FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

SUPPORTING DOCUMENT 1

SAFETY ASSESSMENT

SUMMARY AND CONCLUSIONS

Background

Insect-protected soybean line MON 87701 has been genetically modified (GM) for protection against feeding damage caused by the larvae of a number of insect pest species belonging to the *Lepidoptera* genus. This has been achieved through the introduction of the *cry1Ac* gene, from *Bacillus thuringiensis*, expressing the insecticidal Cry protein, Cry1Ac. The Cry proteins exert their effect on the insect by causing lysis of midgut epithelial cells, which leads to gut paralysis, cessation of feeding and eventual death of the insect.

In conducting a safety assessment of food derived from insect-protected soybean MON 87701, a number of criteria have been addressed including: a characterisation of the transferred genes, their origin, function and stability in the corn 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 proteins 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; or the safety of food derived from the non-GM (conventional) plant.

History of Use

Soybean (*Glycine max* L. Merr.), the host organism is grown as a commercial crop in over 35 countries worldwide. Soybean-derived products have a range of food and feed as well as industrial uses and have a long history of safe use for both humans and livestock. Oil accounts for 94% of the soybean products consumed by humans and is used mainly as a salad and cooking oil, bakery shortening, and frying fat as well as being incorporated into processed products such as margarine.The major food products to be derived from the insect-protected soybean line would likewise be oil, although other fermented and non-fermented soy products such as soymilk, tofu, miso, soy sauce, flour, protein concentrate and dietary supplements (among others) will likely be made from MON 87701.

B. thuringiensis, the donor organism for the *cry1Ac* gene, has been extensively studied and commercially exploited for over 40 years as the active ingredient in a number of insecticide products used in agriculture as well as home gardens. *B. thuringiensis* therefore has had a long history of safe use and the Cry proteins it produces are not known to be toxic to any vertebrates, including humans and other mammals.

Molecular Characterisation

Soybean MON 87701 was generated through the transfer of the *cry1Ac* gene under the control of the *Arabidopsis thaliana* rbcS4 promoter. This promoter directs expression in above ground tissues. The *cry1Ac* gene encodes Cry1Ac consisting of 1182 amino acids with an apparent molecular weight of 133 kDa. The protein sequence is >99% identical to the Cry1Ac protein from *Bacillus thuringiensis* subsp. *kurstaki*.and identical to that found in Bollgard cotton (Application A341). The inserted *cry1Ac* gene contains a four amino acid targeting peptide from *A. thaliana* in frame with the coding region. The targeting peptide *,* known as the chloroplast transit peptide (CTP) ensures trafficking of the expressed Cry1Ac protein to the chloroplast. This transit peptide is cleaved from the mature protein after targeting has occurred.

There are no antibiotic-resistance markers in soybean MON 87701.

Comprehensive molecular analyses of soybean MON 87701 indicate there is one insertion site at a single genetic locus. This site contains one copy of the *cry1Ac* gene. Breeding over five generations has confirmed stability of the introduced genetic elements and segregation data indicate their Mendelian inheritance. Nine potential ORFs are present at junctions associated with the insertion sites. None of these ORFs is likely to be expressed due to the absence of the necessary regulatory sequences, and none contained any similarity to known toxins, allergens or anti-nutrients.

Characterisation of Novel Protein

Soybean MON 87701 expresses one novel protein, Cry1Ac. Cry1Ac is expressed in leaves, pollen/anther, seeds and forage (above ground parts, including immature pods that are harvested for use as feed for livestock). As expected, Cry1Ac protein was not detected in the roots. The average content in mature seeds is 4.7 µg/g dry weight (range 3.4-5.7 µg/g).

A large number of studies have been done with Cry1Ac to confirm its identity and physicochemical and functional properties as well as to determine its potential toxicity and allergenicity. These studies have demonstrated that Cry1Ac conforms in size and amino acid sequence to that expected, does not exhibit any post-translational modification including glycosylation, and demonstrates the predicted insecticidal activity.

In relation to its potential toxicity and allergenicity, it is worth noting that Cry proteins from *B. thuringiensis* are inherently non-toxic to mammals and have exhibited little potential to be allergenic to humans over their long history of use. Bioinformatic studies with the Cry1Ac protein have confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that the protein would be rapidly degraded following ingestion, similar to other dietary proteins. Taken together, the evidence indicates that the Cry1Ac protein is neither toxic nor likely to be allergenic in humans.

Compositional Analyses

Detailed compositional analyses were done to establish the nutritional adequacy of seed-derived products from soybean MON 87701. Seeds were analysed for proximates (ash, fat, moisture, protein), carbohydrates (by calculation), acid detergent fibre (ADF), neutral detergent fibre (NDF), amino acids, fatty acids (C8-C22), trypsin inhibitors, phytic acid, lectin, isoflavones (daidzein, glycitein and genistein), vitamin E, raffinose and stachyose. The levels were compared with levels in the non-GM parent and to those in twenty non-GM commercial soybean cultivars.

These analyses indicated that although several variables differed significantly from the parental strain, in all cases bar one this was not reproducible in separate locations (the exception being Vitamin E). Furthermore, in all cases the differences fell within a 99% tolerance interval established from the conventionally-grown soybean varieties.

In addition, no difference between soybean MON 87701 and the nontransgenic parent were found in allergenicity 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 MON 87701 when compared with conventional soybean cultivars currently on the market.

Nutritional Impact

The detailed compositional studies are considered sufficient to establish the nutritional adequacy of food derived from soybean MON 87701. No significant differences in the nutritional content between MON 87701 and non-GM soybean varieties could be established. As such, the introduction of soybean MON 87701into the food supply would be expected to have little nutritional impact.

Conclusion

No potential public health and safety concerns have been identified in the assessment of insect-protected soybean MON 87701. On the basis of the data provided in the present application, and other available information, food derived from soybean MON 87701 is considered as safe for human consumption as food derived from conventional soybean cultivars.

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1 INTRODUCTION

Insect-protected soybean MON 87701 has been genetically modified (GM) for protection against feeding damage caused by a number of insect larvae. Protection is conferred by expression in the plant of the *cry1Ac* gene, encoding the insecticidal Cry1Ac protein. The *cry1Ac* gene is derived from *Bacillus thuringiensis*, a common soil bacterium. The Cry proteins exert their effect on the insect by causing lysis of midgut epithelial cells, which leads to gut paralysis, cessation of feeding and eventual death of the insect.

Insect-protected MON 87701 soybean is claimed to provide control over a specified number of insect pest larvae within the genus *Lepidoptera*. MON 87701 soybean has been developed for commercial cultivation in South America and is not intended for cultivation in Australia. However, MON87701 may enter the Australian and New Zealand food supply as imported, largely processed food products.

In conducting a safety assessment of food derived from Insect-protected MON 87701 soybean, 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 proteins to be either allergenic or toxic in humans.

2 HISTORY OF USE

2.1 Host Organism

The host organism is soybean (*Glycine max* (L.) Merr.) Soybean is grown as a commercial crop in over 35 countries worldwide and has a long history of safe use amongst both humans and livestock. The major producers of soybeans, accounting for 90% of world production, are the United States, Argentina, Brazil and China. In 2007, soybeans represented 56 percent of total world oilseed production, and 32 percent of those soybeans were produced in the United States where they provided 71 percent of the edible consumption of fats and oils (The American Soybean Association 2008). Australia, while a net importer of soybean, grows crops in latitudes extending from the tropics (16^{o} S) to temperate regions (37^{o} 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 eg United States (91%); Argentina (99%); Brazil (63%); South Africa (87%); Uruguay (99%) (Brookes and Barfoot, 2009).
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.

Soybean 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 eg livestock, pet and poultry food), protein concentrate and isolate (for use in both edible and technical/industrial products), and textured flour (for edible uses). The hulls are used in mill feed.

The soybean cultivar A5547 was used as the parental variety for the MON 87701 insect-protected trait described in this application, and thus is regarded as the near-isogenic line for the purposes of comparative assessment. A5547 was chosen for its superior agronomic performance and suitability for growth in central and South America.

2.2 Donor organism

The organism from which the *cry1Ac* gene is derived is the common soil bacterium, *Bacillus thuringiensis*. The effect of *B. thuringiensis* products on human health and the environment was the subject of a critical review by the WHO International Program on Chemical Safety (WHO, 1999). The review concluded that "*B. thuringiensis* products are unlikely to pose any hazard to humans or other vertebrates or the great majority of non-target invertebrates provided that they are free from non-*B. thuringiensis* microorganisms and biologically active products other than the insecticidal proteins".

B. thuringiensis is a facultative anaerobic, gram-positive spore-forming bacterium that produces characteristic insecticidal proteins, as parasporal crystals, during the sporulation phase. These crystals are predominantly comprised of one or more Crystal (Cry) and Cytolitic (Cyt) toxins, also called δ-endotoxins. These toxins are highly specific to their target insect, are innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo *et al*., 2007). Over 60 subspecies of *B. thuringiensis* have been described. *B. thuringiensis* subspecies can synthesise more than one type of Cry protein, which are specifically toxic to the insect orders Coleoptera, Diptera, Hymenoptera, and Lepidoptera, and also to nematodes. The Cyt toxins are mostly found in *B. thuringiensis* strains that are active against Diptera.

A number of different commercial *B. thuringiensis* formulations have been registered worldwide for use as an insecticide to be applied to foliage, soil, and water or food storage facilities. While the *B. thuringiensis* spores or vegetative cells may persist in the environment for weeks, months or years, the Cry proteins become inactive within hours or days.

Studies on mammals, particularly laboratory animals, demonstrate that *B. thuringiensis* is mostly non-pathogenic and non-toxic. *B. thuringiensis* has been demonstrated to be highly specific in its insecticidal activity and have demonstrated little, if any, direct toxicity to non-target insects.

In the case of humans, the field application of *B. thuringiensis* products can result in considerable aerosol and dermal exposure. With the exception of case reports on ocular and dermal irritation, no adverse health effects have been documented after occupational exposure to *B. thuringiensis* products (McClintock *et al*., 1995). Studies with human volunteers who ingested and inhaled large quantities of a Btk formulation (*B. thuringiensis* subspecies *kurstaki*) did not reveal any adverse effects (Fisher and Rosner, 1959). Similarly, *B. thuringiensis* present in drinking water or food has not been reported to cause adverse effects on human health (WHO, 1999).

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; and the genetic stability of the inserted DNA and any accompanying expressed traits.

Studies submitted:

Sophia M. Arackal, Kim R. Lawry, Zihong Song, Jeanna R. Groat, James F. Rice, James D. Masucci and Qing Tian. Amended Report for MSL0021960: Molecular Analysis of Insect-Protected Soybean MON 87701. Monsanto Study Report MSL0022176.

Andre Silvanovich and Renee Girault. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of the Inserted DNA in MON 87701: Assessment of Putative Polypeptides. Monsanto Study Report MSL0021816.

3.1 Method used in the genetic modification

Soybean cultivar A5547 was transformed with the binary vector PV-GMIR9 consisting of two independent T-DNAs (Figure 1) using standard *Agrobacterium*-mediated transformation methods (Martinell *et al.*, 2002).

