 40.08 (33.82 - 39.26)
Moisture (% fwt)	6.96 (0.16) (6.80 - 7.17)	6.16 (0.16) (5.79 - 6.41)	0.80 (0.22) (0.63 - 1.01)	0.18, 1.42	0.022	4.27, 9.58 (5.50 - 9.23)
Protein	40.88 (0.28) (40.56 - 41.37)	41.99 (0.28) (41.72 - 42.25)	-1.11 (0.38) (-1.54 - -0.63)	-2.15, -0.062	0.042	35.50, 45.19 (37.06 - 43.42)
Total Fat	14.83 (0.38) (14.00 - 15.90)	14.49 (0.38) (14.40 - 14.54)	0.34 (0.44) (-0.53 - 1.49)	-0.89, 1.57	0.487	12.33, 24.10 (15.47 - 21.34)
Fiber (% dwt)						
Acid Detergent Fiber	13.43 (0.43) (12.71 - 14.61)	12.96 (0.43) (12.48 - 13.69)	0.47 (0.61) (-0.71 - 2.13)	-1.21, 2.15	0.480	10.06, 18.04 (12.07 - 17.46)

Table I-10 (continued). Statistical Summary of Site ILWY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Crude Fiber	8.22 (0.52) (7.39 - 9.07)	7.58 (0.52) (7.39 - 7.82)	0.64 (0.74) (-0.14 - 1.68)	-1.40, 2.69	0.430	5.76, 10.76 (6.35 - 11.31)
Neutral Detergent Fiber	15.62 (0.72) (13.84 - 17.83)	13.78 (0.72) (13.44 - 14.00)	1.84 (0.94) (-0.064 - 4.39)	-0.77, 4.45	0.122	11.36, 19.38 (11.66 - 19.45)
Amino Acid (% dwt)						
Alanine	1.74 (0.0076) (1.73 - 1.76)	1.75 (0.0076) (1.75 - 1.76)	-0.014 (0.0093) (-0.024 - -0.0071)	-0.039, 0.012	0.219	1.56, 1.91 (1.59 - 1.86)
Arginine	3.30 (0.037) (3.24 - 3.33)	3.57 (0.037) (3.55 - 3.60)	-0.27 (0.040) (-0.32 - -0.23)	-0.38, -0.16	0.002	2.55, 3.83 (2.88 - 3.74)
Aspartic Acid	4.59 (0.017) (4.55 - 4.61)	4.67 (0.017) (4.67 - 4.68)	-0.089 (0.020) (-0.13 - -0.065)	-0.14, -0.034	0.011	4.04, 5.13 (4.22 - 4.94)
Cystine	0.62 (0.0090) (0.62 - 0.63)	0.61 (0.0090) (0.58 - 0.62)	0.014 (0.0092) (-0.0029 - 0.036)	-0.012, 0.039	0.205	0.50, 0.68 (0.53 - 0.66)

Table I-10 (continued). Statistical Summary of Site ILWY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Glutamic Acid	7.29 (0.030) (7.20 - 7.35)	7.51 (0.030) (7.49 - 7.53)	-0.21 (0.031) (-0.29 - -0.16)	-0.30, -0.13	0.002	6.28, 8.30 (6.69 - 7.92)
Glycine	1.75 (0.0053) (1.74 - 1.76)	1.77 (0.0053) (1.76 - 1.77)	-0.021 (0.0075) (-0.034 - -0.0096)	-0.042, 0.00033	0.052	1.53, 1.92 (1.58 - 1.84)
Histidine	1.05 (0.0032) (1.05 - 1.05)	1.07 (0.0032) (1.06 - 1.07)	-0.017 (0.0046) (-0.027 - -0.0057)	-0.030, -0.0046	0.019	0.93, 1.16 (0.95 - 1.13)
Isoleucine	1.87 (0.012) (1.85 - 1.89)	1.90 (0.012) (1.88 - 1.91)	-0.023 (0.0040) (-0.033 - -0.017)	-0.034, -0.012	0.004	1.65, 2.06 (1.68 - 2.02)
Leucine	3.02 (0.011) (3.00 - 3.04)	3.10 (0.011) (3.09 - 3.13)	-0.077 (0.0062) (-0.086 - -0.061)	-0.094, -0.060	<0.001	2.72, 3.39 (2.80 - 3.27)
Lysine	2.63 (0.0062) (2.63 - 2.64)	2.63 (0.0062) (2.62 - 2.64)	0.0011 (0.0088) (-0.011 - 0.0071)	-0.023, 0.026	0.904	2.33, 2.84 (2.38 - 2.74)

Table I-10 (continued). Statistical Summary of Site ILWY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Methionine	0.59 (0.0074) (0.57 - 0.60)	0.58 (0.0074) (0.56 - 0.59)	0.0079 (0.010) (-0.016 - 0.029)	-0.021, 0.037	0.492	0.50, 0.64 (0.52 - 0.63)
Phenylalanine	2.01 (0.019) (1.96 - 2.06)	2.07 (0.019) (2.05 - 2.10)	-0.061 (0.021) (-0.085 - -0.047)	-0.12, -0.0015	0.046	1.80, 2.30 (1.85 - 2.21)
Proline	1.94 (0.020) (1.93 - 1.96)	2.05 (0.020) (2.01 - 2.09)	-0.10 (0.028) (-0.16 - -0.048)	-0.18, -0.027	0.020	1.65, 2.26 (1.74 - 2.16)
Serine	1.98 (0.013) (1.97 - 2.00)	2.06 (0.013) (2.02 - 2.09)	-0.079 (0.013) (-0.095 - -0.048)	-0.11, -0.044	0.003	1.78, 2.27 (1.90 - 2.18)
Threonine	1.52 (0.0042) (1.51 - 1.53)	1.55 (0.0042) (1.54 - 1.55)	-0.026 (0.0049) (-0.030 - -0.022)	-0.040, -0.013	0.005	1.40, 1.69 (1.47 - 1.64)
Tryptophan	0.44 (0.0083) (0.44 - 0.45)	0.47 (0.0083) (0.45 - 0.48)	-0.025 (0.011) (-0.035 - -0.015)	-0.055, 0.0061	0.089	0.38, 0.52 (0.39 - 0.50)

