![Food Standards Australia New Zealand logo](FOOD STANDARDS Australia New Zealand - Te Mana Kounga Kai – Ahitereiria me Aotearoa)

Supporting document 1

Safety assessment report (Approval)

Application A1063

Food derived from Herbicide-tolerant Soybean MON87708

Summary and conclusions

Background

Monsanto Company (Monsanto) has developed a genetically modified (GM) soybean line known as MON87708 that is tolerant to the herbicide dicamba. This is achieved through expression of the enzyme dicamba mono-oxygenase (DMO) encoded by the *dmo* gene derived from the common soil bacterium *Stenotrophomonas maltophilia*. The DMO protein has not previously been assessed by FSANZ.

In conducting a safety assessment of food derived from soybean line MON87708, a number of criteria have been addressed including: a characterisation of the transferred gene, its origin, function and stability in the soybean genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed protein to be either allergenic or toxic in humans.

This safety assessment report addresses only food safety and nutritional issues. It therefore does not address:

- environmental risks related to the environmental release of GM plants used in food production

- the safety of animal feed or animals fed with feed derived from GM plants

- the safety of food derived from the non-GM (conventional) plant.

History of Use

Soybean (*Glycine max*), the host organism is grown as a commercial crop in over 35 countries worldwide. Soybean-derived products have a range of food and feed uses as well as industrial uses and have a long history of safe use for both humans and livestock. Oil, in one form or another, accounts for the major food use of soybean (Shurtleff and Aoyagi, 2007) and is incorporated in salad and cooking oil, bakery shortening, and frying fat as well as processed products such as margarine.

Molecular Characterisation

Soybean cultivar A3525 was transformed with two gene expression cassettes using an *Agrobacterium*-mediated method. The first cassette contained the *dmo* gene while the second cassette contained, as a marker, the commonly used *cp4 epsps* gene that confers tolerance to the herbicide glyphosate.

Comprehensive molecular analyses of soybean line MON87708 indicate there is a single insertion site comprising a complete copy of the *dmo* expression cassette. The second expression cassette containing the *cp4epsps* gene, that was used in the initial transformation, was deliberately segregated out and so is absent from MON87708.

The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and plasmid backbone analysis shows that no plasmid backbone has been incorporated into the transgenic locus.

Characterisation of Novel Protein

Soybean line MON87708 expresses one novel protein, DMO, which was detected in all plant parts that were analysed. Levels were lowest in the root (approximately 6 µg/g dry weight) and highest in older leaves (approximately 70 µg/g dry weight). The seed contained approximately 47 µg/g dry weight.

Several studies were done to confirm the identity and physicochemical properties of the DMO protein expressed in MON87708. The results of these studies demonstrated that the DMO expressed in MON87708 is actually a mixture of two monomers comprising mature DMO and the DMO precursor protein (designated DMO+27) which is identical to mature DMO except for an additional 27 amino acids at the N terminus which failed to be cleaved off during translocation of the protein to the chloroplast. Both monomers conform in size and amino acid sequence to that expected, and do not exhibit any post-translational modification including glycosylation. The specificity of the MON87708 DMO for the dicamba substrate was demonstrated.

Bioinformatic studies have confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that DMO would be completely digested before absorption in the gastrointestinal tract would occur. As anticipated, a mouse oral toxicity study revealed no treatment-related effects. It was further demonstrated that the MON88708 DMO protein is not stable at elevated temperatures and loses most of its activity above 55° C.

Taken together, the evidence indicates that DMO is unlikely to be toxic or allergenic to humans.

Herbicide Metabolites

The residues generated on soybean line MON87708 as a result of spraying with dicamba are the same as those found on conventional crops sprayed with dicamba. Residue data derived from supervised trials indicate that the residue levels in seed are low and that there is some concentration of residue in hulls, toasted defatted meal and defatted flour but not in other processed commodities. In the absence of any significant exposure to either parent herbicide or metabolites the risk to public health and safety is negligible.

Compositional Analyses

Detailed compositional analyses were done to establish the nutritional adequacy of seed from soybean line MON87708 sprayed with dicamba. Analyses were done of 57 analytes encompassing proximates, fibre, fatty acids, amino acids, isoflavones, anti-nutrients and vitamin E. The levels were compared to levels in the seeds of the non-GM parent A3525.

These analyses indicated that the seeds of soybean line MON87708 are compositionally equivalent to those of the parental line. Out of the analytes tested, there were significant differences between the non-GM control and soybean MON87708 in 27 analytes. In all of these, except for behenic acid, the mean levels observed in seeds of soybean MON87708 were within the range of natural variation either reported in the literature or derived from 18 non-GM commercial varieties grown in the same field trials. For any analyte, the magnitude of the differences observed between MON87708 and A3525 was not as great as the magnitude between the reference varieties.

In addition, no difference between seeds of soybean line MON87708 and A3525 were found in an IgE binding study using sera from soybean-allergic individuals.

The compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key components in seed from soybean line MON87708 when compared with the non-GM control or with the range of levels found in non-GM commercial soybean cultivars.

Conclusion

No potential public health and safety concerns have been identified in the assessment of soybean line MON87708. On the basis of the data provided in the present Application, and other available information, food derived from soybean line MON87708 is considered to be as safe for human consumption as food derived from conventional soybean cultivars.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADF	acid detergent fibre
ADI	Acceptable Daily Intake
ARfD	Acute Reference Dose
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
CCI	Confidential Commercial Information
DCGA	2,5-dichloro-3,6-dihydroxybenzoic acid
DCSA	3,6-dichlorosalicylic acid
DMO	dicamba mono-oxygenase
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FARRP	Food Allergy Research and Resource Program
FASTA	Fast Alignment Search Tool - All
FSANZ	Food Standards Australia New Zealand
GM	genetically modified
HPLC	high performance liquid chromatography
IgE	Immunoglobulin E
ILSI	International Life Sciences Institute
kDa	kilo Dalton
LC/MS	liquid chromatography mass spectrometry
MALDI-TOF	matrix-assisted laser desorption/ionisation–time of flight
NDF	neutral detergent fibre
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
RbcS	Ribulose bisphosphate carboxylase small subunit
RBD	refined, bleached, deodorised
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
TRR	total radioactive residue
U.S.	United States of America

1.   Introduction

A genetically modified (GM) soybean line, MON87708 has been developed that provides tolerance to the herbicide dicamba. This modification will provide soybean growers with a broader weed management strategy (Behrens *et al.*, 2007).

Tolerance to dicamba is achieved through expression of the enzyme dicamba mono-oxygenase (DMO) encoded by the *dmo* gene derived from the common soil bacterium *Stenotrophomonas maltophilia.* The DMO protein has not previously been assessed by FSANZ.

It is anticipated that soybean plants containing event MON87708 may be grown in the United States of America (U.S.) and Canada subject to approval. There is no plan to grow the line in Australia or New Zealand.

2.   History of use

2.1   Host organism

The host organism is a conventional soybean (*Glycine max* (L.) Merr.), belonging to the family Leguminosae. The commercial soybean cultivar A3525 was used as the parent for the genetic modification described in this application, and thus is regarded as the near-isogenic line for the purposes of comparative assessment with soybean MON87708. A3525 is a high-yielding cultivar that was released in the U.S. in 2004 for its cyst nematode resistance combined with multi-race Phytophthora protection, as well as very good brown stem rot and sudden death syndrome tolerance.

Soybean is grown as a commercial food and feed crop in over 35 countries worldwide (OECD, 2001) and has a long history of safe use for both humans and livestock. The major producers of soybeans, accounting for 90% of world production, are the U.S., Argentina, Brazil and China. Australia, while a net importer of soybean, grows crops in latitudes extending from the tropics (16° S) to temperate regions (37° S), mainly in the eastern states and as a rotational crop (James and Rose, 2004). The seed is used mainly to produce meal for use in animal feed (Grey, 2006).

In many soybean producing countries, GM soybean (mainly with a herbicide tolerant trait) accounts for a significant proportion of the total soybean grown e.g. U.S. (91%); Argentina (99%); Brazil (63%); South Africa (87%); Uruguay (99%) (Brookes and Barfoot, 2009). Australia does not currently grow any commercial GM soybean lines[1].

Soybean food products are derived either from whole or cracked soybeans:

*   Whole soybeans are used to produce soy sprouts, baked soybeans, roasted soybeans and traditional soy foods such as miso, tofu, soy milk and soy sauce.
*   Cracked soybeans have the hull (seed coat) removed and are then rolled into flakes which undergo solvent extraction to remove the oil.
*   This crude oil is further refined to produce cooking oil, shortening and lecithins as well as being incorporated into a variety of edible and technical/industrial products. The flakes are dried and undergo further processing to form products such as meal (for use in e.g. livestock, pet and poultry food), protein concentrate and isolate (for use in both

---

[1] See information on approved commercial; releases of GM crops in Australia on the website of the Office of the Gene Technology Regulator - http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/ir-1

edible and technical/industrial products), and textured flour (for edible uses). The hulls are used in mill feed.

Unprocessed (raw) soybeans are not suitable for food use, and have only limited feed uses, as they contain toxicants and anti-nutritional factors, such as lectins and trypsin inhibitors (OECD, 2001). Appropriate heat processing inactivates these compounds.

*S*oybean oil constitutes approximately 30% of global consumption of edible fats and oils (The American Soybean Association, 2011), and is currently the second largest source of vegetable oil worldwide (USDA, 2009). Oil, in one form or another, accounts for the major food use of soybean (Shurtleff and Aoyagi, 2007) and is incorporated in salad and cooking oil, bakery shortening, and frying fat as well as processed products such as margarine.

Another possible food product that can be derived from the soybean plant is bee pollen. This substance is produced by bees during foraging and is taken back to the hive to be fed to larvae and young adult bees (Krell, 1996). It comprises pollen grains that are pelleted by the bee in the corbiculae ('pollen baskets') located on the posterior pair of legs. Beekeepers can collect the pellets by placing a screen at the entrance to a hive; as the bees pass through the screen, the pellets are dislodged and fall into a collection tray. The pellets are frozen or dried for storage and are then packaged for sale as bee pollen, which is generally consumed as the raw product without any further processing. It is highly unlikely that this product would be imported to Australia or New Zealand as domestic supply would satisfy market requirements.

MON87708 is intended primarily for use as a broad-acre commodity (field soybean) to produce products derived from cracked soybeans, and is not intended for vegetable or garden purposes where food-grade products may include tofu, soybean sprouts, soy milk, and green soybean (e.g. edamame). This latter type of soybean generally has a different size, flavour and texture to field soybean (Liu *et al.*, 1995).

2.2   Donor organisms

2.2.1   Stenotrophomonas maltophilia

This bacterium, like the other seven species currently in the genus, has a worldwide distribution and occurs ubiquitously in the environment, being particularly associated with plants (Ryan *et al.*, 2009). It was originally *named Pseudomonas maltophilia* (see Hugh, 1981) but was then changed to *Xanthomonas maltophilia* (Swings *et al.*, 1983) before it was given its own genus (Palleroni and Bradbury, 1993).

S. maltophilia has been isolated from the rhizosphere and internal tissues of a range of plant species (Ryan *et al.*, 2009). It is also an opportunistic coloniser of the environment and has been detected in moist sites in domestic homes and bathrooms such as taps, showers, sinks, washing machines, dishcloths, sponges and kitchen work surfaces (Denton *et al.*, 1998). Although not in itself regarded as an inherently virulent pathogen, *S. maltophilia* has an ability to colonise respiratory tract cells and surfaces of medical devices and, this, coupled with high levels of intrinsic or acquired resistance to antimicrobials, has led to its causing a wide range of sometimes fatal infections in immuno-compromised hospital patients (Al-Jasser, 2006; Looney, 2009).

Additional to the above, *Stenotrophomonas* spp. are considered to have promising applications in bio-and phyto-remediation as they can metabolise a range of organic compounds (see discussion in Ryan *et al.*, 2009).

5

2.2.2 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of soybean MON87708 (refer to Table 1). These non-coding sequences are used to drive, enhance, target or terminate expression of the novel gene. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from plant pathogens are not pathogenic in themselves and do not cause pathogenic symptoms in soybean MON87780.

3. Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

Studies submitted:

Song, Z; Lawry, K.D.; Rice, J.F.; Tian, Q. (2011). Amended report for MSL0022670: Molecular analysis of dicamba-tolerant soybean MON87708. Study ID# MSL0023278, Monsanto Company (unpublished).
Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of DNA sequences flanking the 5' and 3' junctions of inserted DNA in MON87708: Assessment of putative polypeptides. Study ID# MSL0022682, Monsanto Company (unpublished).

3.1 Method used in the genetic modification

Soybean cultivar A3525 was transformed with binary plasmid vector PV-GMHT4355 containing two DNA inserts (Figure 1) using an *Agrobacterium*-mediated method (Martinell *et al.*, 2002). Insert T-DNA I contained the *dmo* gene while insert T-DNA II contained, as a marker, the commonly used *cp4 epsps* gene that confers tolerance to the herbicide glyphosate (see FSANZ, 2011 for the most recent discussion of this gene).

In summary, seeds of A3525 were germinated and shoot meristem tissues were excised from the resulting embryos. 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 a medium that supported shoot and root development. Rooted plants (R_0) with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

Although following transformation both T-DNAs were inserted into the genome, subsequent conventional breeding and segregation (refer to Section 3.3) were used to isolate those plants containing only T-DNA I.

Figure 1:   Genes and regulatory elements contained in plasmid PV-GMHT4355

3.2   Function and regulation of introduced genes

Information on the genetic elements in the T-DNA I insert present in MON87708 is summarised in Table 1.