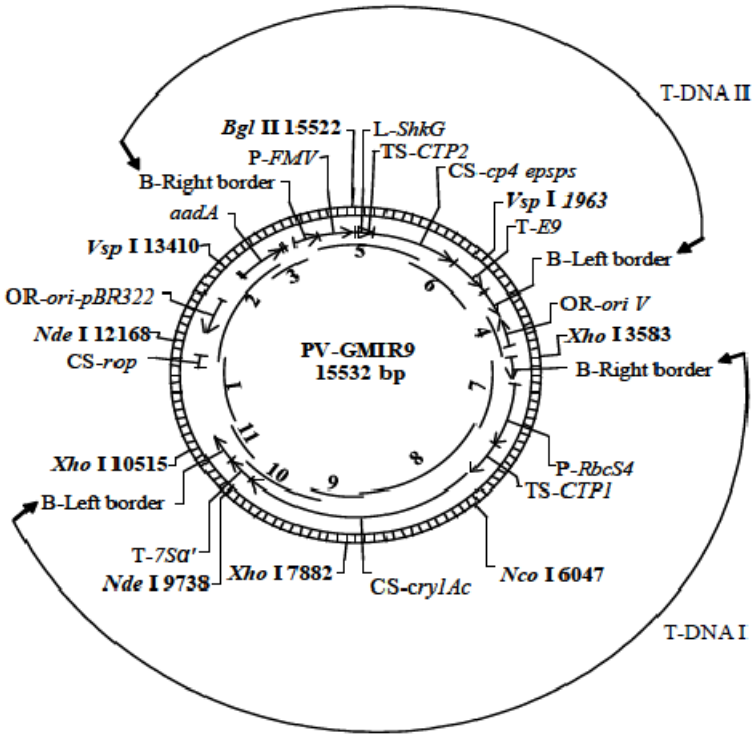

Figure 1: Genetic elements of plant transformation vector PV-GMIR9. Two independent T-DNAs are present, labelled T-DNA I and T-DNA II. T-DNA 1 contains sequences coding for the *B. thuringiensis* Cry1A protein and its regulation within standard left and right border sequences.

T-DNA II contains sequences coding for the *Agrobacterium sp.* EPSPS protein and its regulation. Probes used in Southern blotting for gene characterisation are labelled 1-11 (see section 3.4).

The genetic modification was carried out as follows: Meristem tissues from the embryos of germinated A5547 seeds were cultured in the presence of PV-GMIR9-tranformed *Agrobacterium*. Transformed cells were selected using glyphosate. The meristem material was then cultured and phenotypically normal plants were selected for further growth and analysis. Self-pollination of these R_0 plants led to segregation of T-DNA I and T-DNA II in the R_1 generation. In order to select plants with integrated T-DNA I but not the T-DNA II, a non-lethal dose of glyphosate was applied to the plants. Plants displaying some injury (and thus lacking the *epsps* gene) were selected. Plants homozygous for a single T-DNA I cassette were self-pollinated over several generations. MON 87701 was selected for its superior phenotype and molecular profile (Figure 2).

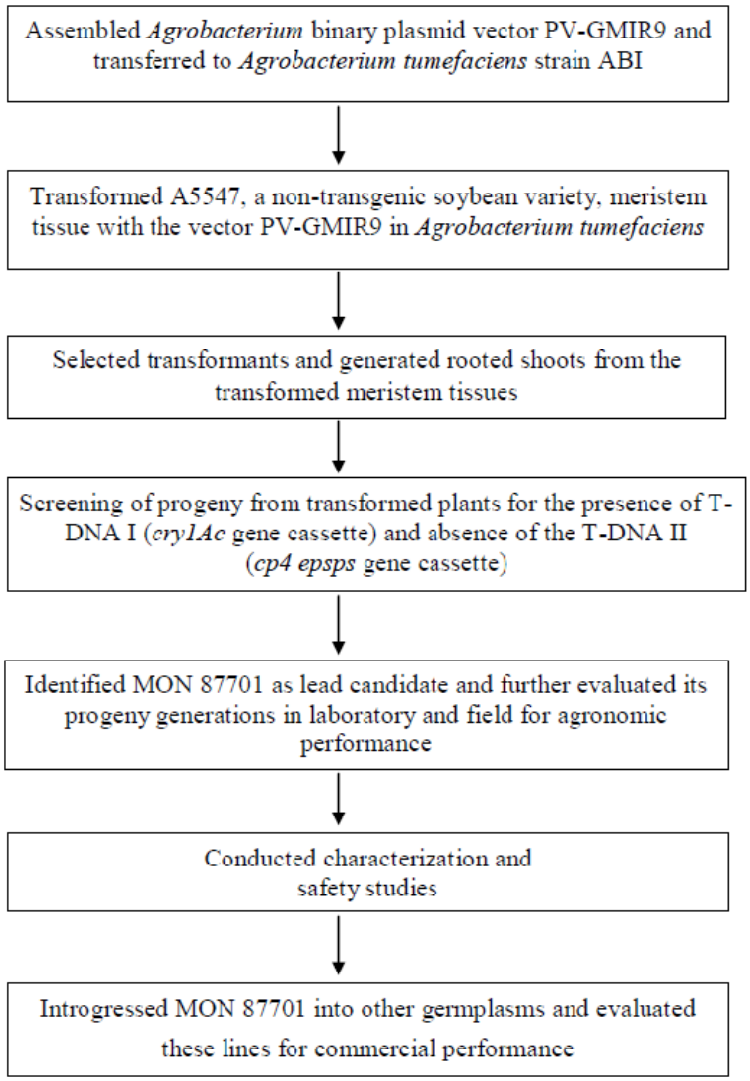

Figure 2: Summary of the transformation and evaluation procedures leading to the selection of soybean MON 87701.

3.2 Breeding of soybean MON 87701

Figure 3 summarises how soybean MON 87701 was produced by genetic modification of the cultivar A5547. The *Agrobacterium*-mediated transformation of meristem tissues and selection for glyphosate resistance led to generation R_0. The R_0 generation was self-pollinated to produce R_1. Plants containing T-DNA I but not T-DNA II were selected and self-pollinated until generation R_5. At this point the plant displaying a single, intact insert and superior phenotypic characteristics was chosen for commercialisation. This plant, MON 87701, was further characterised for its food safety.

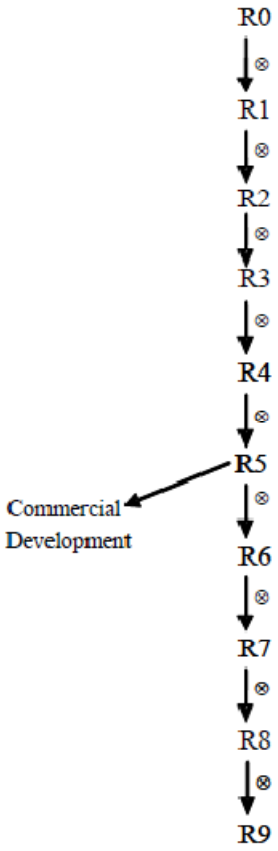

Figure 3: Breeding of MON 87701. All generations were self-pollinated. The seed from generation R_5 was used for molecular characterisation and commercial development. Seeds from R_6 to R_9 were used for analysis of molecular stability.

3.3 Function and regulation of introduced gene sequences

Information on the coding sequences, nucleotide sequences and regulatory elements in the two linear DNA fragments PHP19340A and PHP17752A is given in Table 1.

Table 1: Description of the genetic elements contained in the binary vector PV-GMIR9

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA II (Continued from bp 15,532)		
Intervening sequence	1-14	Sequences used in DNA cloning
L1-ShkG	15-81	5' non-translated leader sequence from the *Arabidopsis ShkG* gene encoding EPSPS (Klee *et al*., 1987) that is involved in regulating gene expression
TS2-CTP2	82-309	Targeting sequence encoding the chloroplast transit peptide from the *ShkG* gene of *Arabidopsis thaliana* encoding EPSPS (Klee *et al*., 1987)
CS3-*cp4-epsps*	310-1677	Codon modified coding sequence of the *aroA* gene from *Agrobacterium sp.* strain CP4 encoding the CP4 EPSPS protein (Barry *et al*., 1997; Padgette *et al*., 2009)
Intervening Sequence	1678-1719	Sequences used in DNA cloning
T4-E9	1720-2362	3' non-translated sequence from *RbcS2* gene of *Pisum sativum* encoding the Rubisco small subunit, which functions to direct polyadenylation of the mRNA (Coruzzi *et al*., 1984)
Intervening Sequence	2363-2409	Sequences used in DNA cloning
B5-Left Border	2410-2851	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker *et al*., 1983)
Vector Backbone		
Intervening Sequence	2852-2937	Sequences used in DNA cloning
OR6-ori V	2938-3334	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker *et al*., 1981)
Intervening Sequence	3335-3595	Sequences used in DNA cloning
T-DNA I		
B-Right Border	3596-3952	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker *et al*., 1982)
Intervening Sequence	3953-4061	Sequences used in DNA cloning
P7-RbcS4	4062-5784	Promoter, leader, and 5' non-translated region of the *Arabidopsis thaliana RbcS4* gene encoding ribulose 1, 5-bisphosphate carboxylase small subunit 1A, (Krebbers *et al*., 1988). Promoter expresses in above ground tissues
TS-CTP1	5785-6048	Targeting sequence encoding the transit peptide of the *Arabidopsis RbcS4* encoding small subunit 1A transit peptide, from *Arabidopsis thaliana*, present to direct the *cry1Ac* protein to the chloroplast (Krebbers *et al*., 1988).
CS-cry1Ac	6049-9585	Codon-modified coding sequence of the Cry1Ac protein of *Bacillus thuringiensis* (Fischhoff and Perlak, 1997)
Intervening Sequence	9586-9594	Sequences used in DNA cloning
T-7S α'	9595-10033	3' region of the *Sphas1* gene of *Glycine max* encoding the 7S *α'* seed storage protein, β-conglycinin, including 35 nucleotides of the carboxyl terminal β -conglycinin coding region with the termination codon and the polyadenylation sequence (Schuler *et al*., 1982). The element functions to terminate transcription and direct polyadenylation of the mRNA.
Intervening	10034-10069	Sequences used in DNA cloning

Sequence		
B-Left Border	10070-10511	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker *et al*., 1983)
Vector Backbone (Continued from bp 3595)		
Intervening Sequence	10512-11786	Sequences used in DNA cloning
CS-rop	11787-11978	Coding sequence for repressor of primer protein derived from ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	11979-12405	Sequences used in DNA cloning
OR-*ori-pBR322*	12406-12994	Origin of replication from pBR322 for maintenance of plasmid in *Escherichia coli* (Sutcliffe, 1978).
Intervening Sequence	12995-13524	Sequences used in DNA cloning
aadA	13525-14413	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3' (9)-O-nucleotidyltransferase from the transposon Tn7 (Fling *et al*., 1985) (GenBank accession) that confers spectinomycin and streptomycin resistance
Intervening Sequence	14414-14549	Sequences used in DNA cloning
T-DNA II (Continued from bp 2851)		
B-Right Border	14550-14906	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker *et al*., 1982)
Intervening Sequence	14907-14939	Sequences used in DNA cloning
P-FMV	14940-15503	Promoter for the 35S RNA from figwort mosaic virus (FMV) (Rogers 2000) that directs transcription in most plant cells
Intervening Sequence	15504-15532	Sequences used in DNA cloning

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

3.3.1 *The cry1Ac coding sequence*

MON 87701 carries a single copy of the *cry1Ac* gene. This gene encodes the Cry1Ac protein, which is derived from the common soil bacterium *Bacillus thuringiensis* subspecies *kurstaki*. As a result of expression of this gene, the soybean is resistant to attack from certain insects of the genus *Lepidoptera*. This protein shares >99% sequence identity with the original bacterial Cry1Ac protein and 100% identity with the Cry1Ac protein from the insect-protected Bollgard cotton. N-terminal and in fusion with the Cry1A sequence is a four amino acid chloroplast targeting peptide (CTP1).