Table I-10 (continued). Statistical Summary of Site ILWY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Tyrosine	1.35 (0.024) (1.32 - 1.41)	1.36 (0.024) (1.34 - 1.40)	-0.013 (0.027) (-0.032 - 0.0095)	-0.090, 0.063	0.651	1.24, 1.50 (1.26 - 1.49)
Valine	1.99 (0.013) (1.95 - 2.01)	2.01 (0.013) (2.01 - 2.02)	-0.022 (0.012) (-0.053 - 0.0028)	-0.057, 0.012	0.149	1.72, 2.20 (1.73 - 2.13)
Fatty Acid (% Total FA)						
16:0 Palmitic	11.26 (0.041) (11.25 - 11.27)	11.04 (0.041) (10.97 - 11.12)	0.22 (0.057) (0.15 - 0.28)	0.063, 0.38	0.017	8.44, 12.56 (9.40 - 11.54)
18:0 Stearic	4.32 (0.063) (4.23 - 4.40)	4.25 (0.063) (4.16 - 4.31)	0.076 (0.089) (0.067 - 0.085)	-0.17, 0.32	0.439	2.90, 5.19 (3.24 - 4.67)
18:1 Oleic	19.52 (0.25) (19.34 - 19.64)	21.14 (0.25) (20.78 - 21.55)	-1.62 (0.36) (-1.97 - -1.43)	-2.62, -0.62	0.010	15.73, 27.19 (17.88 - 25.31)
18:2 Linoleic	53.74 (0.32) (53.55 - 54.06)	52.90 (0.32) (52.33 - 53.20)	0.85 (0.46) (0.47 - 1.22)	-0.43, 2.12	0.139	48.61, 59.37 (50.95 - 56.68)

Table I-10 (continued). Statistical Summary of Site ILWY Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
18:3 Linolenic	10.54 (0.10) (10.51 - 10.59)	10.05 (0.10) (9.89 - 10.14)	0.49 (0.14) (0.39 - 0.65)	0.095, 0.89	0.026	6.01, 12.58 (7.43 - 11.37)
20:0 Arachidic	0.27 (0.0041) (0.26 - 0.27)	0.27 (0.0041) (0.26 - 0.27)	-0.00093 (0.0058) (-0.0057 - 0.0027)	-0.017, 0.015	0.879	0.19, 0.34 (0.20 - 0.30)
20:1 Eicosenoic	0.075 (0.0020) (0.070 - 0.079)	0.076 (0.0020) (0.075 - 0.077)	-0.0014 (0.0023) (-0.0071 - 0.0019)	-0.0076, 0.0049	0.583	0.022, 0.24 (0.065 - 0.17)
22:0 Behenic	0.26 (0.0032) (0.26 - 0.27)	0.28 (0.0032) (0.28 - 0.29)	-0.019 (0.0040) (-0.021 - -0.017)	-0.030, -0.0079	0.008	0.24, 0.40 (0.28 - 0.36)
Vitamin (mg/100g dwt)						
Vitamin E	1.18 (0.037) (1.08 - 1.26)	0.94 (0.037) (0.89 - 0.99)	0.23 (0.053) (0.19 - 0.31)	0.086, 0.38	0.011	0, 3.49 (0.69 - 2.91)

[1]dwt = dry weight; fwt = fresh weight; FA = fatty acid.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-11. Statistical Summary of Site ILWY Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dwt)						
Lectin (H.U./mg dwt)	1.10 (0.44) (0.59 - 1.51)	2.33 (0.44) (1.34 - 3.68)	-1.23 (0.43) (-2.17 - -0.75)	-2.43, -0.035	0.045	0, 7.73 (0.68 - 8.34)
Phytic Acid	1.40 (0.033) (1.33 - 1.46)	1.55 (0.033) (1.47 - 1.61)	-0.14 (0.044) (-0.22 - -0.054)	-0.27, -0.023	0.030	0.77, 1.91 (1.00 - 1.64)
Raffinose	0.37 (0.026) (0.32 - 0.45)	0.41 (0.026) (0.41 - 0.41)	-0.037 (0.036) (-0.086 - 0.040)	-0.14, 0.065	0.371	0.13, 0.70 (0.26 - 0.59)
Stachyose	3.44 (0.19) (3.07 - 4.02)	3.76 (0.19) (3.68 - 3.85)	-0.33 (0.27) (-0.78 - 0.34)	-1.08, 0.43	0.294	2.30, 4.07 (2.50 - 3.94)
Trypsin Inhibitor (TIU/mg dwt)	34.32 (2.07) (29.54 - 39.27)	29.73 (2.07) (25.43 - 32.22)	4.59 (2.89) (-2.68 - 8.72)	-3.43, 12.62	0.187	22.05, 41.12 (22.81 - 44.56)
Isoflavone (µg/g dwt)						
Daidzein	1458.08 (35.08) (1416.31 - 1535.98)	1271.60 (35.08) (1196.71 - 1354.96)	186.48 (31.48) (153.17 - 225.25)	99.09, 273.88	0.004	0, 2271.38 (451.33 - 2033.05)