Table 1: Description of the genetic elements contained in T-DNA 1 of PV GMHT 4355

Genetic element	bp location on PV GMHT 4355	Size (bp)	Source	Orient.	Description & Function	References
RIGHT BORDER	8290 -8646	357				
Intervening sequence	8647 - 8691	45				
PC1SV	8692 - 9124	433	Peanut chlorotic streak caulimovirus	clockwise	• Promoter for the full length transcript • Drives strong constitutive expression of *dmo*	Maiti & Shepherd (1998)
Intervening sequence	9125 - 9144	20				
TEV	9145 - 9276	132	Tobacco etch virus	clockwise	• Leader sequence from the 5' non-translated region • Enhances translation	Niepel & Gallie (1999)
Intervening sequence	9277	1				
RbcS	9278 - 9520	243	*Pisum sativum*	clockwise	• Encodes the transit peptide and the first 24 amino acids of the *RbcS* gene • Directs transport of the DMO precursor protein to the chloroplast	Fluhr et al. (1986)
Intervening sequence	9521 - 9529	9				
dmo	9530 - 10552	1023	*S. maltophilia*	clockwise	• Coding sequence for dicamba mono-oxygenase	Herman et al. (2005); Wang et al. (1997)
Intervening sequence	10553 - 10620	68				
E9	10621 - 11263	643	*Pisum sativum*	clockwise	• 3' non-translated region from the *RbcS* gene • Terminates *dmo* gene expression	Coruzzi et al. (1984)
Intervening sequence	11264 - 11352	89				
LEFT BORDER	1 - 442	442				

3.2.1 dmo *expression cassette*

The *dmo* gene was initially isolated and cloned from the bacterium *S. maltophilia* strain DI-6 (Herman *et al.*, 2005). The gene has been optimized for expression in plants (Feng and Brinker 2007). Expression of the gene confers tolerance to the herbicide dicamba (2-methoxy-3,6-dichlorobenzoic acid) (Dumitru *et al.*, 2009).

The *dmo* coding region is 1,023 bp in length and is driven by the *PC1SV* constitutive promoter from Peanut chlorotic streak caulimovirus. The addition of the leader sequence from the 5' non-translated region of the Tobacco etch virus assists in regulating expression. A transit peptide derived from elements from the small subunit ribulose bisphosphate carboxylase (RbcS) of pea (*Pisum sativum*) targets a DMO precursor protein to the chloroplasts where electrons are available to drive the reaction that leads to demethylation of dicamba (Behrens *et al.*, 2007). The *E9* 3'non-translated region from the pea *RbcS* gene functions to direct polyadenylation of the mRNA.

3.3 Breeding of soybean plants containing transformation event MON87708

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of soybean line MON87708

- ensuring that the MON87708 event is incorporated into elite breeding line(s) for commercialisation of dicamba-tolerant soybean.

The breeding pedigree for the various generations is given in Figure 2.

The originally transformed R_0 plants were self-pollinated to produce R_1 plants to which a non-lethal dose of glyphosate was applied. 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. Subsequently, plants that were homozygous for T-DNA I and that did not contain T-DNA II were identified by quantitative polymerase chain reaction (PCR) analysis. A single plant designated as MON87708 was selected as the lead event at R_1 based on superior phenotypic characteristics, dicamba tolerance, and its molecular profile.

There then followed several rounds of self-pollination and seed bulking. At the R_5 generation, plants were crossed with a number of elite lines for commercial development.

R_0
(Transformation)
$\downarrow \otimes$
R_1
$\downarrow \otimes$
R_2
$\downarrow \otimes$
R_3
$\downarrow \otimes$
R_4
$\downarrow \otimes$
$R_5 \longrightarrow$ Commercial Development
$\downarrow \otimes$
R_6
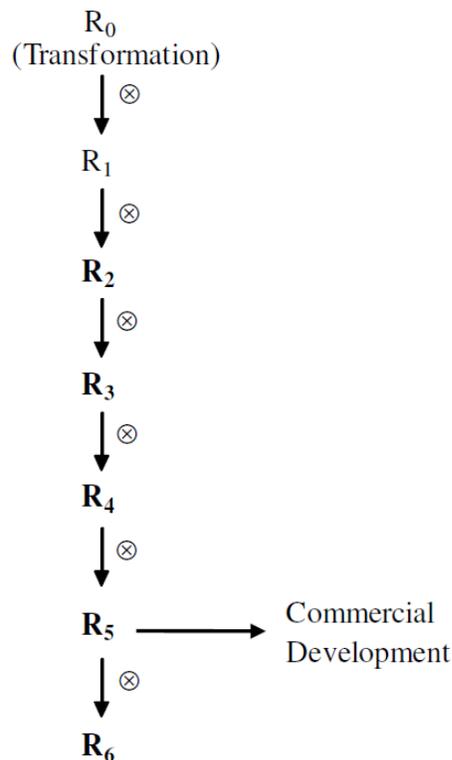
R_0−originally transformed plant; $\otimes$−self pollinated

Figure 2: Breeding strategy for plants containing event MON87708

3.4   Characterisation of the genes in the plant

A range of analyses were undertaken in order to characterise the genetic modification in soybean line MON87708. These analyses focussed on the nature of the insertion of the

introduced genetic elements and whether any unintended genetic re-arrangements may have occurred as a consequence of the transformation procedure.

3.4.1 Transgene copy number and insertion integrity

Total genomic DNA from leaf tissue of certified soybean MON87708 (R_3 generation) and A3525 (negative control) seedlings was used for Southern blot analyses The DNA from soybean MON87708 and A3525 was digested with the same combinations of restriction enzymes. The resulting DNA fragments were separated by agarose gel electrophoresis and transferred to a membrane for sequential hybridisation with ten different overlapping radiolabelled probes that, taken together, spanned the entire T-DNA I sequence, the entire T-DNA II sequence and the backbone sequences of plasmid PV-GMHT4355. A positive control (digested DNA from A3525 spiked with restriction enzyme-digested DNA from plasmid PV-GMHT 4355) was also included in the Southern blot analyses to demonstrate sensitivity of the Southern blots and to confirm that the probes were recognising the target sequences.

The Southern blot analyses indicated that the introduced DNA has been inserted at a single locus and contains one intact copy of T-DNA I (Figure 3). No T-DNA II or backbone sequences were detected.

The negative control showed no hybridisation with any of the probes.

3.4.2 Full DNA sequence of insert

Genomic DNA was obtained from leaf tissue of R_3 generation MON87708 and A3525. The organisation of the genetic elements within the insert and associated flanking regions was determined by amplifying (polymerase chain reaction – PCR) and sequencing (BigDye® Terminator chemistry - http://www.appliedbiosystems.com.au/) two overlapping regions of the DNA.

No PCR product was obtained from the A3525 DNA template.

Consensus sequencing of the products obtained from MON87708 template confirmed that the arrangement and linkage of elements in the inserted DNA were consistent with those in plasmid PV-GMHT4355. The insert is 3003 bp long, beginning at base 8604 in the Right Border region (refer to Table 1) and ending at base 254 in the Left Border region. In addition, there is a 1048 bp sequence flanking the 5' end of the insert and a 1271 bp sequence flanking the 3' end (Figure 3). Precise sequence details are Confidential Commercial Information (CCI).

3.4.3 Analysis of the insertion site

PCR and sequence analysis were carried out on genomic DNA extracted from MON87708 (R_3 generation) and A3525 using one primer specific to the 5' flanking region and one primer specific to the 3' flanking region of the insert.

PCR products were obtained from DNA template from both sources. Products were then sequenced and compared. It was shown that 920 bases from the 5' end of the insert and 1,235 bases from the 3' end of the insert are identical in sequence to bases in the A3525 genome. Additionally, there is an 899 bp deletion and a 128 bp insertion just 5' to the MON87708 insert and a 35 bp insertion just 3' to the MON87708 insert (Figure 3).
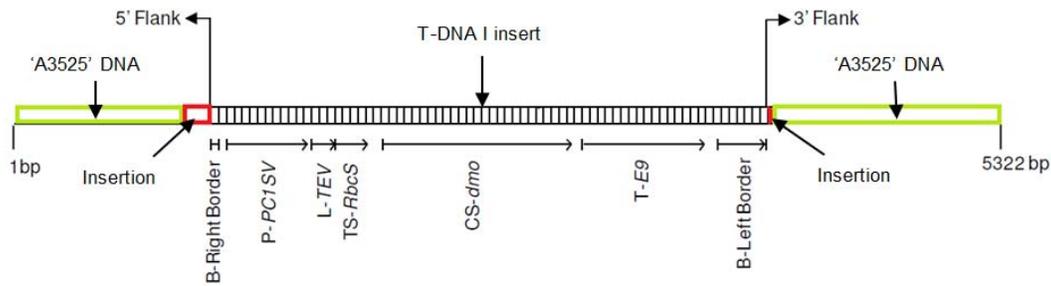
Figure 3: Schematic representation of the insert and flanking regions in MON87708

3.4.4   Open reading frame (ORF) analysis

The transgenic insert has the identical sequence to the T-DNA I of the PV-GMHT4355 plasmid (see Section 3.4.2) and therefore has no unexpected ORFs.

Sequences spanning the two junction regions formed as a result of the insertion of the T-DNA I were translated using DNAStar software (http://www.dnastar.com/) from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames. A total of 20 ORFs were identified, ranging in size from 8 – 120 amino acids. No analysis was done to determine whether any potential regulatory elements were associated with the polypeptides.

The putative polypeptides encoded by the 20 identified ORFs were then analysed using a bioinformatic strategy to determine whether, in the event they were translated, they raise any allergenic or toxicity concerns (refer to Section 4.1).

3.5   Stability of the genetic changes in soybean line MON87708

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification over successive generations, as produced in the initial transformation event. It is best assessed by molecular techniques, such as Southern analysis or PCR, using probes and primers that cover the entire insert and flanking regions. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations. It is often quantified by a trait inheritance analysis to determine Mendelian heritability via assay techniques (chemical, molecular, visual).

3.5.1   Genetic stability

The genetic stability of event MON87708 was evaluated in leaf tissue from individual plants of five different generations, namely R_2, R_3, R_4, R_5 and R_6 (see Figure 2) by Southern analysis. Genomic DNA from each of the generations was digested with two restriction enzymes and the resulting fragments were hybridised with a T-DNA I-specific probe.

There was no hybridisation in an A3525 negative control. The Southern blot analysis confirmed the presence of the expected hybridisation fragments in all tested transgenic DNA samples and therefore confirmed the genetic stability of the insert in MON87708 over different generations.

3.5.2   Phenotypic stability

At each generation up to R_4 the fixed homozygous plants were tested for the expected segregation pattern of 1:0 (presence:absence) for the *dmo* gene using Invader® analysis, a non-PCR based assay that can be used to quantify transgene copy number (Gupta *et al.*, 2011). This segregation pattern was maintained.

Plants from R_4 were then crossed with a non-GM soybean cultivar to produce F_1 hemizygous seed. The resulting F_1 plants were self-pollinated to produce F_2 seed. The F_2 plants were tested for the presence of the *dmo* cassette by Invader® analysis and hemizygous plants were then self-pollinated to produce F_3 seed. This process of self-pollination and selection was continued through to the F_4 generation.

A Chi-square ($\chi2$) analysis was used to compare the observed segregation ratios to the expected ratios according to Mendelian inheritance, at each of the F generations (Table 2). The results showed that there was no significant difference between the expected and observed ratios and therefore, that the *dmo* expression cassette is stably inherited according to Mendelian principles.

Table 2:   *Segregation of the dmo gene over three generations*

Generation	Total plants	Ratio[1]		Probability (P)[2]
		Observed	Expected	
F2	3223	2.97:1	3:1	0.863
F3	118	1:1.8:1.2	1:2:1	0.253
F4	343	1:2.06:1.07	1:2:1	0.899

[1]For the F_2 generation, zygosity of 200 plants could not be determined and therefore the segregation ratio was based on presence:absence of the *dmo* gene with an expected ratio of 3:1.
For the F_3 and F_4 generations segregation was based on zygosity, with an expected ratio of
1 homozygous positive: 2 hemizygous positive: 1 homozygous negative.
[2]Statistical significance is when P≤0.05

3.6   Antibiotic resistance marker genes

No antibiotic marker genes are present in soybean line MON87708. Plasmid backbone analysis shows that no plasmid backbone has been integrated into the soybean genome during transformation, i.e. the *aadA* gene, which was used as a bacterial selectable marker gene, is not present in soybean MON87708.

3.7   Conclusion

Soybean line MON87708 contains the *dmo* gene that encodes a protein conferring tolerance to the herbicide dicamba.

Comprehensive molecular analyses of soybean line MON87708 indicate that there is a single insertion site comprising a complete copy of the *dmo* expression cassette. A second expression cassette containing the *cp4epsps* gene that was used in the initial transformation was segregated out in MON87708.

The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and plasmid backbone analysis shows no plasmid backbone has been incorporated into the transgenic locus.

4.   Characterisation of novel proteins

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, e.g., because they are allergens or anti-nutrients (Delaney *et al.*, 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

Three types of novel proteins were considered:

- Those that may be potentially translated as a result of the creation of ORFs during the transformation process (see Section 0).
- Putative polypeptides that may be encoded unexpectedly by translation of reading frames 1 through 6 of the inserted T-DNA I.
- Those that were expected to be directly produced as a result of the translation of the introduced genes. A number of different analyses were done to determine the identity, physiochemical properties, *in planta* expression, bioactivity and potential toxicity and allergenicity.

4.1   Potential allergenicity/toxicity of ORFs created by the transformation procedure

Study submitted:
Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of DNA sequences flanking the 5' and 3' junctions of inserted DNA in MON87708: Assessment of putative polypeptides. Study ID# MSL0022682, Monsanto Company (unpublished).