3.3.2 *The cry1Ac regulatory sequences*

Expression and localisation of the *cry1Ac* gene is under control of the RbcS4 promoter and CTP1 targeting sequence. Both sequences derive from the *Arabidopsis thaliana* ribulose 1,5-bisphophate carboxylase small subunit 1A gene (Krebbers *et al*., 1988). This results in expression of the protein only in above-ground tissues and localisation of the protein to the chloroplast. The 3' untranslated region (termed 7S a'3') derives from the *Glycine max* 7S seed storage protein gene (Schuler *et al*., 1982). This sequence is necessary for transcription termination and polyadenylation of the *ctpi-cry1Ac* transcript.

3.3.3 *T-DNA borders*

On either side of the *cry1Ac* expression cassette are the right and left T-DNA border sequences. These 24-25 bp sequences are necessary for *Agrobacterium*-mediated T-DNA incorporation into the genome (Barker *et al*., 1983; Depicker *et al*., 1982).

3.4 Characterisation of the genes in the plant

Evaluation of insert copy number, insert integrity and presence/absence of plasmid backbone sequences was done by Southern blot analysis and sequence analysis of plant material R5 generation (see Figure 3) of soybean MON 87701. The parental line, A5547, was used as a control. The probes used in the Southern blot analyses correspond to the numbering system used in the following table:

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

Figure 4: Probes used in the Southern blots analyses. Probe numbers correspond to the position of DNA elements labelled in Figure 1.

3.4.1 *Transgene copy number*

Leaf tissue from individual seedlings of the R5 (commercialised) generation was used for these analyses. In order to determine the number of insertion sites and the transgene copy number, genomic DNA was subjected to restriction digest and gel electrophoresis. The DNA was then transferred to a membrane and incubated with probe 7, 8, 9, 10 or 11. These probes correspond to sequences within T-DNA I (see Figure 1). Upon digestion with restriction enzymes, a single integration event consisting of one copy will give an expected banding pattern corresponding to insertion of the plasmid into the genome. Incorporation of pV-GMIR9 in more than one site, or incorporation of more than one copy of the plasmid within the one site would result in additional bands.

Analysis of restricted genomic DNA by Southern blot demonstrated that one complete copy of T-DNA I was inserted into the soybean genome. The banding pattern was consistent only with a single insertion event.

3.4.2 *Plasmid backbone DNA analysis*

In order to test for the presence of plasmid backbone in the MON 87701 genome, leaf tissue from the R5 generation was isolated, digested with restriction enzymes and subjected to gel electrophoresis as before. Probes 1, 2 and 3 (corresponding to the plasmid backbone between T-DNA I left border and T-DNA II right border) and probe 4 (corresponding to the plasmid backbone between T-DNA I right border and T-DNA II left border) were hybridised to the digested genomic DNA.

None of the four probes hybridised with DNA in either MON 87701 or conventional soybean. Demonstration of the function of the probes was assured through hybridisation with “spiked” conventional soybean (soybean DNA mixed with 0.1 or 1 genome equivalent of the original plasmid). These data demonstrate that the plasmid backbone is not present in MON 87701.

3.4.3 *T-DNA II insertion analysis*

In order to determine if the second T-DNA present in pV-GMIR9 (T-DNA II) had been incorporated into MON 87701, probes 5 and 6 were employed. Neither of these probes hybridised with any sequences in either the MON 87701 or conventional soybean DNA preparations. Again, the veracity of the experiment was verified with hybridisation to “spiked” conventional soybean demonstrated. These data demonstrate that T-DNA II (containing the herbicide resistance gene *cp4-epsps*) is not present in MON 87701.

3.4.4 *Insertion site analysis*

Genomic DNA from the R5 (commercialised line) generation was extracted and subjected to polymerase chain reaction (PCR) analysis. Primers were designed in order to establish the nucleotide sequence of the inserted DNA, as well as to determine the sequence of the soybean genome flanking the insert.

PCR analysis confirmed the presence of T-DNA I in MON 87701. The PCR products were sequenced and assembled into a consensus sequence. Analysis and comparison with the same region from conventional soybean demonstrated that both the 5’ and 3’ regions adjacent to T-DNA I are present in conventional soybean. Thus, no major rearrangements occurred during the creation of MON 87701. However, sequence analysis showed a 32 bp deletion and a 14 bp pair insertion just 5’ to the T-DNA I insertion site.

3.4.5 *Open Reading Frame (ORF) analysis of the junction regions*

The nature of the process by which genetic material is introduced into an organism may result in unintended effects that include the creation of new open reading frames (ORFs) in the genome of the organism. These ORFs may, in turn, lead to the production of novel proteins which could have implications for human safety, particularly regarding toxicity and allergenicity, if ingested.

Using the sequence data encompassing the 5’ and 3’ genomic border sequences, the insertion of T-DNA I into soybean MON 87701 was screened for the presence of ORFs containing stop codons that spanned any novel junctions and that were greater than or equal to 8 amino acids in length. Nine such putative ORFs were identified – five located 5’ to the insertion site and four located 3’ to the insertion site.

Putative polypeptides from each reading frame were compared to three different databases:

(i) AD_2009 database – contains allergen, gliadin and glutenin sequences and was assembled from sequences found on the FARRP allergen database (FARRP 2009). The database contains 1,386 sequences;

(ii) PRT_2009 – this database was used to represent all currently known publicly available protein sequences and consisted of GenBank release 169.0 (December 16, 2008). It contains 14,717,352 sequences; and

(iii) TOX_2009 database – this database was assembled from the above PRT_2009 database. Protein sequences were selected using a keyword and filtered to remove likely non-toxin proteins. The TOX_2009 database contains 7,651 sequences.

Comparison was made of the 6 possible reading frames of each of these peptides with the sequences of known allergens and toxins. A cut-off for sequence similarity was set at E=1x10^{-5}. None of the nine putative peptides showed biologically relevant similarity to known allergens or toxins.

3.4.6 *Open Reading Frame (ORF) analysis of the* cry1Ac *coding sequence.*

In all proteins, there remains the possibility that mutation within a coding sequence could lead to transcription and translation of alternative reading frames. As this principle applies also to the introduced *cry1Ac* gene in MON 87701, an analysis of the other 5 reading frames was carried out. As stated above, the resulting putative polypeptides were compared with the amino acid sequences of known toxins and allergens. Only matches with an *E*-score less than 1x10^{-5} were further considered. Of the five alternative reading frames, only analysis of frames 2 and 5 resulted in alignments of this stringency. The alignments in reading frame 2 contained numerous stop codons and required numerous gaps for alignment – it is therefore highly unlikely that a protein would result from this reading frame, and any potential peptides are unlikely to bear structural conservation. Alignments showing an *E*-score of 1x10^{-5} or less in reading frame 5 all aligned with *Bacillus thuringiensis* proteins, and are therefore not relevant to this analysis.

3.4.7 *Conclusion about gene characterisation*

The applicant has provided comprehensive PCR, bioinformatic and Southern blot analyses. These analyses demonstrate that MON 87701 contains a single, complete copy of the T-DNA I sequence (encoding the *cry1Ac* expression cassette). They have also demonstrated that no part of the second T-DNA (T-DNA II), or the plasmid backbone is present in soybean MON 87701. Finally, the analyses have shown that there are no ORFs present that show any relevant or significant homology with known allergens or toxins.

3.5 Stability of the genetic changes

3.5.1 *Generational stability*

In order to determine if the *cry1Ac* insert is stably inherited, the R5 generation (used for commercialisation) was self-pollinated for a further four generations (see Figure 3). Genomic DNA from leaf tissue from each generation was isolated, digested with two restriction enzymes and subjected to Southern analysis using probe number 8 (see Figure 4), which hybridises to the *cry1Ac* coding region. The results of this experiment show the same banding pattern for each generation, demonstrating that the insert in soy MON 87701 is stable across multiple generations and is not subject to frequent recombination.

3.5.2 *Segregation analysis*

In order to test if the *cry1Ac* locus segregates according to Mendelian principles, a segregation analysis was undertaken. The R1 to R5 generations (see Figure 5) were self-pollinated and the resulting plants of each generation were tested for the segregation of the *cry1Ac* locus. At generation R5, the plants were crossed with conventional soybean, and then self-pollinated for two generations (see Figure 5). The F2 and F3 generations were examined to establish whether the *cry1Ac* locus segregated in the expected 1:2:1 (homozygous positive: hemizygous positive: homozygous negative) ratios. To determine whether the observed segregation ratios deviated significantly from the expected segregation ratios, a Chi-square (χ^2) analysis was undertaken (see Table 2 and Table 3).