Table I-11 (continued). Statistical Summary of Site ILWY Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Isoflavone (µg/g dwt)						
Genistein	898.89 (28.15) (873.39 - 913.50)	860.58 (28.15) (784.27 - 913.26)	38.31 (39.82) (-10.80 - 129.23)	-72.23, 148.86	0.390	78.36, 1869.48 (533.88 - 1726.03)
Glycitein	111.77 (2.15) (109.88 - 113.86)	79.70 (2.15) (77.14 - 81.62)	32.07 (2.23) (31.24 - 32.73)	25.87, 38.27	<0.001	31.24, 233.60 (73.61 - 231.75)

[1]dwt = dry weight; H.U. = Hemagglutinating Units; TIU = Trypsin Inhibitor Units.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-12. Statistical Summary of Site ILWY Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	6.61 (0.23) (6.02 - 7.21)	6.88 (0.23) (6.82 - 6.99)	-0.27 (0.30) (-0.79 - 0.37)	-1.10, 0.56	0.416	3.36, 10.84 (5.20 - 9.81)
Carbohydrates	67.99 (0.77) (66.09 - 69.50)	66.26 (0.77) (65.71 - 66.67)	1.73 (1.08) (-0.30 - 3.78)	-1.28, 4.74	0.185	60.69, 73.46 (62.73 - 71.72)
Moisture (% fwt)	75.17 (0.55) (74.10 - 76.70)	75.40 (0.55) (75.10 - 75.60)	-0.23 (0.65) (-1.40 - 1.10)	-2.04, 1.57	0.737	62.08, 89.80 (70.40 - 84.10)
Protein	20.93 (0.58) (19.27 - 22.70)	21.97 (0.58) (21.81 - 22.13)	-1.03 (0.82) (-2.69 - 0.57)	-3.32, 1.25	0.278	15.69, 26.63 (18.50 - 25.86)
Total Fat	4.44 (0.52) (3.92 - 5.10)	4.91 (0.52) (4.50 - 5.63)	-0.47 (0.35) (-0.67 - -0.19)	-1.44, 0.50	0.252	0, 10.04 (1.57 - 7.99)
Fiber (% dwt)						
Acid Detergent Fiber	29.18 (1.73) (26.72 - 31.00)	27.77 (1.73) (25.12 - 31.00)	1.41 (2.44) (-4.28 - 4.71)	-5.36, 8.19	0.593	16.54, 41.80 (20.98 - 39.23)

Table I-12 (continued). Statistical Summary of Site ILWY Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Neutral Detergent Fiber	28.64 (1.83) (27.22 - 31.26)	33.25 (1.83) (31.89 - 34.06)	-4.61 (2.59) (-6.63 - -0.62)	-11.80, 2.58	0.149	20.28, 44.03 (24.81 - 42.80)

[1]dwt = dry weight; fwt = fresh weight.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-13. Statistical Summary of Site INRC Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	5.03 (0.11) (4.94 - 5.18)	4.95 (0.11) (4.73 - 5.23)	0.075 (0.15) (-0.28 - 0.45)	-0.35, 0.50	0.644	4.74, 6.01 (4.93 - 5.88)
Carbohydrates	37.60 (0.78) (37.15 - 38.07)	35.95 (0.78) (35.27 - 37.04)	1.65 (1.10) (1.03 - 2.05)	-1.41, 4.71	0.208	32.07, 40.08 (33.82 - 39.26)
Moisture (% fwt)	6.74 (0.20) (6.48 - 7.13)	6.53 (0.20) (6.32 - 6.84)	0.21 (0.29) (-0.23 - 0.69)	-0.59, 1.01	0.513	4.27, 9.58 (5.50 - 9.23)
Protein	42.11 (0.53) (41.33 - 42.53)	43.58 (0.53) (43.50 - 43.69)	-1.48 (0.76) (-2.36 - -0.97)	-3.57, 0.62	0.122	35.50, 45.19 (37.06 - 43.42)
Total Fat	15.30 (0.45) (14.54 - 15.95)	15.55 (0.45) (14.52 - 16.10)	-0.25 (0.56) (-0.63 - 0.025)	-1.82, 1.32	0.678	12.33, 24.10 (15.47 - 21.34)
Fiber (% dwt)						
Acid Detergent Fiber	13.22 (0.35) (12.64 - 13.79)	12.77 (0.35) (12.28 - 13.25)	0.46 (0.46) (-0.14 - 1.52)	-0.81, 1.73	0.374	10.06, 18.04 (12.07 - 17.46)