A bioinformatics analysis was performed to assess the similarity to known allergens and toxins of the putative polypeptides encoded by the 20 sequences obtained from the ORF analysis (refer to Section 3.4.4).

To evaluate the similarity to known allergens of proteins that might potentially be produced from translation of the ORFs, an epitope search was carried out to identify any short sequences of amino acids that might represent an isolated shared allergenic epitope. This search compared the sequences with known allergens in the Allergen, Gliadin and Glutenin sequence database, residing in the FARRP (Food Allergy Research and Resource Program) dataset (Version 10) within AllergenOnline (University of Nebraska; http:www.allergenonline.org/). The FASTA algorithm (Pearson and Lipman, 1988), version 3.4t, was used to search the database using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). No alignments with any of the 20 query sequences generated an E (expect)-value[2] of ≤1e^{-5}, no alignment met or exceeded the Codex Alimentarius (Codex,

---

[2] Comparisons between highly homologous proteins yield E-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger E-value indicates a lower degree of similarity. Commonly, for protein-based searches, hits with E-values of 10^{-3} or less and sequence identity of 25% or more are considered significant although any conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxevanis, 2005). In this application an E-value of 10^{-5} or less was set as the high cut-off value for alignment significance.

2003) FASTA alignment threshold for potential allergenicity and no alignments of eight or more consecutive identical amino acids (Metcalfe *et al.*, 1996) were found. It was concluded that the 20 putative polypeptides are unlikely to contain any cross-reactive IgE binding epitopes with known allergens.

The sequences corresponding to the 20 reading frames were also compared with sequences present in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the FASTA algorithm. No significant similarities of the 20 reading frames to any sequences in the databases (including those of known toxins) were found.

It is concluded that, in the unlikely event transcription and translation of the 20 identified ORFs could occur, the encoded polypeptides do not share any significant similarity with known allergens or toxins.

4.2 Potential allergenicity/toxicity of unexpected putative peptides encoded by T-DNA I

Study submitted
Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of the Transfer DNA insert in MON87708 utilizing the AD_2010, TOX_2010 and PRT_2010 databases. Study ID# MSL0022679, Monsanto Company (unpublished).

A bioinformatic analysis, using the same approach as described in Section 4.1, was performed to assess the similarity to known allergens and toxins of putative peptides encoded by translation of reading frames 1 through 6 of the inserted T-DNA I sequence present in MON 87708 sequence. The analyses did not identify any similarity to known allergens or toxins. As expected, in the search of the GenBank database, significant alignments were obtained with an oxygenase from *S. maltophilia* and with ribulose 1,5-bisphosphate carboxylase (see discussion in Section 4.7.2).

4.3 Function and phenotypic effects of the DMO protein

DMO was first purified from *S. maltophilia* isolated from soil at a dicamba manufacturing plant (Krueger *et al.*, 1989).

DMO is categorized as a Rieske (iron-sulphur) non-haem iron oxygenase (see e.g. Ferraro *et al.*, 2005) since it contains Rieske [2Fe-2S] clusters (D'Ordine *et al.*, 2009). Such enzymes are found in soil-dwelling bacteria that often populate hostile and xenobiotic-rich environmental niches (see discussion in Dumitru *et al.*, 2009).

DMO receives electrons originating from an FAD/NADH-dependent reductase via an intermediate ferredoxin (Wang *et al.*, 1997; Chakraborty *et al.*, 2005). Together the oxygenase, ferredoxin and reductase comprise an enzyme complex known as dicamba *O*-demethylase. DMO is involved in the initial step in the degradation of dicamba. The way in which the three components interact to demethylate dicamba to the herbicidally inactive 3,6-dichlorosalicylic acid (DCSA) is shown in Figure 4.
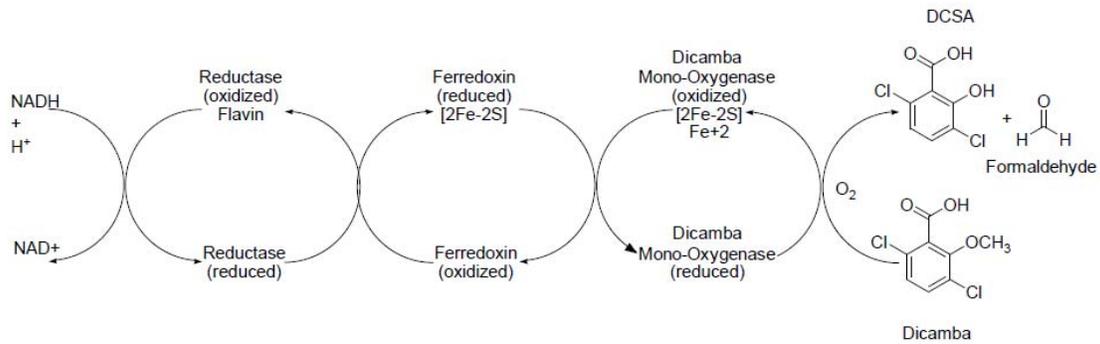
Figure 4: Components of the dicamba O-demethylase system

The functional activity of DMO requires that a homotrimer comprising three DMO monomers is formed (Dumitru *et al.*, 2009; D'Ordine *et al.*, 2009).

4.3.1 The nature of the DMO tri-mer in MON87708

Study submitted:
Morey, M.; Niemeyer, K.E. (2009). Western blot analysis of DMO protein in dicamba-tolerant soybean MON87708 leaf across multiple generations produced in the greenhouse during 2007 and 2008. Study ID# MSL0021459, Monsanto Company (unpublished).

In MON87708 it was anticipated that during translocation into chloroplasts, the chloroplast peptide sequence (RbcS) encoding 24 amino acids, and an intervening sequence encoding three amino acids, would be cleaved resulting in the appropriate amino terminus for mature DMO. However, Western blot analyses of leaf and mature seed tissue during early-stage development of MON87708 revealed the presence of two bands, one corresponding to the mature DMO protein (designated as DMO) and one corresponding to DMO with the additional 27 amino acids (designated as DMO+27). See Section 4.5 for details of the protein characterisation.

The MON87708 DMO monomer has an identical sequence to the wild-type protein (Herman *et al.*, 2005) (Figure 5) except for the loss of the terminal methionine, an additional alanine in position 2 (added for cloning purposes) and a cysteine instead of tryptophan at position 112 (a replacement that early studies indicated made MON87708 DMO more kinetically efficient than the wild type DMO). The absence of the methionine in the DMO monomer suggests it was removed during post-translational processing of the precursor protein. This is not unexpected since the terminal methionine is routinely cleaved from nascent proteins by methionine aminopeptidase (see e.g. Polevoda and Sherman, 2000).

The MON87708 DMO+27 monomer is identical to the MON87708 DMO monomer except that it has an extra N-terminal 27 amino acids and therefore has not lost the methionine (Figure 5).
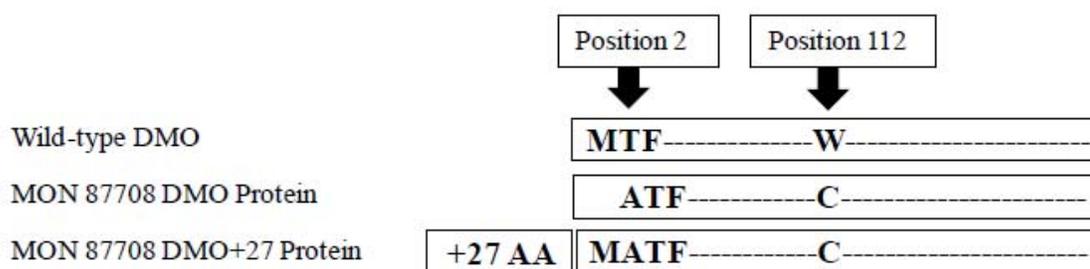
Figure 5: Schematic comparison of DMO from S. maltophilia*, MON87708 DMO monomer and MON87708 DMO+27 monomer.*

In MON87708, the trimer that must be formed in order for DMO to be functional can comprise three DMO monomers, three DMO+27 monomers, or a mixture of DMO and DMO+27 monomers.

Unless stated otherwise, the use of the abbreviation 'DMO' in relation to MON87708 in this application refers to total DMO protein comprising both DMO and DMO+27 monomers.

4.4 Protein expression analysis

Study submitted:
Tauchman, S.; Niemeyer, K.E. (2010). Assessment of total DMO protein levels in soybean tissues collected from MON87708 produced in United States field trials during 2008. Study ID# MSL0022510, Monsanto Company (unpublished).

4.4.1 Novel protein expression in plant tissues

The DMO protein (DMO and DMO+27) is expected to be expressed in all plant tissues since the *dmo* gene is driven by a constitutive promoter (refer to Table 1).

Plants of MON87708 (generation R_5) and A3525 were grown from validated seed lots at five field sites in the U.S. during the 2008 growing season. The identity of subsequent harvested seed from each site was also confirmed using event-specific polymerase chain reaction (PCR). There were three replicated plots at each site. Samples were taken at various stages of growth (Table 3) and the level of DMO protein was determined for each sample type using a validated enzyme linked immunosorbent assay (ELISA). The DMO antibody (which detected both forms of DMO) was a biotinylated goat anti-DMO polyclonal and was detected using NeutrAvidin (Pierce) conjugated to horseradish peroxidase. Quantification of total DMO protein was accomplished by interpolation on a DMO protein standard curve.

DMO was detected in all parts from MON87708 (Table 3); it was lowest in the root (approximately 6 μg/g dry weight) and highest in older leaves (approximately 70 μg/g dry weight). The seed contained approximately 47 μg/g dry weight. Values obtained for tissue from A3525 were all below the limit of quantitation.

Table 3:   Total DMO (DMO and DMO+27) protein content in MON88708 soybean parts at different growth stages (averaged across 5 sites, n = 15)

Growth stage[1]/tissue	µg/g dry weight		Standard Deviation
	Mean	Range	
V3-V4/leaf	17	6.2 - 29	7.7
V5 – V8/leaf	31	12 - 54	13
R2 – V12/leaf	44	25 - 71	44
R5 – V16/leaf	69	23 - 180	46
R6/forage[2]	53	25 - 84	18
R6/root	6.1	3.9 - 11	2.1
R8/seed	47	34 - 59	8.7

[1]For information on soybean growth stages see e.g. NDSU (2004).
[2]Forage is the above ground plant parts used for animal feed.

4.5   DMO characterisation

Studies submitted:

Wang, C.; Hill, S.R.; Burzio, L.A.; Finnessy, J.J. (2010). Characterization of the dicamba mono-oxygenase (DMO) enzyme isolated from the seed of MON87708. . Study ID# MSL0022497, Monsanto Company (unpublished).

Total DMO (DMO and DMO+27) was purified from defatted flour made from validated seed of MON87708 (generation R_6 plants). A number of analytical techniques were then used to identify and characterise the two protein monomers.

4.5.1   Molecular weight

The molecular weights of the two DMO monomer proteins were estimated from densitometric analysis of SDS-PAGE. Calculated molecular weight values of DMO and DMO+27 were averaged from duplicated loads of 0.5, 1.0 and 1.5 µg of total protein. Two predominant bands were obtained with calculated molecular weights of 39.8 kDa and 42 kDa. These values correspond with the predicted molecular weights of DMO and DMO+27 respectively. The molecular weight of native DMO from *S. maltophilia* is 40 kDa (Chakraborty *et al.*, 2005).

4.5.2   Immunoreactivity

Immunoreactivity of DMO was investigated by Western blot analysis. Blotted polyvinylidene fluoride (PVDF) membranes were probed with polyclonal goat anti-DMO followed by a commercially available (Thermo Scientific) rabbit anti-goat enzyme-linked (horseradish peroxidase) secondary antibody.

The anti-DMO antibody recognised two bands migrating at approximately 39 kDa and 42 kDa. No other bands were observed, thus confirming the identity of the two DMO monomer proteins.

4.5.3   MALDI-TOF tryptic mass fingerprint analysis

Mass spectral analysis using matrix-assisted laser desorption/ionisation–time of flight (MALDI–TOF) was performed on trypsin-digested excised bands corresponding to the DMO and DMO+27 monomers obtained by running purified total DMO on SDS-PAGE.

It was estimated that the peptide mapping of the DMO monomer protein identified 77% of the expected protein sequence while 82% of the expected protein sequence of the DMO+27

monomer was identified. The N-terminal peptides of both DMO monomer and DMO+27 monomer were identified and indicated that the N-terminal methionine was missing in DMO monomer (refer to Figure 5) and methylated in DMO+27. The methylation of the methionine in DMO+27 is consistent with the observation of *in vivo* post-translational modification of the amino terminal methionine of the ribulose bisphosphate carboxylase small subunit in pea and other plant species

4.5.4   N-terminal sequence analysis

Edman degradation chemistry was performed on excised bands corresponding to the DMO and DMO+27 monomers obtained by running purified total DMO on SDS-PAGE.

The analysis yielded a 15 amino acid sequence for each monomer. The sequences matched the expected sequences of DMO and DMO+27. The absence of the N-terminal methionine in the DMO monomer and the methylation of the N-terminal methionine in the DMO+27 were confirmed.

4.5.5   Glycosylation analysis

Many eukaryotic proteins are glycoproteins that have been post-translationally modified by the addition of carbohydrate moieties (glycans) covalently linked to the polypeptide backbone. Glycosylation that occurs on side chains of asparagine residues is termed N-glycosylation. The addition of N-acetylglucosamine to the β-hydroxyl of either serine or threonine residues is known as O-glycosylation. The carbohydrate component may represent from <1% to >80% of the total molecular weight of glycoprotein. There is one report in the literature of the expression of non-native proteins in transgenic plants leading to aberrant glycosylation, with the potential to lead in turn to altered immunogenicity (Prescott *et al.*, 2005).