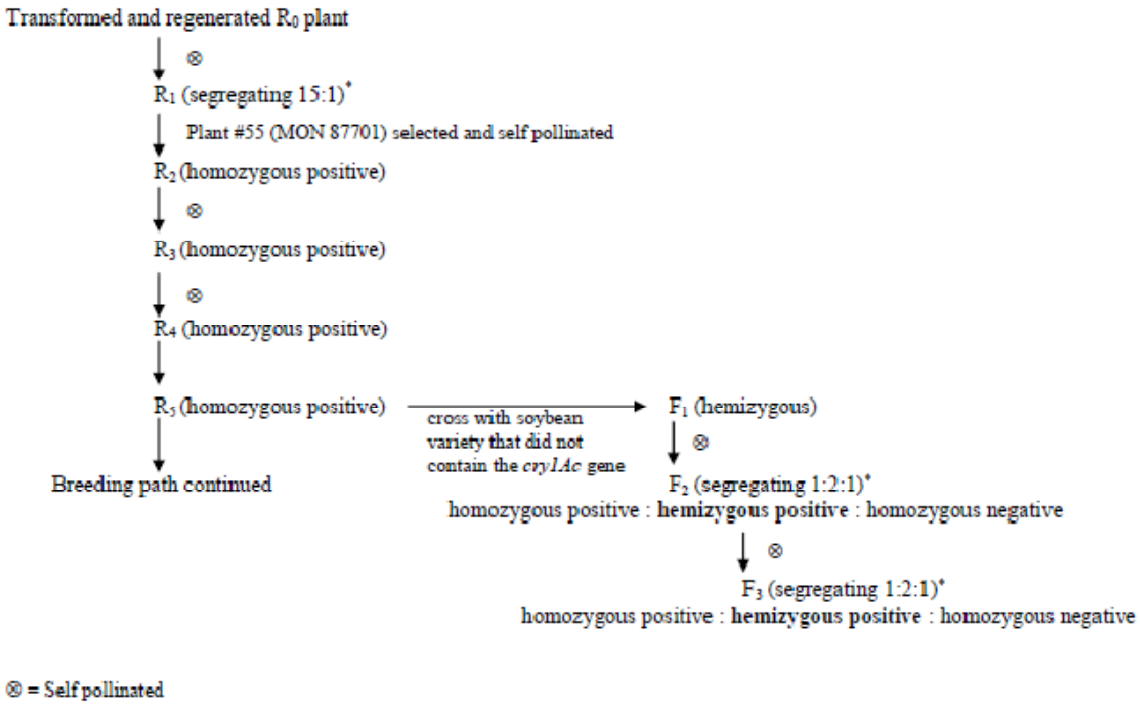

Figure 5: Breeding strategy for the generation of segregation data for MON 87701

Table 2: Segregation of the *cry1Ac* gene during development of MON 87701

Generation	Expected Ratio	Total Plants Tested	Observed # Plants Positive	Observed # Plants Negative	Expected # Plants Positive	Expected # Plants Negative	χ^2	Prob.
R1	15:1	19	18	1	17.8	1.2	0.03	0.8590
R2	1:0	80	80	0	80	0	Fixed	N/A
R3	1:0	48	48	0	48	0	Fixed	N/A
R4	1:0	598	598	0	598	0	Fixed	N/A
R5	1:0	629	629	0	629	0	Fixed	N/A

Table 3: Segregation of the *cry1Ac* gene in F2 and F3 progeny following crossing of MON 87701 with conventional soybean

Generation	Total Plants Tested	Observed # Plants +/+	Observed # Plants +/-	Observed # Plants -/-	Expected # Plants +/+	Expected # Plants +/-	Expected # Plants -/-	χ^2	Prob.
F2	297	79	148	70	74.25	148.5	74.25	0.5	0.76
F3	263	73	121	69	65.75	131.5	65.75	1.8	0.4069

Within generations R1 to R5 (Table 2), the trait was fixed after the first self-pollination. Therefore no segregation was observed. These data correlate with the Southern analysis, demonstrating that the *cry1Ac* locus is stably inherited and present as a single copy in a single site in the genome.

The segregation of *cry1Ac* following crossing with conventional soybean led to segregation of the inserted DNA at ratios consistent with those expected (Table 3). That is, there were no significant differences in the expected and observed segregation ratios. Again, these data correlate with the molecular data presented and demonstrate that the inserted *cry1Ac* gene is present at a single site and as a single copy.

3.5.3 *Conclusion about stability of the genetic change*

Taken together, the molecular data presented (PCR, Southern analysis) and the segregation data suggest that the insert in MON 87701 is stable. No recombination was observed in multiple plants over several generations.

3.6 Antibiotic resistance marker genes

No antibiotic marker genes are present in soybean MON 87701. Molecular data, including several Southern blots, demonstrate that no plasmid backbone has been integrated into the soybean genome during transformation, and the integrated DNA contains no antibiotic resistance gene.

3.7 Conclusion about molecular characterisation

Soybean MON 87701 contains one introduced coding sequence with its associated regulatory elements. This sequence consists of a four amino-acid chloroplast targeting sequence in frame and infusion with the *Bacillus thuringiensis cry1Ac* gene. Expression of this gene is intended to provide protection to MON 87701 from certain species of the *Lepidoptera* genus.

Comprehensive molecular analyses of soybean MON 87701 indicate that there is one insertion site at a single genetic locus. This site contains a single copy of T-DNA I. Breeding over four generations has confirmed stability of the introduced genetic elements and segregation data demonstrate their Mendelian inheritance. Backbone analysis shows that no plasmid backbone has been incorporated. Nine putative ORFs were found at junction regions, but none of these showed biologically relevant homology to known toxins or allergens. Reading frame analysis of the *cry1Ac* sequence itself did not reveal any meaningful alternative polypeptides.

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.

4.1 Description and function of the novel protein

The general mechanism of insecticidal activity of Cry proteins is well understood (Bravo *et al*., 2007; Gill *et al*., 1992; Schnepf *et al*., 1998; Zhuang and Gill, 2003), with the mode of action being characterised principally in lepidopteran insects. The Cry proteins belong to a class of bacterial toxins known as pore-forming toxins (PFT) that are secreted as water-soluble proteins, which after undergoing conformational change, are able to insert into, or translocate across, the cell membranes of their host. There are two main groups of PFT: (i) the α-helical toxins in which the α-helix regions form the trans-membrane pore; and (ii) the β-barrel toxins, that insert into the membrane by forming a β-barrel composed of β-sheet hairpins from each monomer (Parker and Feil, 2005). The Cry proteins belong to the α-helical group of PFT, along with other toxins such as exotoxin A (from *Pseudomonas aeruginosa*) and diphtheria toxin.

The Cry proteins comprise at least 50 subgroups consisting of more than 200 members. Primary sequence identity among different Cry genes is the basis of the nomenclature and categorisation used for the different Cry proteins. Among the Cry proteins, the 3-Domain family is the most commonly used worldwide for insect control and has been the best characterised in terms of mode of action. Protoxins from this family contain three structural domains connected by single linkers plus a C-terminal domain. One large group of protoxins within this family is approximately twice as long as the rest. The C-terminal domain in the long protoxin group is dispensable for toxicity and is believed to play a role in the formation of the crystal inclusion bodies within the bacterium (de Maagd *et al*., 2001). In the 3-Domain family of Cry proteins, the N-terminal domain (domain I) consists of a bundle of seven α-helices which is responsible for membrane insertion and pore formation. Domain II consists of three anti-parallel β-sheets with exposed loop regions, and domain III is a β-sandwich (Boonserm *et al*., 2005; Boonserm *et al*., 2006; Galitsky *et al*., 2001; Grochulski *et al*., 1995; Li *et al*., 1991; Morse *et al*., 2001). Exposed regions in domains II and III are involved in receptor binding (Bravo *et al*., 2005). Domain I shares structural similarities with other PFT like diphtheria toxin, supporting the role of this domain in pore formation. Domain II has structural similarities with several carbohydrate-binding proteins like lectin and domain III shares structural similarity with other carbohydrate binding proteins such as the cellulose-binding domain of 1,4-β-glucanase C (de Maagd *et al*., 2003), suggesting that carbohydrate moieties could have an important role in the mode of action of the 3-Domain Cry toxins (Bravo *et al*., 2007).

The primary action of Cry toxins is to lyse midgut epithelial cells in the target insect by forming pores in the apical microvilli membrane of the cells, which subsequently leads to ion leakage and cell lysis. The crystal inclusions ingested by susceptible larvae dissolve in the alkaline environment of the gut, and the solubilised inactive protoxins are cleaved by midgut proteases yielding 60-70 kDa protease resistant proteins (Bravo *et al*., 2005). Toxin activation involves the proteolytic removal of an N-terminal peptide (25 – 30 amino acids for Cry1 toxins, 58 residues for the Cry3A and 49 for Cry2Aa) and approximately half of the remaining protein from the C-terminus in the case of the long Cry protoxins. The activated toxin then binds to specific receptors on the brush border membrane of the midgut epithelium columnar cells (Bravo *et al*., 2005; de Maagd *et al*., 2001) before inserting into the membrane. Toxin insertion leads to formation of lytic pores in microvilli apical membranes (Aronson and Shai, 2001; Bravo *et al*., 2005). Subsequently cell lysis and disruption of the midgut epithelium releases the cell contents providing spores a germinating medium leading to a severe septicemia and insect death.

The cry protein used in MON 87701 (Cry1Ac) has been included in insect-protected cotton (Bollgard and Bollgard II cotton). These registered products, along with the now deregulated DBT418 corn (which produces the tryptic core of Cry1Ac) and crops containing other Cry proteins, have been available on the market for some time, with no reports of adverse effects, including allergic reactions.

4.2 Identification of the Cry1Ac protein and equivalence of MON 87701-produced and *E. coli*-produced Cry1Ac protein

The amount of Cry1Ac produced in MON 87701 seed was insufficient for safety evaluations. Therefore, the Cry1Ac protein, including the N-terminal four amino acids originating from the CTP was expressed in *E. coli*. In order to establish whether Cry1Ac from *E. coli* is functionally equivalent to MON 87701-expressed Cry1Ac, a range of analytical techniques was employed. These techniques include analysis of molecular weight, protein purity, protein identity (through immunoblot analysis, MALDI-TOF mass spectrometry and N-terminal sequencing), glycosylation analysis and functional activity comparison.

Study submitted:

Erin Bell, Kathleen S. Crowley, Joshua P. Uffman, and Elena A. Rice. Characterization of the Cry1Ac Protein Purified from the Harvested Seed of MON 87701 Soybean and Comparison of the Physicochemical and Functional Properties of the MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins. Monsanto Study Report MSL0021146.

The MON 87701-derived Cry1Ac protein was isolated from harvested seed. The Cry1Ac protein was purified using a combination of ammonium sulphate fractionation, anion exchange chromatography and immunoaffinity chromatography.

4.2.1 *Molecular weight and purity analysis*

The MON 87701-derived and *E.coli*-derived Cry1Ac proteins were separated via SDS-PAGE. The proteins were visualised by Silver Staining. Full-length Cry1Ac protein from MON 87701 migrated at approximately 133.4 kDa. The *E.coli*-produced protein migrated at approximately 131.7 kDa. This difference of 1.3% is within the acceptance criterion of ≤5% difference. Other, smaller, bands were present in both MON 87701 and *E. coli*-derived Cry1Ac preparations. One of these bands was N-terminally sequenced (see Section 4.2.2.3) and found to belong to the Cry1Ac protein.

Purity of the samples was also determined. A previous Western blot had determined which of the bands seen in the SDS-PAGE gels corresponded to MON87701 protein. Summing the intensity of the Cry1Ac protein bands, the purity of the sample was determined to be 77%. The full-length Cry1Ac protein was found to be 43% of total protein.

Calculation on the purity of the *E.coli*-derived Cry1Ac protein was not given. Visual inspection of the gel reveals the Cry1Ac protein band to be the dominant band. However, several other bands are visible on the gel.

4.2.2 *Protein identity*

4.2.2.1 Immunoblot analysis

Immunoblot analysis was carried out to confirm the identity of the MON 87701-derived Cry1Ac protein and compare immunoreactivity with the *E. coli*-derived Cry1Ac protein. Cry1Ac protein from MON 87701 and *E.coli* were loaded onto a tris-glycine polyacrylamide

gel, electrophoresed, transferred to a PVDF membrane and subjected to Western analysis. The membrane was probed with an affinity-purified anti-Cry1Ac antibody and visualised by ECL.