Table I-13 (continued). Statistical Summary of Site INRC Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Crude Fiber	8.06 (0.18) (7.76 - 8.47)	6.89 (0.18) (6.59 - 7.12)	1.17 (0.25) (0.64 - 1.51)	0.47, 1.88	0.009	5.76, 10.76 (6.35 - 11.31)
Neutral Detergent Fiber	15.98 (0.67) (14.97 - 16.49)	14.66 (0.67) (13.24 - 15.56)	1.32 (0.95) (0.93 - 1.73)	-1.32, 3.96	0.237	11.36, 19.38 (11.66 - 19.45)
Amino Acid (% dwt)						
Alanine	1.81 (0.021) (1.80 - 1.83)	1.84 (0.021) (1.80 - 1.87)	-0.032 (0.022) (-0.072 - 0.013)	-0.093, 0.029	0.223	1.56, 1.91 (1.59 - 1.86)
Arginine	3.44 (0.044) (3.39 - 3.50)	3.72 (0.044) (3.64 - 3.81)	-0.27 (0.062) (-0.38 - -0.15)	-0.45, -0.10	0.011	2.55, 3.83 (2.88 - 3.74)
Aspartic Acid	4.76 (0.062) (4.70 - 4.80)	4.89 (0.062) (4.80 - 4.95)	-0.13 (0.078) (-0.23 - 0.0034)	-0.34, 0.087	0.173	4.04, 5.13 (4.22 - 4.94)
Cystine	0.61 (0.0067) (0.61 - 0.61)	0.59 (0.0067) (0.59 - 0.60)	0.020 (0.0094) (0.0087 - 0.029)	-0.0067, 0.046	0.107	0.50, 0.68 (0.53 - 0.66)

Table I-13 (continued). Statistical Summary of Site INRC Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Glutamic Acid	7.66 (0.11) (7.57 - 7.73)	7.93 (0.11) (7.75 - 8.02)	-0.26 (0.14) (-0.45 - -0.018)	-0.66, 0.13	0.138	6.28, 8.30 (6.69 - 7.92)
Glycine	1.82 (0.020) (1.80 - 1.83)	1.85 (0.020) (1.82 - 1.87)	-0.035 (0.027) (-0.072 - 0.013)	-0.11, 0.039	0.257	1.53, 1.92 (1.58 - 1.84)
Histidine	1.09 (0.012) (1.07 - 1.10)	1.11 (0.012) (1.10 - 1.12)	-0.028 (0.016) (-0.056 - -0.0026)	-0.073, 0.018	0.166	0.93, 1.16 (0.95 - 1.13)
Isoleucine	1.94 (0.042) (1.90 - 1.97)	2.00 (0.042) (1.95 - 2.03)	-0.060 (0.057) (-0.11 - 0.025)	-0.22, 0.099	0.354	1.65, 2.06 (1.68 - 2.02)
Leucine	3.16 (0.041) (3.11 - 3.19)	3.24 (0.041) (3.20 - 3.26)	-0.082 (0.052) (-0.14 - -0.0086)	-0.23, 0.061	0.186	2.72, 3.39 (2.80 - 3.27)
Lysine	2.70 (0.030) (2.66 - 2.71)	2.72 (0.030) (2.68 - 2.76)	-0.030 (0.035) (-0.070 - 0.031)	-0.13, 0.068	0.446	2.33, 2.84 (2.38 - 2.74)

Table I-13 (continued). Statistical Summary of Site INRC Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Methionine	0.58 (0.0086) (0.58 - 0.59)	0.59 (0.0086) (0.58 - 0.60)	-0.0055 (0.012) (-0.017 - 0.00070)	-0.039, 0.028	0.674	0.50, 0.64 (0.52 - 0.63)
Phenylalanine	2.15 (0.030) (2.12 - 2.18)	2.20 (0.030) (2.13 - 2.23)	-0.049 (0.040) (-0.11 - 0.048)	-0.16, 0.062	0.289	1.80, 2.30 (1.85 - 2.21)
Proline	2.07 (0.010) (2.06 - 2.09)	2.06 (0.010) (2.05 - 2.07)	0.0045 (0.015) (-0.0071 - 0.016)	-0.036, 0.045	0.771	1.65, 2.26 (1.74 - 2.16)
Serine	2.09 (0.028) (2.06 - 2.12)	2.13 (0.028) (2.13 - 2.14)	-0.038 (0.029) (-0.070 - -0.016)	-0.12, 0.043	0.260	1.78, 2.27 (1.90 - 2.18)
Threonine	1.60 (0.019) (1.57 - 1.62)	1.61 (0.019) (1.60 - 1.62)	-0.014 (0.027) (-0.040 - 0.012)	-0.089, 0.060	0.622	1.40, 1.69 (1.47 - 1.64)
Tryptophan	0.47 (0.013) (0.44 - 0.50)	0.45 (0.013) (0.43 - 0.46)	0.019 (0.018) (-0.015 - 0.064)	-0.032, 0.069	0.365	0.38, 0.52 (0.39 - 0.50)

Table I-13 (continued). Statistical Summary of Site INRC Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Tyrosine	1.38 (0.024) (1.35 - 1.43)	1.44 (0.024) (1.38 - 1.47)	-0.065 (0.022) (-0.12 - -0.036)	-0.13, -0.0024	0.044	1.24, 1.50 (1.26 - 1.49)
Valine	2.05 (0.045) (2.01 - 2.09)	2.12 (0.045) (2.06 - 2.16)	-0.074 (0.063) (-0.14 - 0.026)	-0.25, 0.10	0.305	1.72, 2.20 (1.73 - 2.13)
Fatty Acid (% Total FA)						
16:0 Palmitic	11.42 (0.068) (11.39 - 11.46)	11.19 (0.068) (11.14 - 11.21)	0.23 (0.097) (0.19 - 0.26)	-0.036, 0.50	0.074	8.44, 12.56 (9.40 - 11.54)
18:0 Stearic	4.18 (0.047) (4.11 - 4.27)	4.25 (0.047) (4.16 - 4.31)	-0.064 (0.066) (-0.19 - 0.11)	-0.25, 0.12	0.387	2.90, 5.19 (3.24 - 4.67)
18:1 Oleic	18.78 (0.085) (18.58 - 18.95)	20.19 (0.085) (20.12 - 20.23)	-1.41 (0.093) (-1.64 - -1.27)	-1.66, -1.15	<0.001	15.73, 27.19 (17.88 - 25.31)
18:2 Linoleic	54.98 (0.10) (54.80 - 55.14)	54.43 (0.10) (54.32 - 54.64)	0.54 (0.14) (0.16 - 0.82)	0.15, 0.94	0.019	48.61, 59.37 (50.95 - 56.68)