N-glycosylated proteins are glycosylated on an asparagine residue and commonly contain an asparagine-X-serine/threonine sequence (N-X~(P)-[S/T], where X~(P) indicates any amino acid except proline (Orlando and Yang, 1998). Although rare, the sequence asparagine-X-Cysteine   (N-X-C) can also be an N-glycosylation site (Miletich and Broze Jr., 1990).

Carbohydrate detection was performed directly on the DMO and DMO+27 bands (transferred from SDS-PAGE to a PVDF membrane) using a commercial Glycosylation Detection Module (GE Healthcare). Transferrin, a naturally glycosylated protein was used as a positive control.

Transferrin was detected at the expected molecular weight and in a concentration-dependent manner. No glycosylation signals were detected at the expected molecular weights for either the DMO or DMO+27 monomer.

4.5.6   Enzymatic activity

The DMO enzyme catalyses the formation of DCSA using dicamba as a substrate (refer to Section 4.3). Enzymatic activity was therefore determined by measuring the production of DCSA by high performance liquid chromatography in a reaction mixture containing all the necessary components required for catalysis including ferredoxin and reductase, as well as MON87708 DMO (DMO plus DMO+27). The reaction was initiated by the addition of dicamba and quenched by the addition of sulphuric acid.

The specific activity of MON87708 DMO was determined to be 62.21 nmol DCSA/min/mg of DMO and the result indicates that the MON87708 DMO is active.

4.6 Enzymatic specificity

Studies submitted:

Burzio, L.; McCann, M. (2010). Specificity of dicamba mono-oxygenase for potential endogenous substrates. Study ID# RPN-10-365, Monsanto Company (unpublished).
McCann, M. (2010). Specificity of dicamba mono-oxygenase (DMO) enzyme from MON87708 using *o*-anisic acid as a substrate. Study ID# RPN-10-499, Monsanto Company (unpublished).

The expression of a novel protein in a transformed plant may, in some cases, lead to that protein interacting detectably with substrates in the plant other than those intended. In the case of DMO, compounds structurally similar to dicamba such as phenyl carboxylic acids containing methoxy moieties may be potential candidates. A series of *in vitro* studies were therefore performed to evaluate the specificity of native DMO for dicamba herbicide and potential endogenous substrates.

The DMO used for the assays was obtained from a bacterial (*Escherichia coli*) expression system and was identical in amino acid sequence to wild type DMO (see Figure 5) except for a histidine tag (that aided in purification of the enzyme) at the N-terminal end.

The reaction of DMO with different compounds evaluated as potential substrates was carried out in a reaction mixture containing all the necessary components required for catalysis including ferredoxin and reductase, as well as DMO. The following substrates along with dicamba, were tested: **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]. Ultra high performance liquid chromatography followed by mass spectrometry was used to analyse the reaction mixtures after incubation at 30° C for 15 min. The results indicated that there was no catabolism by DMO of any substrate except dicamba.

In a follow-up study, DMO isolated from defatted flour produced from MON87708 seed was incubated in a complete reaction mixture together with either dicamba or *o*-anisic acid, and analysed as described above. There was no catabolism of *o*-anisic acid. This result indicated that the minor amino acid differences between wild type DMO and MON87708 DMO (refer to Figure 5) did not affect the specificity of the enzyme.

Overall, the results support the conclusion that the DMO protein shows high specificity for dicamba as a substrate.

4.7 Potential toxicity of DMO protein

While the vast majority of proteins ingested as part of the diet are not typically associated with toxic effects, a small number may be harmful to health. Therefore, if a GM food differs from its conventional counterpart by the presence of one or more novel proteins, these proteins should be assessed for their potential toxicity. The main purpose of an assessment of potential toxicity is to establish, using a weight of evidence approach, that the novel protein will behave like any other dietary protein.

The assessment focuses on: whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food;

amino acid sequence similarity with known protein toxins and anti-nutrients; structural properties of the novel protein including whether it is resistant to heat or processing and/or digestion. Appropriate oral toxicity studies in animals may also be considered, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate a concern.

4.7.1   History of human consumption

Rieske non-haem monoxygenases, of which DMO is a member, are found in diverse phyla; the iron and sulphur components present in these proteins are considered to be integral to energy metabolism in present-day organisms (Schmidt and Shaw, 2001). However, they are largely associated with bacteria. *S. maltophilia*, the organism from which the *dmo* gene was isolated, occurs ubiquitously in the environment and is found on plant species many of which are commonly eaten (Ryan *et al.*, 2009). Isolates have been identified in sources such as ready-to-eat salads (Qureshi *et al.*, 2005), fish such as yellowtail (Furushita *et al.*, 2011) and tuna (Ben-Gigirey *et al.*, 2002), drinking water dispensers (Sacchetti *et al.*, 2009) and milk (Cairo *et al.*, 2008).

4.7.2   Similarities with known protein toxins

Bioinformatic analyses are useful for assessing whether introduced proteins share any amino acid sequence similarity with known protein toxins.

> **Study submitted:**
>
> Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of the DMO+27 protein in MON87708 utilizing the AD_2010, TOX_2010 and PRT_2010 databases. Study ID # MSL0022584, Monsanto Company (unpublished).

The DMO+27 protein monomer sequence was compared with sequences present in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the FASTA algorithm (Pearson and Lipman, 1988) and BLOSUM50 scoring matrix. See footnote in Section 4.1 for an explanation of alignment significance.

As expected, the query sequence matched with the DMO protein from *S. maltophilia* as well as with a range of monoxygenases and proteins containing the Rieske [2Fe-2S] cluster domain, particularly vanillate monooxygenase. A secondary group of alignments was also identified with ribulose 1,5-bisphosphate carboxylase small subunit propeptide *from Pisum sativum*. This is consistent with the structure of DMO+27 that contains the first 24 amino acids of the RbcS from *P. sativum*.

There were no matches with any sequences from known protein toxins.

4.7.3   In vitro digestibility

See Section 4.8.3

4.7.4   Thermolability

The thermolability of a protein provides an indication of the stability of the protein under cooking/processing conditions. It is a particularly relevant consideration in soybean-derived products since raw soybeans cannot be consumed by humans because of the presence of anti-nutrient factors that are only destroyed by heat processing (OECD, 2001).

Total DMO protein (DMO and DMO+27) purified from MON87708 seed (taken from validated, generation R6 plants) was incubated at 25°, 37°, 55°, 75°, or 90° for 15 min or 30 min. DMO protein maintained on wet ice was used as a control. Following treatment, the samples were analysed by SDS-PAGE or were tested for enzyme activity (as described in Section 4.5.6).

Analysis by SDS-PAGE indicated that there was no effect on the protein monomers at temperatures up to 55° C. When incubated at 75° C for 15 min there was no apparent effect but when incubated for 30 min at that temperature there was a distinct decrease in band intensity. This decrease was more pronounced at 90° C with incubation for 30 min resulting in less protein being present than in a 10% DMO reference equivalent.

Results of the activity assay indicated that, at temperatures up to 37° C at least 70% activity was maintained. At 55° C and higher, the level of activity was below the limit of quantitation.

Overall, the results demonstrate that the MON88708 DMO protein is not stable at elevated temperatures.

4.7.5   Acute toxicity study

Although not required, since no toxicity concerns were raised in the data considered in Sections 4.5.1 – 4.5.4, the Applicant supplied an acute oral toxicity study.

The study design for each protein is given in Table 4.

Table 4: Study design for acute oral toxicity testing

Test material	Validated DMO protein (DMO and DMO+27) from MON87708
Vehicle	20 mM KPO_4 at pH8.0
Test Species	CD-1 mice (five females and 5 males) – approx. 8 weeks old on day of treatment
Dose	A single dose of test substance by oral gavage on Day 0. Actual total dose of 140 mg protein/kg body weight
Control	Five female and five male mice administered 205 mg protein/kg body weight BSA in 20 mM KPO_4 at pH8.0
Length of study	14 d

The dose of 140 mg DMO/kg body weight that was selected for testing was justified on the basis that it was several orders of magnitude higher than the highest anticipated exposure in humans. Since DMO has been shown to be completely degraded in a simulated gastric juice digestion model the selected dose for testing in mice is considered suitable.

Mice were observed for mortality, body weight gain and clinical signs over 14 days. At the end of the study all animals were killed and examined for organ or tissue damage or dysfunction. All mice in both control and test treatments survived for the duration of the study and gained weight over the duration of the trial. No clinical signs of systemic toxicity were observed in either test or control treatments. No macroscopic abnormalities attributable to the administration of the test proteins were present in the mice at necropsy on day 14.

Under the conditions of the study, oral administration of DMO protein to female and male mice at a dose of 140 mg protein/kg bw produced no test substance-related clinical signs of toxicity, body weight losses, macroscopic abnormalities or mortality.

4.8   Potential allergenicity of DMO protein

The potential allergenicity of novel proteins was evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination. This is because no single criterion is sufficiently predictive of either allergenicity or non-allergenicity (see e.g. Thomas *et al.*, 2009). The assessment focuses on:

- the source of the novel protein
- any significant amino acid sequence similarity between the novel protein and known allergens
- the structural properties of the novel protein, including susceptibility to digestion, heat stability and/or enzymatic treatment
- specific serum screening if the novel protein is derived from a source known to be allergenic or has amino acid sequence similarity to a known allergen, additional *in vitro* and *in vivo* immunological testing may be warranted.

Applying this approach systematically provides reasonable evidence about the potential of the novel protein to act as an allergen.

The allergenic potential of the DMO monomer proteins was assessed by:
- consideration of the source of the gene encoding the protein and history of use or exposure
- bioinformatic comparison of the amino acid sequence of the DMO+27 protein monomer with known protein allergen sequences
- evaluation of the lability of the protein monomers using *in vitro* gastric digestion models; and thermolability.

4.8.1   Source of each protein

As discussed in Section 2.2.1, the DMO protein is derived from a common soil bacterium to which humans have been naturally exposed and which may have been inadvertently ingested on fresh produce. There is therefore a prior history of human exposure to the DMO protein. There are no indications that the DMO protein is associated with any known adverse effects in humans.

4.8.2 Similarity to known allergens

> **Study submitted:**
>
> Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of the DMO+27 protein in MON87708 utilizing the AD_2010, TOX_2010 and PRT_2010 databases. Study ID # MSL0022584, Monsanto Company (unpublished).

Bioinformatic analysis provides part of a 'weight of evidence' approach for assessing potential allergenicity of novel proteins introduced to GM plants (Thomas *et al.*, 2005; Goodman, 2006). It is a method for comparing the amino acid sequence of the introduced protein with sequences of known allergens in order to indicate potential cross-reactivity between allergenic proteins and the introduced protein. As with the bioinformatic analysis that looked at similarities of the novel proteins with known protein toxins (refer to Section 4.7.2), the generation of an *E* value provides an important indicator of significance of matches (Pearson, 2000; Baxevanis, 2005).

The same approach as described in Section 4.1 was used to undertake a bioinformatic evaluation of the relatedness between the DMO+27 monomer and known allergens in the Allergen, Gliadin and Glutenin sequence database.

No alignment generated an E-score of $\leq 1e^{-5}$, no alignment met or exceeded the Codex Alimentarius (Codex, 2003) FASTA alignment threshold for potential allergenicity and no alignments of eight or more consecutive identical amino acids were found.

4.8.3 In vitro digestibility

Typically, food proteins that are allergenic tend to be stable to enzymes such as pepsin and the acidic conditions of the digestive system, exposing them to the intestinal mucosa and leading to an allergic response (Astwood and Fuchs, 1996; Metcalfe *et al.*, 1996; Kimber *et al.*, 1999). Therefore a correlation exists between resistance to digestion by pepsin and potential allergenicity although this may be inconsistent (Thomas *et al.*, 2004; Herman *et al.*, 2007). As a consequence, one of the criteria for assessing potential allergenicity is to examine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an allergic response. However, evidence of slow or limited protein digestibility does not necessarily indicate that the protein is allergenic.

A pepsin digestibility assay (Thomas *et al.*, 2004) was conducted to determine the digestive stability of the two DMO monomers. In addition to the pepsin protocol using simulated gastric fluid (SGF), a second assay was done using simulated intestinal fluid (SIF) containing pancreatin, which is a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. The relevance of the SIF study however, is only meaningful for proteins that are resistant to pepsin digestion because ordinarily an ingested protein would first need to survive passage through the stomach before being subject to further digestion in the small intestine.

> **Study submitted:**
>
> Burge, J.J.; Burzio, L.A.; Finnessy, J.F. (2010). Assessment of the *in vitro* digestibility of the dicamba mon-oxygenase (DMO) enzyme in simulated gastric and simulated intestinal fluids. Study ID# MSL0022502, , Monsanto Company (unpublished).

4.8.3.1 Simulated gastric fluid (SGF)

The *in vitro* digestibility of validated plant-derived total DMO protein in SGF containing pepsin at pH 1.2 was evaluated by incubating samples at 37º for selected times (0, 0.5, 2, 5, 10, 20, 30 and 60 minutes) and then running them on SDS-PAGE. Proteins were visualised by colloidal Brilliant Blue staining of the resulting gel. As pepsin and DMO can have similar mobility in SDS-PAGE, it was found that use of a Tris-glycine 8% polyacrylamide gel allowed optimal resolution.

Western blotting of the SDS-PAGE gels was also performed using a polyclonal goat anti-DMO primary antibody and a commercially available (Thermo Scientific) rabbit anti-goat horseradish peroxidise-linked secondary antibody.

Both the SDS-PAGE gels and Western blots indicated that the two full-length DMO and DMO+27 monomers are digested within 30 s of exposure to SGF. A quantitative analysis of the Western blot showed that more than 98% of each protein monomer was digested within 30 s.