Similarly to SDS-PAGE analysis, the dominant band seen in the Western blot migrated at approximately 133 kDa. This band was seen in lanes from both the MON 87701-derived and *E. coli*-derived protein. Other faint bands were seen, most likely representing aggregation (~250 kDa) and degradation products (below ~133 kDa).

Densitometric analysis was carried out for comparison of the immunoreactivity of the Cry1Ac protein from MON 87701 and *E.coli*. The average intensity of the signal from MON 87701-derived protein was 33.3% less than that for *E.coli*-derived Cry1Ac. This observed difference falls within the accepted limit for variability (±35%).

Taken together, the immunoblot analysis established that the dominant protein seen in section 4.2.1 was indeed the Cry1Ac protein. Furthermore, many of the smaller bands seen in the Silver Stain in 4.2.1 were due to breakdown of the Cry1Ac protein. The Cry1Ac proteins from MON 87701 and *E.coli* migrated at a similar molecular weight and reacted similarly to the anti-Cry1Ac antibody. Thus the two proteins are equivalent with regards to molecular weight and immunoreactivity.

4.2.2.2 MALDI-TOF tryptic mass map analysis

Heat-denatured, reduced and alkylated protein from MON 87701 was trypsinised and subjected to MALDI-TOF analysis. Seventy unique peptides were identified that corresponded to the expected masses of the Cry1Ac trypsin-digested peptides. This resulted in coverage of the Cry1Ac protein of ~67%. Given that coverage of 15% or higher is regarded as reliably identifying a protein (Jensen *et al*., 1997), this analysis has confirmed the identity of the MON 87701-produced protein. No similar analysis was carried out on the *E. coli*-produced Cry1Ac protein.

4.2.2.3 N-terminal sequence analysis

The identity of the Cry1Ac protein was further determined by N-terminal sequencing. The analysis identified seven strong and two tenuous amino acids, as well as one undesignated amino acid (see Table 4). This latter amino acid corresponded to the first in the protein sequence, cysteine, which is known to be unstable during the acid hydrolysis reaction and is not usually observed but rather implied (Inglis and Liu, 1970).

Table 4: Summary of N-terminal sequence analysis of MON 87701-derived Cry1Ac protein

AA #	1	2	3	4	5	6	7	8	9	10
Predicted sequence	C	M	Q	A	M	D	N	N	P	N
Observed sequence	X	M	Q	A	M	D	N	(N)	P	(N)

These results were consistent with those obtained for the *E. coli*-derived Cry1Ac protein.

A second band of approximately 75 kDa was also sequenced. This band, which is immunoreactive with the anti-Cry1Ac antibodies, comprised approximately 10% of the total protein. Sequencing of this band revealed it to have the same N-terminal sequence (although due to weaker signals, only three strong and three tenuous identifications were made) as the full-length protein. This suggests that this 75 kDa protein is a C-terminally truncated Cry1Ac protein.

4.2.3 *Glycosylation analysis*

Glycosylation of a protein is a post-translational modification. Glycoproteins consist of carbohydrate moieties (glycans) covalently linked to a polypeptide backbone and the carbohydrate component may represent from <1% to >80% of the total weight. There is evidence that in transgenic plants, expression of non-native proteins may lead to inauthentic glycosylation and concomitant alteration of immunogenicity (see, for example (Prescott *et al*., 2005)). Characterisation of protein glycosylation in genetically modified plants may therefore assist in informing the risk assessment process.

To assess whether post-translational glycosylation of the MON 87701-derived Cry1Ac protein occurred, glycosylation analysis of the purified protein sample was undertaken using a commercially available glycoprotein staining kit. Positive controls included horseradish peroxidase and transferrin. Whereas both these proteins reacted with the stain, no signal was detected with the MON 87701-derived Cry1Ac protein. Similarly, the *E. coli*-derived Cry1Ac protein was also confirmed to have no glycosylation. This was expected since *E. coli*, like most prokaryotes, lacks the capacity for protein glycosylation (Wacker *et al*., 2002).

4.2.4 *Functional activity*

The functional activity of both the MON 87701 and *E. coli*-produced Cry1Ac proteins was tested. This test was a bioassay of the impact on corn earworm growth fed on a diet including the Cry1Ac protein. The two Cry1Ac protein preparations were compared in parallel on three separate occasions, with insect larvae allowed to feed on a diet including 6x two-fold dilutions between 0.00065 and 0.020 µg Cry1Ac protein/mL diet. Two buffer controls were included. There were 16 larvae per treatment.

Results from these experiments established the half maximal effective concentration (EC_{50}) of Cry1Ac from MON 87701 to be 0.0039 µg Cry1Ac protein/mL diet. The EC_{50} for the *E. coli*-produced Cry1Ac protein was 0.0036 µg Cry1Ac protein/mL diet. Given the high fidelity between these two results, the two protein preparations are functionally equivalent.

4.2.5 *Conclusion*

A range of characterisation methods confirmed the identity and non-glycosylated status of Cry1Ac protein produced either in *E. coli* or in soybean MON 87701. The identity of the Cry1Ac protein from MON 87701 was confirmed by MALDI-TOF analysis and N-terminal sequencing. Furthermore, protein from both sources was found to migrate at very similar molecular weights, to be recognised to a similar degree by anti-Cry1Ac antibodies, to lack glycosylation and to have very similar EC50 values in a bioassay. Thus the Cry1Ac proteins from MON 87701 and *E. coli* can be said to be equivalent. Thus the *E. coli*-derived Cry1Ac protein was used in subsequent studies for the safety assessment.

4.3 Cry1Ac protein expression analysis

Study submitted:

Katherine E. Niemeyer and Andre Silvanovich. Assessment of the Cry1Ac Protein Levels in Soybean Tissues Collected from MON 87701 Produced in U.S. Field Trials During 2007. Monsanto Study Report MSL0021531.

The Cry1Ac protein levels in different plant tissues were determined by a validated enzyme linked immunosorbent assay (ELISA) using a Cry1Ac-specific antibody. Leaf, root, forage (above ground parts that are harvested for use as feed for livestock), pollen/anther and mature seed tissues of soybean MON 87701 and control A5547 conventional soybean were analysed. Leaf samples were collected at four stages (see Table 5): V3-V4 (3-4 leaf stage), V6-V8 (6-8 leaf stage), V10-V12 (10-12 leaf stage) and V14-V16 (14-16 leaf stage).

Pollen/anther was collected at the R2 (full bloom) stage, forage and root material was collected at the R6 (full seed) stage and mature seed was collected at the R8 (full maturity) stage[1].

Table 5: Tissues collected for Cry1Ac expression level analysis

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

*Harvested at or dried to a moisture content of ~10-15%.

Tissues were collected from five separate field sites in North America[2] in 2007. Three replicated plots of MON 87701 as well as the conventional soybean control were planted at each site. Cry1Ac protein levels in each tissue type were determined by ELISA.

The results (see Table 6) indicate that all parts except the roots of soybean MON 87701 were found to express the Cry1Ac protein, with leaf tissues containing the highest concentration. Seeds would be the part most likely to be used as food either directly or derived from seed by-products. The average content of Cry1Ac protein in mature seeds of soybean MON 87701 is 4.7 µg/g dry weight (range 3.4 – 5.7 µg/g).

Table 6: Summary of Cry1Ac protein levels in tissues collected from MON 87701 produced across five sites during the US 2007 growing season.

Tissue Type	Cry1Ac µg/g fwt (SD)[1,3]	Range[4] (µg/g fwt)	Cry1Ac µg/g dwt (SD)[2]	Range (µg/g dwt)	LOQ/LOD (µg/g fwt)
OSL-1	30 (8.5)	12-40	220 (70)	110-350	2.5/0.74
OSL-2	38 (16)	18-80	260 (100)	130-500	2.5/0.74
OSL-3	34 (17)	14-77	240 (110)	94-480	2.5/0.74
OSL-4	53 (36)	15-110	340 (290)	78-960	2.5/0.74
Root	< LOD	< LOD	NA[5]	NA[5]	0.4/0.347
Forage	9.0 (8.8)	2.5-32	34 (36)	8.2-140	2.0/0.55
Harvested seed	4.2 (0.73)	3.1-5.0	4.7 (0.79)	3.4-5.7	1.0/0.47
Pollen/ anther6	2.3 (0.58)	1.8-3.1	NA[7]	NA[7]	ND[8]

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

[1] For full descriptions of soybean growth stages, please see:http://www.ag.ndsu.edu/pubs/plantsci/rowcrops/a1174/a1174-2.htm

[2] Sites were located in Baldwin County (AL), Jackson County (AR), Clarke County (GA), Jackson County (IL) and Wayne County (NC).

4.4 Potential toxicity of the Cry1Ac 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.

4.4.1 *History of human consumption*

As outlined in section 2.2, the donor organism, *Bacillus thuringiensis*, is an organism that has a long history of safe use. Agricultural sprays including sporulated *B.t.* have been used for decades on crops including fruits and vegetables (which are often ingested raw). Naturally-occurring *Bacillus thuringiensis* strains, as well as residues of *B.t.*- containing sprays have been found on and in many food stuffs (Frederiksen *et al*., 2006). However, extensive testing has yet to establish harm to humans following ingestion of either of the whole organism (Baum *et al*., 1999; Betz *et al*., 2000; EPA 1998; EPA, 1998; McClintock *et al*., 1995; Mendelsohn *et al*., 2003; Siegel, 2001) or of transgenic plants containing Cry proteins (Betz *et al*., 2000; OECD 2007; Shelton *et al*., 2002). At least 11 corn or cotton crops containing Cry1Ab or Cry1Ac proteins have been approved for commercial use between 1995 and 2009 (EPA 2009) and both the Cry1Ab and Cry1Ac have received an exemption from the requirement for a tolerance from the EPA (40 CFR § 174.511 and 40 CFR § 174.510 respectively). FSANZ has approved food from corn and cotton plants including a *cry1Ab* or *cry1Ac* gene on 7 occasions between 2000 and 2006. There has been no evidence to suggest any safety concerns following consumption of Cry protein-containing crops.

4.4.2 *Structural similarity of Cry1Ac to known toxins or other proteins*

Study submitted:

Renee Girault and J. Scott McClain. Bioinformatics Evaluation of the Cry1Ac Protein Present in MON 87701 Soybean Utilizing the AD8, TOXIN6, and PROTEIN Databases. Monsanto Study Report MSL0021658.

Bioinformatic analyses are useful for assessing whether introduced proteins share any amino acid sequence similarity with known protein toxins. The Cry1Ac sequence was compared with sequences included in the TOXIN6 database (see section 3.4.5). This database consists of 7,176 sequences associated with known toxins.

The FASTA sequence alignment tool was employed, with the *E*-score set to $1x10^{-5}$. This parameter (the *E* value (Baxevanis, 2005)) represents the probability that a particular alignment is due to chance. 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.