Table I-13 (continued). Statistical Summary of Site INRC Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
18:3 Linolenic	10.03 (0.050) (9.89 - 10.10)	9.31 (0.050) (9.26 - 9.40)	0.71 (0.070) (0.60 - 0.84)	0.52, 0.91	<0.001	6.01, 12.58 (7.43 - 11.37)
20:0 Arachidic	0.26 (0.0029) (0.26 - 0.27)	0.27 (0.0029) (0.26 - 0.27)	-0.0053 (0.0041) (-0.013 - 0.0051)	-0.017, 0.0062	0.270	0.19, 0.34 (0.20 - 0.30)
20:1 Eicosenoic	0.072 (0.0020) (0.069 - 0.076)	0.071 (0.0020) (0.069 - 0.076)	0.0010 (0.0025) (-0.00013 - 0.0025)	-0.0059, 0.0079	0.701	0.022, 0.24 (0.065 - 0.17)
22:0 Behenic	0.28 (0.0033) (0.27 - 0.29)	0.29 (0.0033) (0.29 - 0.30)	-0.014 (0.0047) (-0.023 - 0.00015)	-0.027, -0.0012	0.038	0.24, 0.40 (0.28 - 0.36)
Vitamin (mg/100g dwt)						
Vitamin E	1.28 (0.054) (1.25 - 1.30)	1.16 (0.054) (1.10 - 1.23)	0.12 (0.067) (0.018 - 0.18)	-0.065, 0.31	0.146	0, 3.49 (0.69 - 2.91)

[1]dwt = dry weight; fwt = fresh weight; FA = fatty acid.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table I-14. Statistical Summary of Site INRC Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dwt)						
Lectin (H.U./mg dwt)	3.03 (0.32) (2.75 - 3.45)	2.56 (0.32) (2.33 - 3.02)	0.47 (0.45) (-0.26 - 1.13)	-0.79, 1.74	0.357	0, 7.73 (0.68 - 8.34)
Phytic Acid	1.27 (0.071) (1.22 - 1.34)	1.19 (0.071) (1.09 - 1.36)	0.078 (0.087) (-0.022 - 0.15)	-0.16, 0.32	0.421	0.77, 1.91 (1.00 - 1.64)
Raffinose	0.37 (0.023) (0.33 - 0.43)	0.40 (0.023) (0.36 - 0.43)	-0.032 (0.033) (-0.098 - 0.069)	-0.12, 0.060	0.384	0.13, 0.70 (0.26 - 0.59)
Stachyose	3.14 (0.077) (3.12 - 3.17)	3.46 (0.077) (3.33 - 3.67)	-0.32 (0.11) (-0.51 - -0.21)	-0.62, -0.015	0.043	2.30, 4.07 (2.50 - 3.94)
Trypsin Inhibitor (TIU/mg dwt)	29.17 (1.79) (26.09 - 33.09)	29.28 (1.79) (25.22 - 31.77)	-0.12 (1.83) (-4.76 - 3.09)	-5.21, 4.97	0.952	22.05, 41.12 (22.81 - 44.56)
Isoflavone (µg/g dwt)						
Daidzein	1683.50 (67.03) (1593.24 - 1777.49)	1419.40 (67.03) (1416.92 - 1421.55)	264.10 (94.79) (173.52 - 360.58)	0.92, 527.28	0.049	0, 2271.38 (451.33 - 2033.05)

Table I-14 (continued). Statistical Summary of Site INRC Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Isoflavone (µg/g dwt)						
Genistein	1033.37 (50.59) (963.43 - 1092.19)	862.03 (50.59) (840.10 - 890.94)	171.34 (71.54) (108.39 - 204.37)	-27.29, 369.97	0.074	78.36, 1869.48 (533.88 - 1726.03)
Glycitein	111.51 (3.23) (110.91 - 112.28)	98.42 (3.23) (89.42 - 103.14)	13.10 (3.31) (7.77 - 21.94)	3.91, 22.29	0.016	31.24, 233.60 (73.61 - 231.75)