A faint, transiently stable fragment with molecular weight of approximately 21 kDa not detected previously (Section 4.4.1) was observed in SDS-PAGE but not the Western blot. The protein in this band was N-terminally sequenced but its identity could not be established although it was confirmed that the sequence did not match that of either DMO or DMO+27. The likely explanation for the presence of this protein is that it was not entirely removed during extraction and purification of DMO from soybean MON87708.

4.8.3.2 Simulated intestinal fluid (SIF)

The *in vitro* digestibility of plant-derived validated total DMO protein in SIF (U.S.Pharmacopeia, 1990) containing pancreatin at pH 7.5 was assessed by incubating samples at 37° C for specified time intervals (0, 5, 15, 30, 60, 120, 240, 480 and 1,440 minutes), and analysing by Western blotting using appropriate antibodies (see Section 4.6.3.1).

The Western blot analysis showed that at least 95% of each protein monomer was digested within 5 min.

4.9   Conclusion

Soybean line MON87708 expresses one novel protein, DMO, which was detected in all plant parts that were analysed. Levels were lowest in the root (approximately 6 µg/g dry weight) and highest in older leaves (approximately 70 µg/g dry weight). The seed contained approximately 47 µg/g dry weight.

Several studies were done to confirm the identity and physicochemical properties of the DMO protein expressed in MON87708. The results of these studies demonstrated that the DMO expressed in MON87708 is actually a mixture of two monomers comprising mature DMO and the DMO precursor protein (designated DMO+27) which is identical to mature DMO except for an additional 27 amino acids at the N terminus which failed to be cleaved off during translocation of the protein to the chloroplast. Both monomers conform in size and amino acid sequence to that expected, and do not exhibit any post-translational modification including glycosylation. The specificity of the MON87708 DMO for the dicamba substrate was demonstrated.

Bioinformatic studies have confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that DMO would be completely digested before absorption in the gastrointestinal tract would occur. As anticipated a mouse oral toxicity study revealed no treatment-related effects. It was further demonstrated that the MON88708 DMO protein is not stable at elevated temperatures and loses most of its activity above 55° C.

Taken together, the evidence indicates that the DMO protein, comprising both DMO and DMO+27 monomers, is unlikely to be toxic or allergenic to humans.

5. Herbicide metabolites

There are essentially three strategies available for making plants tolerant to herbicides:

- detoxifying the herbicide with an enzyme which transforms the herbicide, or its active metabolite, into biologically inactive products
- inducing mutation(s) in the target enzyme so that the functional enzyme is less sensitive to the herbicide, or its active metabolite
- inducing over-expression of the sensitive enzyme so that the concentration of target enzyme in the plant is sufficient in relation to the inhibiting herbicide such as to have enough functional enzyme available despite the presence of the herbicide.

In the case of herbicide-tolerant GM lines, such as MON87708, that involve the first strategy described above, there is the possibility that novel metabolites are produced following application of the herbicide and these metabolites may be present in the final food. It is therefore necessary for those lines incorporating a herbicide/gene combination not previously assessed to establish whether such metabolites occur. If they do, their toxicity needs to be determined in order to enable the establishment of an appropriate health-based guidance value (e.g. Acute Reference Dose – ARfD; Acceptable Daily Intake – ADI). Residue data also need to be considered to confirm the concentration of the novel GM trait-specific metabolites relative to the parent herbicide in the final food.

Studies submitted:

Miller, M.J.; Mierkowski, M.J. (2010). Metabolism of dicamba in dicamba-tolerant soybeans. Study ID# MSL0022659, Monsanto Company (unpublished).

Moran, S.J.; Foster, J.E. (2010). Magnitude of residues of dicamba in soybean raw agricultural and processed commodities after application to MON87708. Study ID # MSL0023061, Monsanto Company (unpublished).

Foster, J.E.; Mierkowski, M.; Miller, M.J. (2010). Analytical method for the determination of dicamba and its major metabolites in soy matrices by LC/MS/MS. Study ID # MSL0022661, Monsanto Company (unpublished).

5.1 Metabolism of dicamba

Seeds of a GM soybean line (GM_A90617) containing the same *dmo* expression cassette present in MON87708 were sown in 12-inch pots (4 seeds per pot) in two glasshouses. A number of treatments were included to determine the nature of the residues found in/on agricultural commodities derived from plants following treatment with [phenyl-U-^{14}C] dicamba. These treatments were:

- PRE-T (pre-emergence treatment). Surface of pots sprayed directly on the day after seeds were planted. The application rate was approximately 2.8 kg ae/ha[3], which is marginally higher than the maximum intended seasonal rate in the U.S. of 2.24 ae kg/ha.
- POE-T (post-emergence treatment). Plants sprayed at the R1 growth stage[4] (first flower), 29 days after sowing. Application rate was as for PRE-T.
- UNT-C (untreated control).

At 14 days after planting, 29 pots designated PRE-T and 32 pots designated POE-T were thinned to two plants per pot. Immature foliage (pre-forage) was collected from UNT-C and PRE-T plants 14 days after planting and the pre-emergence application. Forage samples were collected 7 d after post-emergence application and 36 d after pre-emergence application. Hay was collected 27 d after post-emergence treatment (56 d after pre-emergence treatment). Seed was collected 83 d after post-emergence treatment (112 d after pre-emergence treatment).

Identification and quantitation of the residues in pre-forage, forage, hay and seed extracts were accomplished by using reverse phase high performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC/MS).

The following identified or characterised dicamba metabolites and radioactive residue components were found
- Sugars
- DCGA[5] glucoside: 2,5-dichloro-3-(β-D-glucopyranosyloxy)-6-hydroxybenzoic acid
- DCGA malonylglucoside: 3-[6-O-(2carboxyacetyl)-β-D-glucopyranosyloxy]-2,5-dichloro-6-hydroxybenzoic acid
- DCSA glucoside: 3,6-dichloro-2-(β-D-glucopyranosyloxy)benzoic acid
- DCSA HMGglucoside: 2-[[6-O-(4-carboxy-3-hydroxy-3-methylbutyryl)-β-D-glucopyranosyl-oxy]-3,6-dichlorobenzoic acid
- Unknown DCSA/DCGA conjugates
- Unknown DCSA/DCGA glucose conjugates
- DCSA: 3,6-dichlorosalicylic acid
- Dicamba: 3,6-dichloro-2-methoxybenzoic acid
- Triglycerides

DCSA glucoside was the major metabolite in foliage (pre-forage, forage and hay) accounting for up to 75% of the total radioactive residue (TRR). Other metabolites in forage were present at less than approximately 5% of TRR except in POE-T forage and hay where some 24% and 12% respectively of TRR was dicamba. Washing experiments indicated that this dicamba was almost entirely surface residue.

In seeds, sugars, triglycerides, DCSA HMGglucoside and DCSA glucoside each accounted for 9%-15% of TRR. The sugar and triglyceride residues were assumed to be the result of uptake of $^{14}CO_2$ from metabolism of dicamba in the soil and plant.

The proposed pathways for the metabolism of dicamba in GM dicamba-tolerant soybean are shown in Figure 6. Both pathways are consistent with those found in soil, animals and non-GM plants (Roberts, 1998). DCSA is the major metabolite produced by soil bacteria (Dumitru

---

[3] Herbicide application rates are expressed as acid equivalents (ae).The acid equivalent is the theoretical yield of parent acid from a pesticide active ingredient that has been formulated as a derivative.
[4] For information on soybean growth stages see e.g. NDSU (2004).
[5] DCGA = 2,5-dichloro-3,6-dihydroxybenzoic acid

et al., 2009) and has been shown to be produced in cows (Oehler and Ivie, 1980). In wheat, which is naturally tolerant to dicamba, the major metabolite is the glucoside conjugate of 5-hydroxydicamba but DCSA is a minor metabolite (Broadhurst *et al.*, 1966)
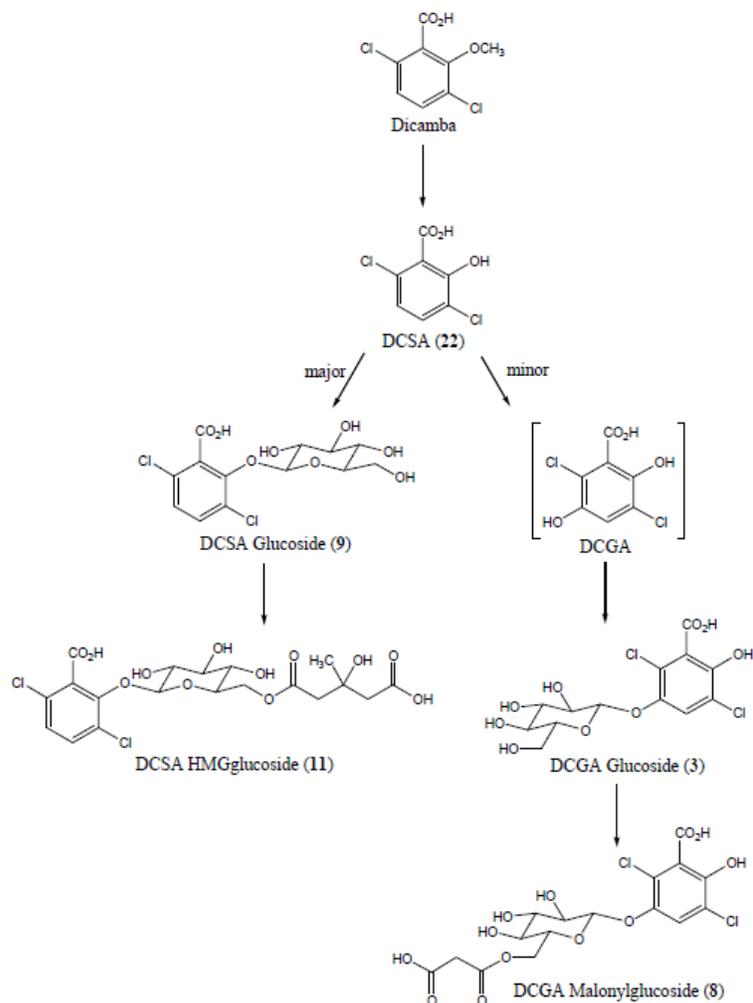
Figure 6: Proposed pathways for the metabolism of dicamba in dicamba-tolerant soybean

5.2 Dicamba residue chemistry studies

Field trials of MON87708 were conducted at 22 sites in the U.S. representative of commercial soybean-producing areas. There was an untreated control at each site. For other treatments, dicamba was applied, at a combination of pre-emergence, V3 stage or R1/R2 stage, as MON54140 (formulated as the diglycolamine salt and identical to Clarity®) or MON11955 (formulated as the monoethanolamine salt) as outlined in Table 5. Treatment 2 represents the application rates and timing that will be recommended on the proposed label for use in the U.S.

Table 5:   Treatments for testing dicamba residue levels

Treatment	No. of sites	Formulation	Application rate (kg ae/ha)			
			Pre-emergence	V3	R1/R2	Total
1	22	unsprayed	-	-	-	0
2	22	MON11955	1.12	0.56	0.56	2.24
3	6	MON11955	-	1.12	1.12	2.24
4	4	MON54140	-	1.12	1.12	2.24
5	2	MON54140	1.12	-	2.24	3.36

For Treatments 2, 3 and 4 the rate of application was equivalent to the intended maximal seasonal rate of 2.24 kg ae/ha. In Treatment 5, the application rate was increased with the objective of producing seed with measurable residues for processing. All applications were made, using ground based spray equipment.

Samples were collected for analysis of seed, forage and hay. Results in Table 6 are those for Treatment 2 only, and were obtained through LC/MS-MS.

Table 6: Levels of dicamba and its metabolites in raw agricultural commodity of MON87708 sprayed with dicamba

	Residue levels in raw agricultural commodity of MON87708 (ppm)					
	Seed		Forage		Hay	
Metabolite	Median	Range	Median	Range	Median	Range
Dicamba	<0.013	<0.013	0.068	<0.021 – 2.62	0.051	<0.014 – 1.16
DCSA	0.031	0.009 – 0.41	14	8.34 – 47.9	29.8	11.4 – 57.1
5-hydroxy dicamba	<0.021	<0.021	<0.005	<0.005 – 0.01	<0.014	<0.014
DCGA	0.017	0<0.011 – 0.136	1.95	0.359 – 5.95	2.01	0.169 – 7.33

Seed from Treatment 4 was processed into a number of fractions. Results for levels of DCSA and DCGA are given in Table 7. These show that there is minimal concentration of the metabolites during processing with hulls, toasted defatted meal and defatted flour showing the highest level of concentration.

Table 7: Concentration factor of DCSA and DCGA residues in processed fractions of MON87708 seed

Fraction	Average DCSA concentration factor	Average DCGA concentration factor
Hulls	1.4	0.99
Toasted defatted meal	1.35	1.3
Degummed oil	<0.11	<0.13
RBD oil[6]	<0.11	<0.13
Crude lecithin	<0.18	<0.13
Defatted flour	1.26	1.25
Protein isolate	<0.16	<0.15
Protein concentrate	<0.12	<0.14
Soymilk	<0.12	<0.13
Tofu	<0.12	<0.13

---

[6] RBD oil = refined, bleached and deodorised oil

5.3   ADI for dicamba

As no novel herbicide metabolites are present in dicamba-sprayed soybean line MON87708, the existing health-based guidance value (i.e. Acceptable Daily Intake - ADI) for dicamba is appropriate and relevant for assessing dietary risk with soybean line MON87708. In Australia the ADI for dicamba is 0.03 mg/kg bw/day[7].