The results from the search using the Cry1Ac protein sequence returned 15 alignments entries with *E*-values below $1x10^{-5}$. The top alignment displayed 98.981% identity with a delta-endotoxin. "Delta-endotoxin" is a synonym for the *B.t.* Cry proteins, and was not considered relevant for human toxicity. The delta endotoxin referred to shares 99% identity with sequences from *Bacillus thuringiensis* Cry1Ac protein. Thus, this homology is truly representative of a *B.t.* sequence and not of another, unrelated toxin. The 14 subsequent alignments identified similarly with other Cry proteins.

Thus none of the proteins returned from the bioinformatic alignment search with the Cry1Ac protein sequence is associated with known toxins of human health significance.

4.4.3 *Acute oral toxicity study*

An acute oral toxicity study using mice was conducted to examine the potential toxicity of the Cry1Ac protein. For this purpose, the *E. coli*-derived Cry1Ac protein was employed. The Cry1Ac from *E. coli* was shown in rigorous testing to be equivalent to plant-derived Cry1Ac (see Section 4.2).

Study submitted:

Jason W. Smedley. An Acute Toxicity Study of Cry1Ac Protein Administered by the Oral (Gavage) Route to Mice. Monsanto Study Report CRO-2007-325.

Initially, ten male and ten female mice received a dose of Cry1Ac protein administered by oral gavage of 1290 mg/kg body weight (as two doses separated by four hours). Ten male and ten female control mice were administered bovine serum albumin (BSA) at a dose of 1280 mg/kg (as two doses separated by four hours) by oral gavage.

Mice were observed for clinical signs, body weight, body weight changes and food consumption for 14 days. At the end of the study all animals were killed and examined post mortem for gross necroscopy findings.

All mice survived through the duration of the study. No clinical signs of systemic toxicity were observed. No gross lesions were present in the mice at necropsy on day 14.

A statistically significant difference in the body weight of males emerged from the study. A similar finding was not observed in female mice. In order to further investigate this finding, a further group of ten male mice were administered a higher dose of Cry1Ac (1460 mg/kg body weight), with ten control male mice being administered 1620 mg/kg body weight BSA). No evidence of a decrease in body weight was observed. Therefore this observation was not considered related to treatment with the Cry1Ac protein. These results support the conclusion that the Cry1Ac protein is not acutely toxic.

4.5 Potential allergenicity of the Cry1Ac protein

The potential allergenicity of novel proteins is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. 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; and specific

serum screening if the novel protein is derived from a source known to be allergenic or has amino acid sequence similarity with a known allergen. In some cases, such as where the novel protein has 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 Cry1Ac protein was assessed by:

- consideration of the *cry1Ac* gene source and history of use or exposure
- calculation of the Cry1Ac protein as a proportion of total protein
- bioinformatic comparison of the amino acid sequence of the Cry1Ac protein with known protein allergen sequences
- evaluation of the stability of the microbially produced and purified Cry1Ac protein from *E. coli* using *in vitro* gastric and intestinal digestion models
- Assessment of human IgE binding to MON 87701, control and conventional soybean extracts

4.5.1 *Source of protein*

The Cry1Ac protein is derived from the ubiquitous bacterium *Bacillus thuringiensis*. This bacterium and its Cry proteins are not considered to be allergens, and have a long history of safe use, both as an insecticide (since 1958) and latterly in insect-protected crops (see sections 2.2 and 4.4.1).

4.5.2 *Similarity to known allergens*

Study submitted:

Renee Girault and J. Scott McClain. Bioinformatics Evaluation of the Cry1Ac Protein Present in MON 87701 Soybean Utilizing the AD8, TOXIN6, and PROTEIN Databases. Monsanto Study Report MSL0021658.

Bioinformatic analysis provides part of a 'weight of evidence' approach for assessing potential allergenicity of novel proteins introduced to GM plants. 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 Cry1Ac with known protein toxins (see Section 4.4.2), the generation of a small *E* value provides an important indicator of significance of matches (Baxevanis, 2005; Pearson, 2000).

To determine whether the Cry1Ac protein has significant sequence identity to any proteins known or suspected to be allergens, the amino acid sequence of Cry1Ac was compared to a subset of sequences from the Food Allergy Research and Resource Program (FARRP, University of Nebraska) Allergen Database (the AD8 database (see section 3.4.5). This database contains the amino acid sequences of known and putative allergenic proteins (www.allergenonline.com/about.asp) using established criteria (Codex Alimentarius, 2003). Potential similarities between the introduced protein in soybean MON 87701 and proteins in the allergen database were evaluated using the FASTA sequence alignment program. Alignments were inspected for identities greater than or equal to 35% over 80 or greater residues. The Cry1Ac protein was also evaluated for any eight or greater contiguous identical amino acid matches to entries in the AD8 database. These two approaches aim to identify both short contiguous regions of identity that could potentially correspond to shared IgE binding epitopes, as well as longer stretches of sequence similarity that may infer a potential cross-reactive protein structure.

None of the proteins in the AD8 database exceeded the 35% threshold over 80 or greater amino acids. In addition, further sequences were examined based on their *E* score. No sequence had an *E*-score less than 1. Furthermore, there were no eight or greater contiguous identical amino acid stretches in common between the Cry1Ac protein sequence and any of the protein sequences in the AD8 database. The results indicate that the Cry1Ac protein does not show significant sequence identity with known allergens.

4.5.3 *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; Kimber *et al*., 1999; Metcalfe *et al*., 1996). Therefore a correlation exists between resistance to digestion by pepsin and potential allergenicity. 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 a response.

A pepsin digestibility assay was conducted to determine the digestive stability of the Cry1Ac protein. In addition to the pepsin protocol using simulated gastric fluid (SGF), a second digestibility study was done using simulated intestinal fluid (SIF) containing pancreatin, which is a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. A sequential study of digestion with SGF and SIF was also carried out.

Study submitted:

Brian E. Goertz, Erin Bell and Elena A. Rice. Assessment of the *In Vitro* Digestibility of the Cry1Ac Protein in Simulated Gastric and Simulated Intestinal Fluids. Monsanto Study Report MSL0021376.

4.5.3.1 Simulated gastric fluid (SGF) study

The *in vitro* digestibility of the *E. coli*-derived Cry1Ac protein in SGF containing pepsin at pH ~1.2 (Thomas *et al*., 2004)) was evaluated by colloidal Brilliant Blue G-stained SDS-PAGE and Western blot. Digestibility of the protein in SGF was measured by incubating samples at 36.6ºC for selected times (0, 0.5, 2, 5, 10, 20, 30 and 60 minutes) and subjecting these to SDS-PAGE. Protein was visualised by staining the gel with colloidal Brilliant Blue G or by transfer to a nitrocellulose membrane and incubation with an anti-Cry1Ac antibody. The SGF was pre-tested with acidified haemoglobin to determine that its activity was within the normal range.

Within 30 seconds of exposure to the SGF, the full-length Cry1Ac protein became visually indistinguishable in both the protein gel and the Western blot. Given the known limits of detection of the proteins, this corresponds to a digestion rate of at least 99% and at least 95% respectively. However, a small (~4 kDa) band was visible and stable after digestion with SGF. N-terminal sequencing of this band revealed it to be a mixture of two Cry1Ac peptides, one from AA 415-424 and the other from AA 882-896.

These results suggest that the Cry1Ac protein is rapidly digested in SGF, but that two small peptides (of 10 and 15 AA) remain undigested over the course of the incubation.

4.5.3.2 Simulated intestinal fluid (SIF) study

The digestibility of *E. coli*-derived Cry1Ac protein in SIF containing pancreatin (Sigma, MO) was assessed using SDS-PAGE. Digestibility of the protein in SIF was measured by incubating samples with SIF containing porcine pancreatin for specified time intervals (0, 5, 15, 30 and 1 hour, 2 hours, 4 hours, 8 hours, 24 hours), and analysing by Western blot. The activity of the SIF was confirmed prior to Cry1Ac analysis using resorufin-labeled casein.

Within five minutes the full-length Cry1Ac protein was undetectable by Western blot. However, a smaller Cry1Ac fragment (~55 kDa) was identifiable and stable for the entire incubation period. This corresponds to the trypsin-resistant core of the Cry1Ac protein, which is biologically active in the target insect species. This result is of little significance, however, because the study detailed in section 4.5.3.1 demonstrates that the Cry1Ac protein is digested down to fragments no larger than 4 kDa when subjected to SGF. Thus, this trypsin-resistant core is already digested before it encounters the SIF.

4.5.3.3 Digestibility of the Cry1Ac protein in SGF followed by SIF

To determine if the entire Cry1Ac protein is digested in a normal digestive process, the Cry1Ac protein was subjected to sequential SGF and SIF digestion. For this experiment, Cry1Ac protein was incubated in SGF for two time points (0 minutes, 2 minutes) and then quenched with 0.7 M sodium carbonate buffer. This mixture was then added to pre-heated SIF and incubated for 0, 0.5, 2, 5, 10, 30, 60 or 120 minutes. The samples were then analysed by SDS-PAGE and Western blot.

As expected, the Cry1Ac protein was digested to small fragments (~4 kDa) within 2 minutes of incubation with SGF. The full-lengthCry1Ac protein was visually indistinguishable in the colloidal Brilliant Blue G-stained gel at this time point. Subsequent incubation with SIF demonstrated the digestion of the remaining Cry1Ac fragments below the limit of detection. Western blot analysis confirmed the digestion of the full-length Cry1Ac protein below the limit of detection within the first 2 minutes of incubation with SGF.

4.6 Conclusion from characterisation of novel protein

Soybean MON 87701 expresses one novel protein Cry1Ac. The Cry1Ac protein was derived from the ubiquitous bacterium, *Bacillus thuringiensis*, which has a long history of safe use and consumption. The Cry1Ac protein is expressed at low levels in the seed (0.0012% of total protein).

Extensive studies have been done to confirm the identity and physicochemical and functional properties of the expressed Cry1Ac protein, as well as to determine its potential toxicity and allergenicity. The protein conforms in size and amino acid sequence to that expected and demonstrates the predicted insecticidal activity. It does not exhibit any glycosylation.

Bioinformatic studies with the Cry1Ac protein have confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that the protein would be rapidly degraded following ingestion, similar to other dietary proteins. An acute oral toxicity study in mice with the Cry1Ac has also confirmed the absence of toxicity. Taken together, the evidence indicates that Cry1Ac protein is neither toxic, nor likely to be allergenic, in humans.

5 COMPOSITIONAL ANALYSES

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 such 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).

5.1 Key components

For soybean there are a number of components that are considered to be important for compositional analysis (EuropaBio, 2003; 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 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.

5.2 Study design and conduct for key components

Studies submitted:

Kristina H. Berman, Susan G. Riordan, Michelle N. Smith and Roy Sorbet. Compositional Analyses of Forage and Seed Collected from MON 87701 Grown in United States during 2007. Monsanto Study Report MSL0021413.

Ideally, the comparator in compositional analyses should be the near isogenic parental line grown under identical conditions (OECD, 2001). In the case of soybean MON 87701, the control was the parental line A5547. These, along with 20 unique commercial conventional soybean varieties were grown in 2007 at five field locations in North America representative of the range of growing regions for soybean[3]. Plants were grown in a randomised complete block design comprising three replicates per block. The reference cultivars were planted, harvested, processed and analysed using the same methods as used for soybean MON 87701 and the parental control line Any statistically significant differences between soybean MON 87701 and the control could be compared to the reference range to assess whether the differences were likely to be biologically meaningful.