[1]dwt = dry weight; H.U. = Hemagglutinating Units; TIU = Trypsin Inhibitor Units.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-15. Statistical Summary of Site INRC Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	6.45 (0.24) (5.94 - 7.05)	6.95 (0.24) (6.84 - 7.07)	-0.50 (0.26) (-0.89 - 0.11)	-1.23, 0.23	0.128	3.36, 10.84 (5.20 - 9.81)
Carbohydrates	65.19 (0.69) (63.10 - 66.54)	63.82 (0.69) (62.91 - 64.44)	1.38 (0.98) (-1.34 - 3.03)	-1.34, 4.10	0.232	60.69, 73.46 (62.73 - 71.72)
Moisture (% fwt)	73.00 (0.32) (72.40 - 73.70)	72.27 (0.32) (71.60 - 72.70)	0.73 (0.39) (-0.10 - 1.30)	-0.34, 1.80	0.129	62.08, 89.80 (70.40 - 84.10)
Protein	21.78 (0.41) (20.99 - 22.51)	23.33 (0.41) (22.64 - 24.11)	-1.55 (0.41) (-2.26 - -0.73)	-2.69, -0.41	0.019	15.69, 26.63 (18.50 - 25.86)
Total Fat	6.54 (0.27) (6.12 - 7.34)	5.88 (0.27) (5.39 - 6.19)	0.66 (0.39) (-0.069 - 1.96)	-0.42, 1.73	0.164	0, 10.04 (1.57 - 7.99)
Fiber (% dwt)						
Acid Detergent Fiber	26.46 (1.50) (23.30 - 31.06)	23.83 (1.50) (22.93 - 25.53)	2.63 (2.13) (-0.51 - 8.13)	-3.27, 8.54	0.283	16.54, 41.80 (20.98 - 39.23)

Table I-15 (continued). Statistical Summary of Site INRC Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Neutral Detergent Fiber	27.20 (1.62) (24.21 - 31.27)	26.11 (1.62) (23.91 - 29.42)	1.08 (2.04) (0.30 - 1.85)	-4.59, 6.76	0.623	20.28, 44.03 (24.81 - 42.80)

[1]dwt = dry weight; fwt = fresh weight.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-16. Statistical Summary of Site PAHM Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	5.33 (0.11) (5.27 - 5.39)	5.05 (0.11) (4.96 - 5.17)	0.28 (0.16) (0.15 - 0.44)	-0.16, 0.72	0.148	4.74, 6.01 (4.93 - 5.88)
Carbohydrates	38.30 (0.51) (37.69 - 38.65)	35.23 (0.51) (34.49 - 35.75)	3.07 (0.64) (2.23 - 4.07)	1.29, 4.84	0.008	32.07, 40.08 (33.82 - 39.26)
Moisture (% fwt)	7.84 (0.22) (7.38 - 8.47)	10.50 (0.22) (10.40 - 10.60)	-2.66 (0.22) (-3.12 - -2.13)	-3.27, -2.04	<0.001	4.27, 9.58 (5.50 - 9.23)
Protein	40.25 (0.41) (39.00 - 41.05)	43.69 (0.41) (43.46 - 43.85)	-3.43 (0.51) (-4.84 - -2.70)	-4.86, -2.01	0.002	35.50, 45.19 (37.06 - 43.42)
Total Fat	16.10 (0.63) (14.95 - 18.03)	16.05 (0.63) (15.64 - 16.85)	0.050 (0.89) (-1.90 - 2.37)	-2.41, 2.51	0.957	12.33, 24.10 (15.47 - 21.34)
Fiber (% dwt)						
Acid Detergent Fiber	12.91 (0.35) (12.45 - 13.21)	11.96 (0.35) (11.62 - 12.17)	0.96 (0.43) (0.37 - 1.44)	-0.24, 2.15	0.089	10.06, 18.04 (12.07 - 17.46)

Table I-16 (continued). Statistical Summary of Site PAHM Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Crude Fiber	8.30 (0.63) (6.23 - 9.65)	6.61 (0.63) (6.05 - 6.98)	1.68 (0.71) (0.18 - 2.67)	-0.30, 3.66	0.077	5.76, 10.76 (6.35 - 11.31)
Neutral Detergent Fiber	13.97 (0.36) (13.43 - 14.97)	13.11 (0.36) (12.63 - 13.62)	0.86 (0.50) (-0.18 - 1.88)	-0.54, 2.25	0.164	11.36, 19.38 (11.66 - 19.45)
Amino Acid (% dwt)						
Alanine	1.75 (0.017) (1.74 - 1.77)	1.86 (0.017) (1.82 - 1.90)	-0.11 (0.024) (-0.16 - -0.054)	-0.17, -0.042	0.010	1.56, 1.91 (1.59 - 1.86)
Arginine	3.25 (0.053) (3.09 - 3.36)	3.88 (0.053) (3.83 - 3.93)	-0.63 (0.074) (-0.83 - -0.47)	-0.83, -0.42	0.001	2.55, 3.83 (2.88 - 3.74)
Aspartic Acid	4.56 (0.041) (4.45 - 4.63)	4.94 (0.041) (4.90 - 5.01)	-0.38 (0.057) (-0.56 - -0.27)	-0.54, -0.22	0.002	4.04, 5.13 (4.22 - 4.94)
Cystine	0.62 (0.0062) (0.60 - 0.63)	0.59 (0.0062) (0.59 - 0.60)	0.028 (0.0081) (0.0092 - 0.039)	0.0061, 0.051	0.024	0.50, 0.68 (0.53 - 0.66)