5.4   Conclusion

The residues generated on soybean line MON87708 as a result of spraying with dicamba are the same as those found on conventional crops sprayed with dicamba. Residue data derived from supervised trials indicate that the residue levels in seed are low and that there is some concentration of residue in hulls, toasted defatted meal and defatted flour but not in other processed commodities. In the absence of any significant exposure to either parent herbicide or metabolites the risk to public health and safety is negligible.

6.   Compositional analysis

The main purpose of compositional analysis is to determine if any unexpected changes in composition have occurred to the food and to establish its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where there has been a deliberate change to the composition of food.

The classic approach to the compositional analysis of GM food is a targeted one; rather than analysing every single constituent, which would be impractical. The aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health (eg solanine in potatoes).

6.1   Key components

For soybean there are a number of components that are considered to be important for compositional analysis (OECD, 2001). As a minimum, the key nutrients of soybean seed appropriate for a comparative study include the proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids and fatty acids. In addition, mineral and vitamin levels may be considered and international guidance also suggests that levels of the key anti-nutrients phytic acid, trypsin inhibitors, lectins, isoflavones and stachyose and raffinose should be determined for new varieties of soybean. The reasons for focussing on these particular anti-nutrients are:
- Phytic acid causes chelation of mineral nutrients (including calcium, magnesium, potassium, iron and zinc) thereby making them unavailable to monogastric animals, including humans
- Trypsin inhibitors interfere with digestion of protein; lectins are proteins that bind to carbohydrate-containing molecules. Both trypsin inhibitors and lectins can inhibit

---

[7]ADIs are established by the Office of Chemical Safety within the Department of Health and Ageing. The most recent (July 2011) list can be found at
http://www.health.gov.au/internet/main/publishing.nsf/Content/E8F4D2F95D616584CA2573D700770 C2A/$File/ADI-report-july11.pdf

animal growth. The activity of trypsin inhibitors and lectins is heat-labile and they are inactivated during processing of soybean protein products and soybean meal so that the final edible soybean product should contain minimal levels of these anti-nutrients.

- Isoflavones are reported to possess biochemical activity including estrogenic, anti-estrogenic and hypocholesterolaemic effects that have been implicated in adversely affecting animal reproduction. Major isoflavones in soybeans include daidzein, genistein, glycitein and coumestrol.
- Stachyose and raffinose are low molecular weight carbohydrates (oligosaccharides) that are associated with production of intestinal gas and resulting flatulence when they are consumed.

6.2   Study design and conduct for key components

Studies submitted:

Harrigan, G.G.; Riordan, S.G.; Miller, K.D.; Sorbet, R. (2010). Compositional analysis of soybean forage and seed collected from dicamba-treated MON87708 grown in the United States during 2008. Study ID # MSL0022378, Monsanto Company (unpublished).
Harrigan, G.G.; Riordan, S.G. (2011). Amended Report: Statistical re-analysis of compositional data of soybean forage and seed collected from MON87708 grown in the United States. Study ID # RAR-10-407. Monsanto Company (unpublished).

The test (MON87708 seed from generation R_5), and control (A3525) were grown under typical production conditions at five field sites across North America[8] during the 2008 growing season. A3525 is the original transformed line and therefore represents the isogenic control line for the purposes of the comparative analyses. Additionally, four different commercial lines were grown at each site in order to generate tolerance ranges for each analyte i.e. there were twenty reference lines in total. Of these, two were GM lines. In the first study (Harrigan et al, 2010) the data that were analysed included those for the GM lines. In the second study (Harrigan & Riordan, 2011). The data were re-analysed without those for the GM lines. The statistical information discussed in this Safety Assessment deals only with the second study, but information such as trial design and methods for determining analyte levels has been taken from the first study.

All lines were sprayed with maintenance pesticides as necessary during the growing season. Additionally, MON87708 plants were sprayed with dicamba at the V2-V3 growth stage at a rate of 0.56 kg ae/ha.

Seed was harvested at normal maturity and samples were analysed for proximates, fibre, fatty acids, amino acids, isoflavones, anti-nutrients and vitamin E. Key analyte levels for forage were also obtained but are not reported here; it is noted, however, that the only significant difference found was in acid detergent fibre, the mean of which was higher in MON87708 than in A3525 but was not outside either the literature range or the tolerance range (see discussion in Section 6.3). Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods. A total of 57 analytes were measured in seed.

6.3   Analyses of key components in seed

The identity of harvested seed from the test and control lines was verified by event specific PCR.

---

[8] The five sites were: Jefferson County, IA ; Stark County, IL; Clinton County IL; Parke County IN; Berks County, PA

For each analyte 'descriptive statistics' were generated i.e. a mean (least square mean) and standard error averaged over all sites. The values thus calculated are presented in Tables 8 – 14. [Note that in the Tables, mauve shading represents MON87708 means that are significantly lower than the control means while orange shading represents MON87708 means that are significantly higher].

Of the 57 analytes measured, 14 had more than half of the observations below the assay limit of quantitation. The remaining 42 analytes (carbohydrate was calculated rather than being measured) were analysed using a mixed model analysis of variance. Data were transformed into Statistical Analysis Software[9] (SAS) data sets and analysed using SAS® software. In assessing the significance of any difference between means, a P-value of 0.05 was used (i.e. a P- value of ≥0.05 was not significant).

As mentioned, two of the 20 reference lines, were commercial GM lines. Data from these GM lines were excluded from the statistical analyses used to generate the 99% tolerance range reported in Tables 11 – 18. Data from the 18 non-GM lines were combined across all sites to calculate the tolerance range.

Any statistically significant differences between MON87708 and the A3525 control have been compared to the tolerance range compiled from the results of the non-GM commercial cultivars, to assess whether the differences are likely to be biologically meaningful. Additionally, the results for MON87708 and A3525 have been compared to a combined literature range for each analyte, compiled from published literature for commercially available soybean[10]. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within soybean (Harrigan *et al.*, 2010). Therefore, even if means fall outside the published range, this is not necessarily a concern.

6.3.1   *Proximates and fibre*

Results of the proximate and fibre analysis are shown in Table 8. Total fat was the only analyte for which there was no significant difference between MON87708 and A3525. Mean protein levels were significantly lower in MON87708 than in A3525, while all other analyte levels were significantly higher in MON87708. However, the means for all analytes for both MON87708 and A3525 were within the range reported in the literature as well as within the tolerance range of the commercial cultivars. Additionally, the magnitude of any differences in means between MON87708 and A3525 were small.

Table 8:   *Mean (±standard error) percentage dry weight (%dw) of proximates and fibre in seed from MON87708 and A3525.*

Analyte	MON87708[1] (%dw)	A3525 (%dw)	Overall treat effect (P-value)	Tolerance range (%dw)	Combined literature range (%dw)
Ash	5.24±0.067	5.12±0.067	0.031	5.00 – 5.88	3.89 – 6.99
Protein	40.86±0.39	42.41±0.39	0.016	37.06 – 43.42	32.0 – 45.5
Total Fat	15.97±0.59	15.84±0.59	0.691	15.47 – 21.34	8.10 – 24.7
Carbohydrate[1]	37.93±0.5	36.64±0.5	0.012	34.8 - 41.6	29.6 – 50.2
ADF	13.55±1.9	12.86±0.4	0.009	12.07 – 17.46	7.81 – 18.6

[9] SAS website - http://www.sas.com/technologies/analytics/statistics/stat/index.html
[10] Published literature for soybean includes Codex (2001); Douglas (1996); ILSI (2010); Kakade et al. (1972); Liener (1994); Novak & Haslberger (2000); OECD (2001); Vaidehi & Kadam (1989).

Analyte	MON87708[1] (%dw)	A3525 (%dw)	Overall treat effect (P-value)	Tolerance range (%dw)	Combined literature range (%dw)
NDF	15.29±0.59	14.34±0.59	0.028	11.66 – 19.45	5.0 – 21.3
Crude Fibre	8.29±0.26	7.37±0.26	<0.001	6.35 – 11.31	4.12 – 13.87

[1] Carbohydrate calculated as 100% - (protein %dw+ fat %dw + ash %dw)

6.3.2   Fatty Acids

The levels of 23 fatty acids were measured. Of these, the following were below the limits of quantitation - C8:0 caprylic, C10:0 capric, C12:0 lauric, C14:0 myristic, C14:1 myristoleic, C15:0 pentadecanoic, C15:1 pentadecenoic,C16:1 palmitoleic, C17:0 heptadecanoic, C17:1 heptadecanoic, C18:3 gamma linolenic, C20:2 eicosadienoic, C20:3 eicosatrienoic, C20:4 arachidonic, and C22:1 erucic. Results for the remaining eight fatty acids are given in Table 9 and can be summarised as follows:

- There was no significant difference between the means of MON87708 and A3525 for stearic, arachidic and eicosenoic acids.
- The mean levels of oleic and behenic acids were significantly lower in seeds of MON87708 soybean compared with seeds of A3525. The mean for oleic acid in MON87708 fell within both the tolerance range and the combined literature range while that for behenic acid was marginally lower than both the tolerance range and literature range.
- The mean levels of palmitic, linoleic and linolenic acids were significantly higher in seeds of MON87708 compared with seeds of A3525. All means fell within the combined literature range; the means for all but palmitic acid in MON87708 fell within the tolerance range.

Table 9:   Mean (±standard error) percentage composition, relative to total fat, of major fatty acids in seed from MON87708 and 'A3525'.

Analyte	MON87708 (%total)	A3525 (%total)	Overall treat effect (P-value)	Tolerance range (%total)	Combined literature range (%total)
Palmitic acid (C16:0)	11.59±0.16	11.33±0.16	0.002	9.42 – 11.54	7.00 - 15.8
Stearic acid (C18:0)	4.06±0.1	4.04±0.1	0.584	3.24 – 4.67	2.00 - 5.88
Oleic acid (C18:1)	19.2±0.3	20.91±0.3	<0.001	17.88 – 25.31	14.3 – 34.0
Linoleic acid (C18:2)	54.4±0.37	53.59±0.37	0.01	50.95 – 56.68	48 - 60.0
Linolenic acid (C18:3)	10.12±0.27	9.49±0.27	<0.001	7.43 – 10.65	2.00 – 12.52
Arachidic acid (C20:0)	0.26±0.0052	0.26±0.0052	0.707	0.20 – 0.30	<0.1 – 0.48
Eicosenoic acid (C20:1)	0.093±0.00.017	0.090±0.017	0.495	0.065 – 0.17	0.14 - 0.35
Behenic acid (C22:0)	0.27±0.0038	0.28±0.0038	0.001	0.28 – 0.35	0.277-0.595

6.3.3 Amino acids

Levels of 18 amino acids were measured. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine. Results (Table 7) can be summarised as follows:

- There was no significant difference between the control and soybean MON87708 for the means of alanine, lysine, methionine, serine, threonine, tryptophan and tyrosine.
- The mean level of cystine in MON87708 seeds was higher than the mean level in seeds of A3525 but was within both the tolerance range and literature range.
- The mean levels of the remaining 10 amino acids were significantly lower in MON87708 than in A3525 but all were within both the tolerance and literature ranges. These differences would account for the significantly lower mean protein level in MON87708 when compared to A3525 (refer to Section 6.3.1).

Table 10: Mean percentage dry weight (dw), relative to total dry weight, of amino acids in seed from 'MON87708 and 'A3525'.

Analyte	MON87708 (%dw)	A3525 (%dw)	Overall treat effect (P-value)	Tolerance range (%dw)	Combined literature range (%dw)
Alanine	1.76±0.018	1.80±0.018	0.059	1.59 – 1.86	1.51 - 2.10
Arginine	3.30±0.069	3.58±0.069	0.006	2.88 – 3.74	2.17 - 3.40
Aspartate	4.63±0.044	4.78±0.044	0.016	4.22 – 4.94	3.81 - 5.12
Cystine	0.61±0.0049	0.59±0.0049	<0.001	0.53 – 0.64	0.37 - 0.81
Glutamate	7.38±0.085	7.69±0.085	0.01	6.69 – 7.92	5.84 - 8.2
Glycine	1.76±0.016	1.81±0.016	0.02	1.58 – 1.84	1.46 - 2.27
Histidine	1.06±0.0095	1.09±0.0095	0.017	0.95 – 1.13	0.84 - 1.22
Isoleucine	1.88±0.019	1.95±0.019	0.006	1.68 – 2.02	1.54 – 2.32
Leucine	3.06±0.029	3.17±0.029	0.008	2.8 – 3.27	2.2 - 4.0
Lysine	2.64±0.019	2.68±0.019	0.11	2.38 – 2.74	1.55 - 2.86
Methionine	0.58±0.0053	0.58±0.0053	0.985	0.52 – 0.63	0.43 - 0.76
Phenylalanine	2.06±0.028	2.13±0.028	0.034	1.85 – 2.21	1.60 - 2.39
Proline	1.99±0.021	2.05±0.021	0.017	1.74 – 2.16	1.69 - 2.33
Serine	2.04±0.023	2.09±0.023	0.105	1.90 – 2.18	1.11 - 2.48
Threonine	1.56±0.015	1.58±0.015	0.169	1.47 – 1.64	1.14 - 1.89
Tryptophan	0.47±0.0085	0.46±0.0085	0.494	0.39 – 0.50	0.36 - 0.67
Tyrosine	1.37±0.018	1.42±0.018	0.048	1.26 – 1.49	0.1 - 1.62
Valine	1.98±0.02	2.06±0.02	0.002	1.73 – 2.13	1.50 - 2.44

6.3.4 Isoflavones

In total, there are 12 different soybean isoflavone isomers, namely three parent isoflavones (genistein, daidzein and glycitein), their respective β-glucosides (genistin, daidzin, and glycitin), and three β-glucosides each esterified with either malonic or acetic acid (Messina, 2005). The parent isoflavones are also referred to as free or aglycon isoflavones, while the glucosides and their esters are also referred to as conjugated isoflavones.