Data from the commercial cultivars were used to calculate population tolerance intervals for each compositional component. Tolerance intervals are expected to contain, with 95% confidence, 99% of the values contained in the population of commercial lines. The population tolerance interval, together with the combined range of values for each analyte available from the published literature, (International Life Sciences Institute, 2003; Kim *et al*., 2005; OECD, 2001; Taylor *et al*., 1999), were used to interpret the compositional data for soybean MON 87701. Any mean value for a soybean MON 87701 analyte that fell within the tolerance interval and/or the combined literature range was considered to be within the normal variability of commercial soybean cultivars even if the mean value was statistically different from the parental conventional soybean control.

Statistical evaluation of the compositional data, using a standard computer programme, compared the forage and seed from the soybean MON 87701 population to the parental conventional soybean control population. Data were analysed using a linear mixed model analysis of variance. The five replicated sites were analysed both separately and combined.

In assessing the significance of any difference between the mean analyte value for soybean MON 87701 and the parental control, a P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

For those comparisons in which the soybean MON 87701 test result was statistically different from the control, the test mean was compared to the 99% tolerance interval derived from the commercial cultivars.

5.3 Analyses of key components

Although the Applicant provided results for the compositional analyses of forage, the focus of this assessment is necessarily on the food uses of soybean and therefore the forage data are not presented in this report. In short, no significant differences were found between MON 87701 and the conventional soybean control in the combined site analysis. Compositional analyses of the soybean seed included proximates (protein, fat, carbohydrates, moisture and

[3] Sites were located in Baldwin County, (AL), Jackson County, (AR), Clarke County (GA), Jackson County (IL) and Wayne County (NC).

ash), acid detergent fibre (ADF), neutral detergent fibre (NDF), fatty acids, amino acids, isoflavones, anti-nutrients (stachyose, raffinose, lectins, phytic acid and trypsin inhibitor), isoflavones (daidzein, genistein and glycitein), minerals and vitamin E.

5.3.1 *Proximates and fibre*

Results of the proximate and fibre analysis of soybean MON 87701 seed are shown in Table 7. Methods for the analysis of proximates and fibre are published in the literature - Ash: (AOAC, 2000a), Carbohydrates: (USDA, 1973), Fat: (AOAC, 2000c) moisture: (AOAC, 2000b), Protein: (Foss-Tecator, 1999), Acid Detergent Fibre/ Neutral Detergent Fibre: (Ankom Technology, 1999).

In the combined site analysis, statistically significant differences between soybean MON 87701 and the parental control (p-value <0.05) were observed for carbohydrates (34.22%, p=0.037) and protein (39.27%, p=0.023). The commercial tolerance interval for these proximates was 28.17 – 40.99 (carbohydrates) and 35.30 – 45.38 (protein). The mean value for soybean MON 87701 for each of these analytes was within the statistical tolerance intervals for commercial soybean cultivars and the ranges reported in the literature (International Life Sciences Institute 2003; Taylor *et al*., 1999). No statistically significant differences were observed between soybean MON 87701 and the control mean values for fat, moisture, ash, ADF or NDF.

Proximates found to have statistically significant differences in one or more (but not all) sites were: ash (one site): 5.42%, p=0.039 (commercial tolerance interval: 3.74 – 6.45) and carbohydrates: 36.65%, p=0.024 (commercial tolerance interval: 28.17 – 40.99).

Table 7: Statistical summary of combined-site soybean seed proximate and fibre content for MON 87701 vs the conventional control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Interval[2]]
			Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	
Proximate (% DW, unless noted)						
Ash	5.20 (0.18)	5.14 (0.18)	0.054 (0.043)	-0.046, 0.15	0.246	(4.32 - 5.62)
	[4.70 - 5.90]	[4.70 - 5.88]	[-0.14 - 0.21]			[3.74, 6.45]
Carbohydrates	34.22 (1.50)	36.44 (1.50)	-2.22 (1.02)	-4.31, -0.14	0.037	(31.97 - 38.00)
	[21.58 - 39.61]	[29.88 - 43.48]	[-15.00 - 2.56]			[28.17, 40.99]
Moisture (%FW)	7.52 (0.38)	6.84 (0.38)	0.68 (0.47)	-0.28, 1.64	0.159	(5.48 - 11.70)
	[5.86 - 10.70]	[5.44 - 8.74]	[-0.96 - 4.70]			[1.45, 12.81]
Protein	39.27 (0.86)	37.80 (0.86)	1.46 (0.54)	0.24, 2.68	0.023	(38.14 - 42.66)
	[36.49 - 42.23]	[32.29 - 41.87]	[-0.73 - 6.49]			[35.30, 45.38]
Total Fat	20.29 (0.78)	20.12 (0.77)	0.17 (0.39)	-0.71, 1.05	0.670	(17.90 - 23.56)
	[17.33 - 23.08]	[17.24 - 22.55]	[-1.82 - 1.98]			[14.74, 25.18]
Fibre (%DW)						
Acid detergent fibre	15.58 (0.49)	15.62 (0.49)	-0.042 (0.58)	-1.37, 1.28	0.943	(12.79 - 17.98)
	[13.53 - 17.05]	[14.00 - 19.02]	[-2.84 - 1.88]			[0.30, 0.67]
Neutral detergent fibre	17.33 (0.70)	17.28 (0.70)	0.057 (0.74)	-1.67, 1.78	0.940	(13.32 - 23.57)
	[15.06 - 21.80]	[15.02 - 22.45]	[-6.43 - 4.47]			[7.24, 28.70]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval.
[2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero. Statistically significant differences between MON 87701 and the control are highlighted in green.

5.3.2 *Fatty Acids*

The levels of 23 fatty acids in soybean MON 87701 and parental control seed were measured. Methods for measuring fatty acids followed AOAC 2000.

No data are presented for 9 fatty acids[4] that were below the limit of quantitation. Results of the analysis for the remaining fatty acids are given in Table 8. In the combined site analysis, one fatty acid was significantly different from the parental control: 22:0 behenic acid (0.56% total FA), p=0.022. However, this fatty acid was within the commercial tolerance interval (0.30 – 0.67). This fatty acid was also found to be significantly different from the parental control in more than one individual site: site AR: 0.47% total FA, p=0.037; site GA: 0.60% total FA, p=0.029. Both of these findings fall within the commercial tolerance interval.

Six fatty acids were significantly different from the parental control in one site: 16:0 palmitic acid (11.53% total FA, p=0.025); 16:1 palmitoleic acid (0.086%, p=0.012); 18:0 stearic acid (4.42% total FA, p=0.038; 18:1 oleic acid (19.78% total FA, p=0.047); 18:2 linoleic acid (54.21% total FA, p=0.046); 20:1 eicosenoic acid (0.24% total FA, p=0.035). All of these values fell within their appropriate commercial tolerance intervals.

[4] Lauric acid, caprylic acid, myristoleic acid, pentadecanoic acid (C15:0), pentadecanoic acid (C15:1), gamma linolenic acid, arachidonic acid, erucic acid.

Table 8: Statistical summary of combined-site soybean seed fatty acid content for MON 87701 vs the conventional control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Interval[2]]
			Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	
Fatty Acid (% DW)						
10:0 Capric Acid	0.20 (0.014)	0.21 (0.014)	-0.010 (0.020)	-0.053, 0.032	0.607	(0.15 - 0.27)
	[0.14 - 0.25]	[0.16 - 0.26]	[-0.11 - 0.048]			[0.065, 0.34]
14:0 Myristic Acid	0.093 (0.0031)	0.094 (0.0031)	-0.00056 (0.0019)	-0.0048, 0.0037	0.769	(0.064 - 0.097)
	[0.082 - 0.10]	[0.083 - 0.11]	[-0.0085 - 0.0025]			[0.052, 0.12]
16:0 Palmitic Acid	11.80 (0.12)	11.88 (0.12)	-0.079 (0.081)	-0.27, 0.11	0.359	(9.80 - 12.38)
	[11.32 - 12.30]	[11.50 - 12.13]	[-0.72 - 0.40]			[8.88, 13.53]
16:1 Palmitoleic Acid	0.092 (0.0033)	0.095 (0.0033)	-0.0028 (0.0029)	-0.0097, 0.0041	0.372	(0.073 - 0.14)
	[0.073 - 0.11]	[0.078 - 0.11]	[-0.018 - 0.015]			[0.037, 0.15]
17:0 Heptadecanoic Acid	0.094 (0.0021)	0.093 (0.0021)	0.0011 (0.0018)	-0.0030, 0.0052	0.553	(0.076 - 0.10)
	[0.084 - 0.10]	[0.082 - 0.099]	[-0.0064 - 0.0074]			[0.066, 0.11]
17:1 Heptadecenoic Acid	0.041 (0.0032)	0.041 (0.0032)	-0.00009 (0.0040)	-0.0092, 0.0090	0.981	(0.020 - 0.064)
	[0.023 - 0.048]	[0.019 - 0.047]	[-0.020 - 0.022]			[0.0058, 0.083]
18:0 Stearic Acid	4.59 (0.22)	4.70 (0.22)	-0.12 (0.11)	-0.38, 0.14	0.328	(3.21 - 5.24)
	[3.97 - 5.36]	[4.03 - 5.36]	[-0.57 - 0.29]			[1.88, 6.25]
18:1 Oleic Acid	22.35 (1.28)	22.71 (1.28)	-0.36 (0.49)	-1.51, 0.79	0.486	(16.69 - 35.16)
	[19.21 - 26.64]	[20.34 - 28.78]	[-3.16 - 2.04]			[5.01, 42.01]
18:2 Linoleic Acid	52.16 (0.95)	51.76 (0.95)	0.40 (0.38)	-0.48, 1.29	0.320	(44.17 - 57.72)
	[49.32 - 54.63]	[47.18 - 54.07]	[-1.35 - 2.68]			[38.57, 66.94]
18:3 Linolenic Acid	7.24 (0.45)	7.11 (0.45)	0.13 (0.12)	-0.13, 0.40	0.276	(4.27 - 8.81)
	[5.55 - 8.41]	[5.34 - 8.26]	[-0.40 - 0.68]			[2.69, 10.81]
20:0 Arachidic Acid	0.51 (0.025)	0.51 (0.025)	-0.0027 (0.013)	-0.032, 0.026	0.836	(0.36 - 0.55)
	[0.41 - 0.58]	[0.41 - 0.57]	[-0.044 - 0.047]			[0.23, 0.64]
20:1 Eicosenoic Acid	0.24 (0.012)	0.23 (0.012)	0.0044 (0.010)	-0.020, 0.029	0.683	(0.21 - 0.30)
	[0.19 - 0.28]	[0.18 - 0.28]	[-0.065 - 0.046]			[0.16, 0.33]
20:2 Eicosadienoic Acid	0.040 (0.0030)	0.042 (0.0030)	-0.0024 (0.0042)	-0.012, 0.0068	0.585	(0.016 - 0.054
	[0.020 - 0.054]	[0.020 - 0.047]	[-0.024 - 0.011]			[0.0029, 0.083]
22:0 Behenic Acid	0.56 (0.028)	0.54 (0.028)	0.023 (0.0084)	0.0041, 0.042	0.022	(0.38 - 0.59)
	[0.46 - 0.65]	[0.45 - 0.65]	[-0.00071 - 0.078]			[0.30, 0.67]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval. [2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero. Statistically significant differences between MON 87701 and the control are highlighted in green.