Table I-16 (continued). Statistical Summary of Site PAHM Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Glutamic Acid	7.28 (0.081) (7.06 - 7.40)	8.00 (0.081) (7.91 - 8.14)	-0.73 (0.11) (-1.09 - -0.54)	-1.05, -0.41	0.003	6.28, 8.30 (6.69 - 7.92)
Glycine	1.73 (0.015) (1.69 - 1.75)	1.86 (0.015) (1.83 - 1.89)	-0.13 (0.022) (-0.20 - -0.076)	-0.19, -0.066	0.004	1.53, 1.92 (1.58 - 1.84)
Histidine	1.05 (0.0089) (1.02 - 1.06)	1.13 (0.0089) (1.11 - 1.14)	-0.085 (0.013) (-0.12 - -0.053)	-0.12, -0.050	0.002	0.93, 1.16 (0.95 - 1.13)
Isoleucine	1.85 (0.026) (1.79 - 1.90)	2.00 (0.026) (1.94 - 2.04)	-0.15 (0.036) (-0.24 - -0.046)	-0.25, -0.051	0.014	1.65, 2.06 (1.68 - 2.02)
Leucine	3.03 (0.027) (2.96 - 3.09)	3.28 (0.027) (3.24 - 3.32)	-0.24 (0.038) (-0.36 - -0.15)	-0.35, -0.14	0.002	2.72, 3.39 (2.80 - 3.27)
Lysine	2.60 (0.023) (2.53 - 2.65)	2.75 (0.023) (2.71 - 2.77)	-0.15 (0.032) (-0.23 - -0.091)	-0.24, -0.060	0.009	2.33, 2.84 (2.38 - 2.74)

Table I-16 (continued). Statistical Summary of Site PAHM Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Methionine	0.58 (0.0090) (0.57 - 0.60)	0.58 (0.0090) (0.57 - 0.60)	-0.00056 (0.0072) (-0.0027 - 0.00085)	-0.021, 0.020	0.942	0.50, 0.64 (0.52 - 0.63)
Phenylalanine	2.04 (0.027) (2.00 - 2.07)	2.21 (0.027) (2.16 - 2.27)	-0.18 (0.038) (-0.27 - -0.083)	-0.28, -0.069	0.010	1.80, 2.30 (1.85 - 2.21)
Proline	1.98 (0.024) (1.94 - 2.00)	2.10 (0.024) (2.08 - 2.13)	-0.13 (0.032) (-0.14 - -0.11)	-0.21, -0.038	0.016	1.65, 2.26 (1.74 - 2.16)
Serine	2.04 (0.035) (2.01 - 2.06)	2.16 (0.035) (2.06 - 2.21)	-0.12 (0.050) (-0.19 - 0.0063)	-0.26, 0.018	0.073	1.78, 2.27 (1.90 - 2.18)
Threonine	1.55 (0.015) (1.52 - 1.57)	1.62 (0.015) (1.60 - 1.64)	-0.069 (0.021) (-0.10 - -0.032)	-0.13, -0.011	0.029	1.40, 1.69 (1.47 - 1.64)
Tryptophan	0.47 (0.012) (0.44 - 0.48)	0.46 (0.012) (0.45 - 0.46)	0.010 (0.017) (-0.0098 - 0.023)	-0.036, 0.057	0.567	0.38, 0.52 (0.39 - 0.50)

Table I-16 (continued). Statistical Summary of Site PAHM Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dwt)						
Tyrosine	1.35 (0.027) (1.28 - 1.43)	1.49 (0.027) (1.48 - 1.52)	-0.14 (0.032) (-0.20 - -0.088)	-0.23, -0.052	0.011	1.24, 1.50 (1.26 - 1.49)
Valine	1.95 (0.029) (1.89 - 2.00)	2.13 (0.029) (2.05 - 2.17)	-0.17 (0.041) (-0.27 - -0.050)	-0.29, -0.061	0.012	1.72, 2.20 (1.73 - 2.13)
Fatty Acid (% Total FA)						
16:0 Palmitic	11.74 (0.12) (11.39 - 12.07)	11.49 (0.12) (11.38 - 11.55)	0.25 (0.15) (-0.15 - 0.52)	-0.17, 0.67	0.169	8.44, 12.56 (9.40 - 11.54)
18:0 Stearic	3.85 (0.12) (3.60 - 4.12)	3.76 (0.12) (3.67 - 3.91)	0.093 (0.14) (-0.078 - 0.42)	-0.30, 0.48	0.544	2.90, 5.19 (3.24 - 4.67)
18:1 Oleic	18.58 (0.31) (17.85 - 19.42)	20.01 (0.31) (19.60 - 20.32)	-1.43 (0.35) (-1.74 - -0.90)	-2.40, -0.45	0.015	15.73, 27.19 (17.88 - 25.31)
18:2 Linoleic	54.89 (0.41) (53.59 - 55.67)	54.68 (0.41) (54.18 - 54.99)	0.21 (0.53) (-0.59 - 0.68)	-1.25, 1.67	0.708	48.61, 59.37 (50.95 - 56.68)

Table I-16 (continued). Statistical Summary of Site PAHM Soybean Seed Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
18:3 Linolenic	10.33 (0.21) (9.91 - 10.88)	9.47 (0.21) (9.13 - 9.68)	0.85 (0.16) (0.59 - 1.20)	0.40, 1.31	0.006	6.01, 12.58 (7.43 - 11.37)
20:0 Arachidic	0.25 (0.0063) (0.23 - 0.26)	0.24 (0.0063) (0.24 - 0.25)	0.0034 (0.0071) (-0.0079 - 0.020)	-0.016, 0.023	0.652	0.19, 0.34 (0.20 - 0.30)
20:1 Eicosenoic	0.091 (0.015) (0.073 - 0.12)	0.072 (0.015) (0.068 - 0.075)	0.018 (0.022) (-0.0014 - 0.050)	-0.042, 0.078	0.445	0.022, 0.24 (0.065 - 0.17)
22:0 Behenic	0.27 (0.0046) (0.26 - 0.28)	0.27 (0.0046) (0.27 - 0.28)	-0.0067 (0.0034) (-0.012 - 0.0024)	-0.016, 0.0028	0.121	0.24, 0.40 (0.28 - 0.36)
Vitamin (mg/100g dwt)						
Vitamin E	1.32 (0.10) (1.21 - 1.54)	1.23 (0.10) (1.11 - 1.40)	0.097 (0.022) (0.049 - 0.14)	0.037, 0.16	0.010	0, 3.49 (0.69 - 2.91)