The Applicant used an acid hydrolysis method to extract the isoflavones. This method results in the hydrolysis of all isoflavones to aglycons and therefore the results in Table 11 are expressed as total aglycon equivalents. The mean level of daidzein equivalents was

significantly higher in MON87708 than in A3525 but was within both the tolerance and literature ranges.

Table 11:   Mean weight (µg/g dry weight) of isoflavones in MON87708 and A3525 seed

Analyte	MON87708 (µg/g dw)	A3525 (µg/g dw)	Overall treat effect (P-value)	Tolerance range (µg/g dw)	Combined literature range (µg/g dw)
Daidzein equivalents	1494.97±154.94	1340.71±90.36	0.046	451.33 – 2033.05	60 - 2453
Genistein equivalents	967.01±90.36	886.57±1.8	0.062	533.88 – 1726.03	144 - 2837
Glycitein equivalents	108.01±5.24	95.85±5.24	0.116	73.61 – 231.75	15.3 - 310

6.3.5   Anti-nutrients

Levels of key anti-nutrients are given in Table 12. No significant differences between means were obtained for lectin or trypsin inhibitor. For phytic acid, raffinose and stachyose the mean levels were significantly lower in seeds of MON87708 when compared to levels in seeds of A3525 but the means were all within the tolerance and literature ranges.

Table 12:   Mean levels of anti-nutrients in MON87708 and A3525 seed.

Analyte	MON87708	A3525	Overall treat effect (P-value)	Tolerance range	Combined literature range
Lectin (hemagglutinat. units/mg)	3.17±0.76	3.16±0.76	0.984	0.68 – 8.34	0.11 - 129
Phytic acid (%dw)	1.30±0.071	1.39±0.071	0.043	1.00 – 1.64	0.634 - 2.74
Raffinose (%dw)	0.43±0.038	0.47±0.038	0.045	0.26 – 0.59	0.11 – 1.28
Stachyose (%dw)	3.36±0.078	3.62±0.078	0.011	2.23 – 2.96	1.21 – 6.3
Trypsin inhibitor (trypsin inhibitor units/mg)	32.27±1.4	30.37±1.4	0.319	23.37 – 44.56	19.6 - 119

6.3.6   Vitamins

α-tocopherol, one of the vitamers of vitamin E, was the only vitamin measured. The results are given in Table 13 and show that the mean level in MON87708 was significantly higher than the level in A3525 but was within the tolerance and literature ranges.

Table 13:   Mean weight (mg/100 g dry weight) of vitamin E in seed from MON87708 and A3525.

Analyte	MON87708 (mg/100 g dw)	A3525 (mg/100g dw)	Overall treat effect (P-value)	Tolerance range (mg/100g dw)	Combined literature range (mg/100g dw)
α-tocopherol	1.41±0.18	1.23±0.18	0.001	0.69 – 2.91	0.19 – 6.17

6.3.7   Summary of analysis of key components

Statistically significant differences in the analyte levels found between seed of MON87708 and A3525 are summarised in Table 14. These differences do not raise safety concerns for a number of reasons. Firstly, for all analytes except behenic acid, the soybean MON87708 means fall within the combined literature range and for all analytes except palmitic and behenic acids the MON87708 means fall within the tolerance range. Secondly, it is noted that the percentage differences between the lowest and highest levels in the tolerance range obtained from the commercial non-GM lines are higher than the percentage differences between MON87708 and A3525 means for any analyte. Finally, there are no trends in the results.

Table 14:   *Summary of analyte levels found in seed of soybean MON87708 that are significantly (P < 0.05) different from those found in seed of A3525.*

Analyte	Unit of measure.	MON87708	A3525	% difference between means	MON87708 within tolerance range	MON87708 within literature range
Ash	%dw	5.24	5.12	2.3	yes	yes
Protein	%dw	40.86	42.41	3.8		
Carbohydrate	%dw	37.93	36.64	3.5	yes	yes
ADF	%dw	13.55	12.86	5.4	yes	yes
NDF	%dw	15.29	14.34	6.6	yes	yes
Crude Fibre	%dw	8.29	7.37	12.5	yes	yes
Palmitic acid (C16:0)	% total fat	11.59	11.33	2.3	no	yes
Oleic acid (C18:1)	% total fat	19.2	20.91	8.9	yes	yes
Linoleic acid (C18:2)	% total fat	54.4	53.59	1.5	yes	yes
Linolenic acid (C18:3)	% total fat	10.12	9.49	6.6	yes	yes
Behenic acid (C22:0)	% total fat	0.27	0.28	3.7	no	no
Arginine	%dw	3.30	3.58	8.4	yes	yes
Aspartate	%dw	4.63	4.78	3.2	yes	yes
Cystine	%dw	0.61	0.59	3.4	yes	yes
Glutamate	%dw	7.38	7.69	4.2	yes	yes
Glycine	%dw	1.76	1.81	2.8	yes	yes
Histidine	%dw	1.06	1.09	2.8	yes	yes
Isoleucine	%dw	1.88	1.95	3.7	yes	yes
Leucine	%dw	3.06	3.17	3.6	yes	yes
Phenylalanine	%dw	2.06	2.13	3.4	yes	yes
Proline	%dw	1.99	2.05	3.0	yes	yes
Valine	%dw	1.98	2.06	4.0	yes	yes
Daidzein	µg/g dw	1494.97	1340.71	11.5	yes	yes
Phytic acid	%dw	1.30	1.39	6.9	yes	yes
Raffinose	%dw	0.43	0.47	9.3	yes	yes
Stachyose	%dw	3.36	3.62	7.7	yes	yes
α-tocopherol	mg/100 g dw	1.41	1.23	14.6	yes	yes

6.4   Assessment of endogenous allergenic potential

Soybean naturally contains allergenic proteins and is one of a group of known allergenic foods including milk, eggs, fish, shellfish, wheat, peanuts, tree nuts and sesame. This group of foods accounts for approximately 90% of all food allergies (Metcalfe *et al.*, 1996). The presence of allergenic proteins in the diet of hypersensitive individuals can cause severe adverse reactions. The allergenic effect of soybeans is attributed to the globulin fraction of soybean proteins that comprise about 85% of total protein (OECD, 2001). Soybean-allergic individuals will also be allergic to soybean MON87708.

Since soybean is associated with allergic effects in susceptible individuals, a study was done to assess whether seed from soybean line MON87708 may have an endogenous allergen content that is different from the non-GM parent line, as measured by IgE binding using sera from soybean allergic individuals. This method does not provide a direct measure of endogenous allergen content and is purely comparative.

Study submitted:

Bhakta, T.; Finnessy, J.; Meng, C.; Bannon, G. (2010). Quantitative ELISA assessment of human IgE binding to MON87708, control, and reference soybean using sera from soybean-allergic subjects. Study ID # MSL0022502, Monsanto Company (unpublished).

Aqueous protein extracts were prepared from seeds of soybean MON87708, the non-GM parent, A3525 and 17 non-GM commercial soybean varieties, and were incubated with sera from 13 clinically documented soybean-allergic subjects and pooled sera from five non-allergic subjects for a quantitative, validated ELISA soybean-specific IgE antibody binding assay. The level of IgE binding provides an estimate of the amount of endogenous soybean allergens present in the seeds. Comparison of the binding values of protein from a GM source with the binding values of protein from an equivalent non-GM source has been shown to be a valid approach (Sten *et al.*, 2004).

Sera from the 13 soybean-allergic subjects yielded positive IgE values for all soybean extracts; none of the sera from non-allergic subjects showed IgE binding. To compare levels of IgE binding in the soybean-allergic subjects, the ELISA values were statistically analysed. The values obtained for the 17 reference soybean extracts were used to calculate a 99% tolerance interval for each serum. The IgE binding levels obtained for the protein extract from MON87708 soybean were compared to the calculated tolerance intervals. The results showed that IgE values for the MON87708 and' A3525' were within the established tolerance intervals obtained for each serum.

This study suggests that the levels of endogenous allergens in MON87708 soybean are comparable to those in soybean varieties currently available for human food uses.

6.5   Conclusion

Detailed compositional analyses were done to establish the nutritional adequacy of seed from soybean line MON87708 sprayed with dicamba. Analyses were done of 57 analytes encompassing proximates, fibre, fatty acids, amino acids, isoflavones, anti-nutrients and vitamin E. The levels were compared to levels in the seeds of the non-GM parent A3525.

These analyses indicated that the seeds of soybean line MON87708 are compositionally equivalent to those of the parental line. Out of the analytes tested, there were significant differences between the non-GM control and soybean MON87708 in 27 analytes. In all of these, except for behenic acid, the mean levels observed in seeds of soybean MON87708 were within the range of natural variation either reported in the literature or derived from 18

non-GM commercial varieties grown in the same field trials. For any analyte, the magnitude of the differences observed between MON87708 and A3525 was not as great as the magnitude between the reference varieties.

In addition, no difference between seeds of soybean line MON87708 and A3525 were found in IgE binding studies using sera from soybean-allergic individuals.

The compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key components in seed from soybean line MON87708 when compared with the non-GM control or with the range of levels found in non-GM commercial soybean cultivars.

7.   Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (OECD, 2003; EFSA, 2008). Soybean line MON87708 is the result of a simple genetic modification to confer herbicide tolerance with no intention to significantly alter nutritional parameters in the food. In addition, the extensive compositional analyses of seed that have been undertaken to demonstrate the nutritional adequacy of MON87708, indicate it is equivalent in composition to conventional soybean cultivars. The introduction of food from soybean line MON87708 into the food supply is therefore expected to have little nutritional impact and as such no additional studies, including animal feeding studies, are required.

However, the Applicant provided two feeding studies which FSANZ considered as additional supporting information. The studies – a 90 day in rats and a 42-day in broilers – used processed soybean meal produced from MON87708 and the conventional control (A3525). Meal from a number of conventional lines was also compared for reference.

Studies submitted:

Davis, S.W. (2010). Comparison of Broiler Performance and Carcass Parameters When Fed Diets Containing Soybean Meal Produced from MON87708, Control or Reference Soybean. Monsanto Study Number: CQR-09-045 (MSL-22551)

Kirkpatrick, J.B. (2010). A 90-day Feeding Study in Rats with Processed Soybean Meal from MON87708 Soybeans. Sponsor study number WI-08-291. Monsanto Study Number MSL-22868.

In the broiler study, no significant differences were observed in growth performance (body weight gain, mortality, feed efficiency) or carcass yield (breast, thigh, leg, wing and abdominal fat) in birds fed meal derived from MON87708 and those fed meal from the control or six reference lines.

The major parameters considered in the rat study were survival, clinical observations, feed intake, clinical pathology, and anatomical pathology. No differences attributable to consumption of meal from MON87708 were found.

The results of these two studies are consistent with the findings from the compositional analysis.

References[11]

Al-Jasser, A.M. (2006) *Stenotrophomonas maltophilia* resistant to trimethoprim – sulfamethoxazole: an increasing problem. *Annals of Clinical Microbiology and Antimicrobials* 5:23 (ePub).

Astwood, J.D. and Fuchs, R.L. (1996) Allergenicity of foods derived from transgenic plants. *Monographs in Allergy* 32 (Highlights in Food Allergy):105-120.

Baxevanis, A.D. (2005) Assessing Pairwise Sequence Similarity: BLAST and FASTA. In: Baxevanis, A.D. and Ouellette, B.F.F. eds. *Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins.* Chapter 11. John Wiley & Sons, Inc., pp. 295-324.

Behrens, M.R., Mutlu, N., Chakraborty, S., Dumitru, R., Jiang, W.Z., LaVallee, B.J., Herman, P.L., Clemente, T.E. and Weeks, D.P. (2007) Dicamba Resistance: Enlarging and Preserving Biotechnology-Based Weed Management Strategies. *Science* 316(5828):1185-1188.

Ben-Gigirey, B., Vieites, J.M., Kim, S.H., An, H., Villa, T.G. and Barros-Velásquez, J. (2002) Specific detection of *Stenotrophomonas maltophilia* strains in albacore tuna (*Thunnus alalunga*) by reverse dot-blot hybridization. *Food Control* 13:293-299.

Broadhurst, N.A., Montgomery, M.L. and Freed, V.H. (1966) Metabolism of 2-methoxy-3,6-dichlorobenzoic acid (dicamba) by wheat and bluegrass plants. *Journal of Agricultural and Food Chemistry* 14(6):585-588.

Brookes, G. and Barfoot, P. (2009) *GM crops: global socio-economic and environmental impacts 1996-2007*. PG Economics Ltd. http://www.pgeconomics.co.uk/pdf/2009globalimpactstudy.pdf.

Cairo, R.C., Silva, L.R., de Andrade, C.F., de Andrade Barberino, M.G., Bandeira, A.C., Santos, K.P. and Diniz-Santos, D.R. (2008) Bacterial contamination in milk kitchens in pediatric hospitals in Salvador, Brazil. *Brazilian Journal of Infectious Diseases* 12(3):

Chakraborty, S., Behrens, M.R., Herman, P.L., Arendsen, A.F., Hagen, W.R., Carlson, D.L., Wang, X.-Z. and Weeks, D.P. (2005) A three-component dicamba O-demethylase from *Pseudomonas maltophilia*, strain DI-6: Purification and characterization. *Archives of Biochemistry and Biophysics* 437(1):20-28.

Codex (2001) *Codex Standard for Named Vegetable Oils.* Report No. CX-STAN 210 – 1999, Codex Alimentarius. http://www.codexalimentarius.net/web/standard_list.do?lang=en.

Codex (2003) *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants.* Report No. CAC/GL 45-2003, Codex Alimentarius. http://www.codexalimentarius.net/web/standard_list.do?lang=en.