[1]dwt = dry weight; fwt = fresh weight; FA = fatty acid.
[2]MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-17. Statistical Summary of Site PAHM Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dwt)						
Lectin (H.U./mg dwt)	2.81 (0.80) (2.08 - 3.34)	3.85 (0.80) (3.28 - 4.45)	-1.03 (1.03) (-1.73 - 0.051)	-3.88, 1.81	0.370	0, 7.73 (0.68 - 8.34)
Phytic Acid	1.35 (0.077) (1.13 - 1.51)	1.50 (0.077) (1.41 - 1.62)	-0.15 (0.092) (-0.28 - -0.054)	-0.40, 0.11	0.179	0.77, 1.91 (1.00 - 1.64)
Raffinose	0.47 (0.047) (0.32 - 0.55)	0.54 (0.047) (0.49 - 0.57)	-0.072 (0.066) (-0.24 - 0.058)	-0.26, 0.11	0.339	0.13, 0.70 (0.26 - 0.59)
Stachyose	3.29 (0.18) (3.19 - 3.47)	3.36 (0.18) (3.07 - 3.90)	-0.075 (0.25) (-0.69 - 0.40)	-0.77, 0.62	0.777	2.30, 4.07 (2.50 - 3.94)
Trypsin Inhibitor (TIU/mg dwt)	32.53 (4.33) (27.64 - 36.16)	30.39 (4.33) (26.59 - 33.33)	2.14 (5.59) (1.05 - 2.83)	-13.38, 17.66	0.721	22.05, 41.12 (22.81 - 44.56)
Isoflavone (µg/g dwt)						
Daidzein	1918.51 (144.27) (1565.54 - 2305.26)	1642.38 (144.27) (1510.07 - 1729.91)	276.14 (204.03) (-121.61 - 795.19)	-290.35, 842.62	0.247	0, 2271.38 (451.33 - 2033.05)

Table I-17 (continued). Statistical Summary of Site PAHM Soybean Seed Anti-nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Isoflavone (µg/g dwt)						
Genistein	1196.16 (107.80) (976.03 - 1496.78)	1101.98 (107.80) (983.22 - 1162.01)	94.18 (152.45) (-185.98 - 513.56)	-329.09, 517.45	0.570	78.36, 1869.48 (533.88 - 1726.03)
Glycitein	110.37 (7.95) (93.26 - 119.09)	81.44 (7.95) (68.68 - 90.51)	28.93 (11.25) (2.75 - 50.41)	-2.30, 60.16	0.061	31.24, 233.60 (73.61 - 231.75)

[1]dwt = dry weight; H.U. = Hemagglutinating Units; TIU = Trypsin Inhibitor Units.
[2] MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error).
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

Table I-18. Statistical Summary of Site PAHM Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dwt)						
Ash	7.51 (0.26) (7.18 - 8.13)	7.88 (0.32) (7.67 - 8.09)	-0.37 (0.41) (-0.86 - -0.49)	-1.67, 0.94	0.438	3.36, 10.84 (5.20 - 9.81)
Carbohydrates	70.95 (1.04) (69.23 - 73.31)	65.81 (1.16) (65.74 - 66.41)	5.14 (1.03) (3.49 - 6.90)	1.85, 8.43	0.015	60.69, 73.46 (62.73 - 71.72)
Moisture (% fwt)	74.27 (0.63) (73.40 - 75.40)	74.91 (0.63) (73.80 - 74.90)	-0.64 (0.15) (-0.90 - -0.40)	-1.11, -0.18	0.021	62.08, 89.80 (70.40 - 84.10)
Protein	17.47 (1.26) (15.23 - 19.58)	21.96 (1.45) (21.49 - 21.91)	-4.49 (1.45) (-6.26 - -2.34)	-9.10, 0.12	0.053	15.69, 26.63 (18.50 - 25.86)
Total Fat	3.97 (0.26) (3.82 - 4.21)	4.18 (0.31) (4.30 - 4.35)	-0.20 (0.31) (-0.42 - -0.14)	-1.19, 0.78	0.553	0, 10.04 (1.57 - 7.99)
Fiber (% dwt)						
Acid Detergent Fiber	26.97 (1.95) (25.46 - 29.89)	26.02 (2.39) (21.79 - 30.24)	0.96 (3.08) (-4.78 - 8.09)	-8.86, 10.77	0.776	16.54, 41.80 (20.98 - 39.23)

Table I-18 (continued). Statistical Summary of Site PAHM Soybean Forage Nutrients for MON 87708 vs. Conventional Control

Analytical Component (Units)[1]	MON 87708[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87708 minus Control) Mean (S.E.) (Range)	95% Confidence Interval	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dwt)						
Neutral Detergent Fiber	29.45 (1.01) (27.00 - 32.07)	30.20 (1.24) (30.00 - 30.40)	-0.75 (1.60) (-3.40 - -0.71)	-5.83, 4.34	0.672	20.28, 44.03 (24.81 - 42.80)

[1]dwt = dry weight; fwt = fresh weight.
[2] MON 87708 was treated with dicamba.
[3]Mean (S.E.) = least-square mean (standard error)
[4]Control refers to the near isogenic conventional soybean control A3525.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference soybean varieties. Negative limits set to zero.

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