---

[11] All website references were current as at 6 January 2012

Coruzzi, G., Broglie, R., Edwards, C. and Chua, N.H. (1984) Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. *EMBO Journal* 3(8):1671-1679.

D'Ordine, R.L., Ryde, T.J., Storek, M.J., Sturman, E.J., Moshiri, F., Bartlett, R., Brown, G.R., Eilers, R.J., Dart, C., Qi, Y., Flasinski, S. and Franklin, S.J. (2009) Dicamba monooxygenase: Structural insights into a dynamic Rieske oxygenase that catalyzes an exocyclic monooxygenation. *Journal of Molecular Biology* 392(2):481-497.

Delaney, B., Astwood, J.D., Cunny, H., Eichen Conn, R., Herouet-Guicheney, C., MacIntosh, S., Meyer, L.S., Privalle, L.S., Gao, Y., Mattsson, J., Levine, M. and ILSI. (2008) Evaluation of protein safety in the context of agricultural biotechnology. *Food and Chemical Toxicology* 46:S71-S97.

Denton, M., Todd, N.J., Kerr, K.G., Hawkey, P.M. and Littlewood, J.M. (1998) Molecular epidemiology of *Stenotrophomonas maltophilia* isolated from clinical specimens from patients with cystic fibrosis and associated environmental samples. *Journal of Clinical Microbiology* 36(7):1953-1958.

Douglas, J.S. (1996) Recommended compositional and nutritional parameters to test in soybean.   Technical Assessment Services, Washington.

Dumitru, R., Jiang, W.Z., Weeks, D.P. and Wilson, M.A. (2009) Crystal structure of dicamba monooxygenase: a Rieske nonheme oxygenase that catalyzes oxidative demethylation. *Journal of Molecular Biology* 392(2):498-510.

EFSA. (2008) Safety and nutritional assessment of GM plants and derived food and feed: The role of animal feeding trials. *Food and Chemical Toxicology* 46:S1-S70, doi:10.1016/j.fct.2008.02.008.

Feng, P.C.C. and Brinker, R.J. (2007) Methods for weed control. (WO 2007/143690 A2): http://www.patentlens.net/imageserver/getimage/WO_2007_143690_A2.pdf?id=17943007&page=all.

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

Fluhr, R., Moses, P., Morelli, G., Coruzzi, G. and Chua, N.-H. (1986) Expression dynamics of the pea rbcS multigene family and organ distribution of the transcripts. *EMBO Journal* 5:2063-2071.

FSANZ (2011) *A1049 - Food derived from herbicide-tolerant, high oleic acid soybean line MON87705*. Report preapred by Food Standards Australia New Zealand. http://www.foodstandards.gov.au/foodstandards/applications/applicationa1049food4840.cfm.

Furushita, M., Okamoto, A., Maeda, T., Ohta, M. and Shiba, T. (2011) Isolation of multidrug-resistant *Stenotrophomonas maltophilia* from cultured yellowtail (Seriola quinqueradiata) from a marine fish farm. *Applied and Environmental Microbiology* 71(9):5598-5600.

Goodman, R.E. (2006) Practical and predictive bioinformatics methods for the identification of potentially cross-reactive protein matches. *Molecular Nutrition and Food Research* 50:655-660.

Grey, D. (2006) Growing Soybeans in Northern Victoria. State of Victoria, Department of Primary Industries, http://aof.clients.squiz.net/__data/assets/pdf_file/0008/7667/Growing_Soybeans_in_Northern_Victoria.pdf.

Gupta, M., Nirunsuksiri, W., Schulenberg, G., Hartl, T., Novak, S., Bryan, J., Vanopdorp, N., Bing, J. and Thompson, S. (2011) A non-PCR-based Invader® assay quantitatively detects single-copy genes in complex plant genomes. *Molecular Breeding* 21(173):181.

Harrigan, G.G., Lundry, D., Drury, S., Berman, K., Riordan, S.G., Nemeth, M.A., Ridley, W.P. and Glenn, K.C. (2010) Natural variation in crop composition and the impact of transgenesis. *Nature Biotechnology* 28(5):402-404.

Henikoff, S. and Henikoff, J.G. (1992) Amino acid substitution matrices from protein blocks. *Proceedings of the National Academy of Sciences* 89:10915-10919.

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

Herman, R.A., Woolhiser, M.M., Ladics, G.S., Korjagin, V.A., Schafer, B.W., Storer, N.P., Green, S.B. and Kan, L. (2007) Stability of a set of allergens and non-allergens in simulated gastric fluid. *International Journal of Food Sciences and Nutrition* 58:125-141.

Hugh, R. (1981) *Pseudomonas maltophilia* sp. nov., nom. rev. *International Journal of Systematic Bacteriology* 31(2):195.

ILSI (2010) *International Life Sciences Institute Crop Composition Database Version 4.0*. http://www.cropcomposition.org/query/index.html.

James, A.T. and Rose, I.A. (2004) Integrating crop physiology into the Australian soybean improvement program. *In: 4th International Crop Science Congress, Brisbane, Australia, September 2004*. http://www.cropscience.org.au/icsc2004/poster/3/4/6/327_jamesat.htm#TopOfPage.

Kakade, M.L., Simons, N.R., Liener, I.E. and Lambert, J.W. (1972) Biochemical and nutritional assessment of different varieties of soybeans. *Journal of Agricultural and Food Chemistry* 20(1):87-90.

Kimber, I., Kerkvliet, N.I., Taylor, S.L., Astwood, J.D., Sarlo, K. and Dearman, R.J. (1999) Toxicology of protein allergenicity: prediction and characterization. *Toxicological Sciences* 48(2):157-162.

Krell, R. (1996) *Value-Added Products from BeeKeeping*. Chapter 3: Pollen, FAO Agricultural Services Bulletin No. 124, Food and Agriculture Organization of the United Nations, available online at http://www.fao.org/docrep/w0076e/w0076e00.htm#con.

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

Liener, I.E. (1994) Implications of antinutritional components in soybean foods. *Critical Reviews in Food Science and Nutrition* 34(1):31-67.

Liu, K.S., Orthoefer, F. and Thompson, K. (1995) The case for food-grade soybean varieties. *INFORM* 6(5):593-599.

Looney, W.J. (2009) *Stenotrophomonas maltophilia*: an emerging opportunist human pathogen. *The Lancet Infectious Diseases* 9(5):312-323.

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

Martinell, B.J., Julson, L.S., Elmer, C.A., Huang, Y., McCabe, D.E. and Williams, E.J. (2002) Soybean Agrobacterium transformation method. (US 6384301 B1): United States. http://www.patentlens.net/imageserver/getimage/US_6384301.pdf?id=1505465&page=all.

Messina, M. (2005) Isoflavones. In: Coates, P., Blackman, M.R., Cragg, G., Levine, M., Moss, J., and White, J. eds. *Encyclopedia of Dietary Supplements*. Informa Healthcare.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L. and Fuchs, R.L. (1996) Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition* 36 Suppl:S165-S186.

Miletich, J.P. and Broze Jr., G.J. (1990) b Protein C is not glycosylated at asparagine 329. *The Journal of Biological Chemistry* 265:11397-11404.

NDSU. (2004) Soybean Growth and Management Quick Guide: Reproductive Stages. Publication A-1174, North Dakota State University Agriculture and University Extension, http://www.ag.ndsu.edu/pubs/plantsci/rowcrops/a1174/a1174-2.htm.

Niepel, M. and Gallie, D.R. (1999) Identification and characterization of the functional elements within the tobacco etch virus 5' leader required for cap-independent translation. *Journal of Virology* 73:9080-9088.

Novak, W.K. and Haslberger, A.G. (2000) Substantial equivalence of antinutrients and inherent plant toxins in genetically modified novel foods. *Food and Chemical Toxicology* 38:473-483.

OECD (2001) *Consensus document on compositional considerations for new varieties of soybean: key food and feed nutrients and anti-nutrients*. Series on the Safety of Novel Foods and Feeds No. 2. Report No. ENV/JM/MONO(2001)15, Organisation for Economic Co-operation and Development, Paris.

OECD (2003) *Considerations for the safety assessment of animal feedstuffs derived from genetically modified plants*. Report No. ENV/JM/MONO(2003)10, Organisation for Economic Co-operation and Development, Paris. http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=ENV/JM/MONO(2003)10&doclanguage=en.

Oehler, D.D. and Ivie, G.W. (1980) Metabolic fate of the herbicide dicamba in a lactating cow. *Journal of Agricultural and Food Chemistry* 28(4):685-689.

Orlando, R. and Yang, Y. (1998) Analysis of Glycoproteins. In: Larsen, B.S. and McEwen, C.N. eds. *Mass Spectrometry of Biological Materials*. 2nd ed, Chapter 9. Marcel Dekker, pp. 216-246.

Palleroni, N.J. and Bradbury, J.F. (1993) Stenotrophomonas maltophilia, a new bacterial genus for Xanthomonas maltophilia (Hugh 1980) Swings et al. 1983. *International Journal of Systematic Bacteriology* 43(3):606-609.

Pearson, W.R. (2000) Flexible Sequence Similarity Searching with the FASTA3 Program Package. In: Misener, S. and Krawetz, S.A. eds. *Methods in Molecular Biology, Volume 132: Bioinformatics Methods and Protocols.* Chapter 10. Human Press Inc., Totowa, NJ, pp. 185-219.

Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences* 85(8):2444-2448.

Polevoda, B. and Sherman, F. (2000) Na-terminal Acetylation of Eukaryotic Proteins. *Journal of Biological Chemistry* 275(47):36479-36482.

Prescott, V.A., Campbell, P.M., Moore, A., Mattes, J., Rothenberg, M.E., Foster, P.S., Higgins, T.J.V. and Hogan, S.P. (2005) Transgenic expression of bean a-amylase inhibitor in peas results in altered structure and immunogenicity. *Journal of Agricultural and Food Chemistry* 53:9023-9030.

Qureshi, A., Mooney, L., Denton, M. and Kerr, K.G. (2005) *Stenotrophomonas maltophilia* in salad. *Emerging Infectious Diseases* 11(7):1157-1158.

Roberts, T.e. (1998) Dicamba. In: *Metabolic pathways of agrochemicals. Part One: herbicides and plant growth regulators.* The Royal Society of Chemistry, Cambridge, pp. 148-151.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van der Lelie, D. and Dow, J.M. (2009) The versatility and adaptation of bacteria from the genus *Stenotrophomonas. Nature Reviews Microbiology* 7:514-525.

Sacchetti, R., De Luca, G. and Zanetti, F. (2009) Control of *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* contamination of microfiltered water dispensers with peracetic acid and hydrogen peroxide. *International Journal of Food Microbiology* 132:162-166.

Schmidt, C.L. and Shaw, L. (2001) A comprehensive phylogenetic analysis of Rieske and Rieske-type iron-sulfur proteins. *Journal of Bioenergetics and Biomembranes* 33:9-26.

Shurtleff, W. and Aoyagi, A. (2007) History of soybean crushing: Soy oil and soybean meal. In: *History of soybeans and soyfoods: 1100 B.C. to the 1980s.* SoyInfo Center, available online at http://www.soyinfocenter.com/HSS/soybean_crushing1.php, Lafayette, California.

Sten, E., Skov, P.S., Andersen, S.B., Torp, A.M., Olesen, A., Bindslev-Jensen, U., Poulsen, L.K. and Bindslev-Jensen, C. (2004) A comparative study of the allergenic potency of wild-type and glyphosate-tolerant gene-modified soybean cultivars. *Acta Pathologica, Microbiologica et Immunologica* 112:21-28.

Swings, J., Devos, P., Vandenmooter, M. and Deley, J. (1983) Transfer of *Pseudomonas maltophilia* Hugh 1981 to the genus *Xanthomonas* as *Xanthomonas maltophilia* (Hugh 1981) comb. nov. *International Journal of Systematic Bacteriology* 33:409-413.

The American Soybean Association. (2011) Soy Stats 2011. http://www.soystats.com/2011/Default-frames.htm.

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

Thomas, K., Bannon, G., Hefle, S., Herouet, C., Holsapple, M., Ladics, G., MacIntosh, S. and Privalle, L. (2005) In silico methods for evaluating human allergenicity to novel proteins: International Bioinformatics Workshop Meeting Report February 23 - 24, 2005. *Toxicological Sciences* 88:307-310.

Thomas, K., MacIntosh, S., Bannon, G., Herouet-Guicheney, C., Holsapple, M., Ladics, G., McClain, S., Vieths, S., Woolhiser, M. and Privalle, L. (2009) Scientific advancement of novel protein allergenicity evaluation: An overview of work from the HESI Protein Allergenicity Technical Committee (2000 - 2008). *Food and Chemical Toxicology* 47:1041-1050.

U.S.Pharmacopeia. (1990) *United States Pharmacopeia, Volume 23.* United States Pharmacopeia Convention, Inc, Rockville, MD, p1788-1789.

USDA (2009) *Oilseeds: World markets and trade.* Circular Series FOP1-09. Foreign Agricultural Service, United States Department of Agriculture. http://www.fas.usda.gov/oilseeds/circular/2009/January/Oilseedsfull0109.pdf.

Vaidehi, M.P. and Kadam, S.S. (1989) Soybeans. In: Salunkhe, D.D. and Kadam, S.S. eds. *Handbook of world food legumes, Volume III.* Chapter 1. CRC Press Inc., Boca Raton, pp. 1-21.

Wang, X.-Z., Li, B., Herman, P.L. and Weeks, D.P. (1997) A three-component enzyme system catalyzes the O Demethylation of the herbicide dicamba in *Pseudomonas maltophilia* DI-6. *Applied and Environmental Microbiology* 63:1623-1626.