5.3.3 *Amino acids*

Levels of 18 amino acids were measured in seed from soybean MON 87701 and the parental control. 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. The method for determining amino acid levels followed standard procedures (Hong, 1994; Tagers and Pesti, 1990; Waters Corporation 2009).

Nine amino acids in MON 87701 were found in the combined sites analysis to be significantly different from the parental control (see Table 9). These were: alanine (1.72%, p=0.027), glycine (1.75%, p=0.007), histidine (1.12%, p=<0.0001), isoleucine (1.81%, p=0.031), leucine (3.04%, p=0.046), lysine (2.74%, p=0.012), serine (2.03%, p=0.004), threonine (1.60%, p=0.024) and valine (1.92%, p=0.040). All of these values were within their commercial tolerance intervals.

Three amino acids showed statistically significant difference from the parental control in more than one site. These were: arginine (site GA: 2.80%, p=0.011; site IL: 2.61%, p=0.045), histidine (site GA: 1.15%, p=0.019; site IL: 1.11%, p=0.036), tyrosine (site AL: 1.32%, p=0.034; site IL: 1.10%, p=0.003). Again, all of these values fall within the commercial tolerance intervals.

Five amino acids differed significantly from the parental control in one site only. These were isoleucine (1.81%, p=0.029), leucine (3.04%, p=0.014), proline (2.00%, p=0.025), tryptophan (0.49%, p=0.006) and valine (1.91%, p=0.035). These values were within their commercial tolerance intervals.

Table 9: Statistical summary of combined-site soybean seed amino acid content for MON 87701 vs the conventional control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Interval[2]]
			Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	
Amino Acid (% DW)						
Alanine	1.72 (0.029)	1.69 (0.029)	0.036 (0.016)	0.0044, 0.068	0.027	(1.66 - 1.93)
	[1.66 - 1.84]	[1.59 - 1.82]	[-0.034 - 0.099]			[1.49, 2.02]
Arginine	2.68 (0.069)	2.58 (0.069)	0.096 (0.058)	-0.039, 0.23	0.138	(2.54 - 2.99)
	[2.36 - 3.00]	[2.37 - 2.89]	[-0.16 - 0.31]			[2.22, 3.25]
Aspartic Acid	4.90 (0.10)	4.85 (0.10)	0.053 (0.055)	-0.059, 0.17	0.339	(4.74 - 5.50)
	[4.61 - 5.26]	[4.46 - 5.34]	[-0.23 - 0.31]			[4.22, 5.96]
Cystine	0.62 (0.014)	0.61 (0.014)	0.0051 (0.014)	-0.024, 0.034	0.718	(0.53 - 0.68)
	[0.57 - 0.67]	[0.56 - 0.69]	[-0.11 - 0.066]			[0.45, 0.77]
Glutamic Acid	7.65 (0.15)	7.53 (0.15)	0.12 (0.084)	-0.056, 0.29	0.177	(7.53 - 8.72)
	[7.25 - 8.21]	[6.89 - 8.26]	[-0.29 - 0.46]			[6.60, 9.37]
Glycine	1.75 (0.026)	1.70 (0.026)	0.049 (0.017)	0.014, 0.083	0.007	(1.67 - 1.99)
	[1.63 - 1.89]	[1.64 - 1.85]	[-0.0052 - 0.12]			[1.49, 2.09]
Histidine	1.12 (0.015)	1.08 (0.015)	0.043 (0.011)	0.021, 0.064	<0.001	(1.04 - 1.24)
	[1.05 - 1.18]	[1.03 - 1.15]	[-0.00077 - 0.090]			[0.94, 1.31]
Isoleucine	1.81 (0.037)	1.76 (0.037)	0.052 (0.020)	0.0061, 0.098	0.031	(1.73 - 2.02)
	[1.68 - 1.99]	[1.64 - 1.96]	[-0.044 - 0.12]			[1.54, 2.14]
Leucine	3.04 (0.066)	2.94 (0.066)	0.095 (0.040)	0.0018, 0.19	0.046	(2.93 - 3.32)
	[2.82 - 3.36]	[2.73 - 3.29]	[-0.044 - 0.23]			[2.64, 3.52]
Lysine	2.74 (0.060)	2.62 (0.060)	0.12 (0.046)	0.028, 0.21	0.012	(2.35 - 3.15)
	[2.48 - 2.99]	[2.42 - 2.91]	[-0.12 - 0.39]			[2.05, 3.47]
Methionine	0.53 (0.012)	0.53 (0.012)	0.0043 (0.014)	-0.023, 0.032	0.754	(0.49 - 0.62)
	[0.48 - 0.58]	[0.47 - 0.59]	[-0.094 - 0.080]			[0.42, 0.68]
Phenylalanine	2.15 (0.056)	2.04 (0.056)	0.11 (0.052)	-0.013, 0.23	0.073	(1.97 - 2.44)
	[1.91 - 2.48]	[1.91 - 2.38]	[-0.036 - 0.41]			[1.66, 2.64]
Proline	2.01 (0.035)	1.96 (0.035)	0.042 (0.021)	-0.0069, 0.091	0.082	(1.92 - 2.25)
	[1.86 - 2.16]	[1.85 - 2.12]	[-0.058 - 0.11]			[1.73, 2.35]
Serine	2.03 (0.032)	1.96 (0.032)	0.060 (0.019)	0.020, 0.10	0.004	(1.96 - 2.30)
	[1.90 - 2.19]	[1.87 - 2.13]	[0.010 - 0.14]			[1.75, 2.38]
Threonine	1.60 (0.020)	1.55 (0.020)	0.046 (0.016)	0.0078, 0.084	0.024	(1.54 - 1.74)
	[1.50 - 1.72]	[1.49 - 1.68]	[-0.016 - 0.13]			[1.40, 1.83]
Tryptophan	0.51 (0.0068)	0.50 (0.0068)	0.011 (0.0067)	-0.0024, 0.025	0.102	(0.47 - 0.55)
	[0.47 - 0.54]	[0.46 - 0.53]	[-0.039 - 0.075]			[0.43, 0.59]

Tyrosine	1.13 (0.034)	1.10 (0.034)	0.039 (0.029)	-0.028, 0.11	0.213	(1.04 - 1.31)
	[0.96 - 1.33]	[0.98 - 1.22]	[-0.11 - 0.25]			[0.85, 1.48]
Valine	1.92 (0.032)	1.86 (0.032)	0.053 (0.022)	0.0029, 0.10	0.040	(1.83 - 2.13)
	1.80 - 2.07]	[1.76 - 2.04]	[-0.033 - 0.12]			[1.64, 2.22]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval. [2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero. Statistically significant differences between MON 87701 and the control are highlighted in green.

5.3.4 *Isoflavones*

Three isoflavones were measure in soybean MON 87701: the three aglycons genistin, daidzein and glycitein. The method for measuring their quantities followed standard procedures (Pettersson and Kiessling, 1984). The results for isoflavone content are presented in Table 10.

One isoflavone showed a significant difference in the combined site analysis from the parental control. This was daidzein (667.54 mg/kg, p=0.040). This was within the commercial tolerance interval of 0 – 1585.14. The same isoflavone showed significant differences in more than one individual site: site AR (767.90 mg/kg, p=0.031; site EL (890.96 mg/kg, p=0.042). Again, these values are within the commercial tolerance interval.
One isoflavone showed a significant difference in one site only. This was genistin (807.35 mg/kg, p=0.007). This was within the commercial tolerance interval of 0 – 1352.86.

Table 10: Statistical summary of combined-site soybean seed isoflavone content for MON 87701 vs the conventional control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Interval[2]]
			Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	
Isoflavones (% DW)						
Daidzein	667.54 (108.30)	604.88 (108.30)	62.65 (25.68)	3.56, 121.74	0.040	(213.98 - 1273.94)
	[188.96 - 983.26]	[198.95 - 830.65]	[-27.87 - 178.54]			[0, 1585.14]
Genistin	655.57 (88.52)	594.58 (88.52)	60.99 (36.27)	-23.10, 145.09	0.132	(148.06 - 1024.50)
	[214.73 - 863.84]	[244.95 - 760.87]	[-30.22 - 178.22]			[0, 1352.86]
Glycitein	164.87 (21.23)	156.93 (21.23)	7.94 (13.22)	-22.57, 38.44	0.564	(32.42 - 208.45)
	[61.08 - 228.79]	[61.28 - 227.25]	[-49.56 - 88.71]			[0, 272.12]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval. [2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero. Statistically significant differences between MON 87701 and the control are highlighted in green.

5.3.5 *Anti-nutrients*

Levels of five key anti-nutrients in seeds from soybean MON 87701 and the control were tested using standards protocols: trypsin inhibitor:(Anonymous, 1997); phytic acid:(Anonymous, 1988); lectins:(Leiner, 1954), stachyose/raffinose: (Anonymous, 1985; Johansen *et al*., 1996). One anti-nutrient, trypsin inhibitor, was found in the combined analysis to be significantly different from its parental control (26.06 TIU/mg DW, p=0.014) (see Table 11). This value was within the commercial tolerance interval of 13.58-46.02. One antinutrient, stachyose, was found to be significantly different from the parental control in more than one individual site (site AL: 1.84%, p=0.024; site NC: 4.56, p=0.006). Both of these levels are within the commercial tolerance interval. One anti-nutrient, trypsin inhibitor, was found to be significantly different from the parental control at one site only (23.28 TIU/mg DW, p=0.005). This value was within the commercial tolerance interval of 13.58-46.02.

Table 11: Statistical summary of combined-site soybean seed antinutrient content for MON 87701 vs the conventional control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Interval[2]]
			Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	
Antinutrient (%DW, unless noted)						
Lectin (H.U./mg FW)	0.96 (0.19)	0.72 (0.19)	0.24 (0.25)	-0.27, 0.74	0.354	(0.090 - 2.47)
	[0.062 - 2.01]	[0.28 - 1.28]	[-0.88 - 1.42]			[0, 3.40]
Phytic Acid	1.85 (0.12)	1.97 (0.12)	-0.11 (0.097)	-0.34, 0.11	0.276	(1.10 - 2.32)
	[1.39 - 2.29]	[1.31 - 2.66]	[-0.53 - 0.31]			[0.54, 3.05]
Raffinose	1.33 (0.19)	1.34 (0.19)	-0.0086 (0.074)	-0.18, 0.16	0.910	(0.52 - 1.62)
	[0.49 - 1.70]	[0.43 - 1.85]	[-0.32 - 0.19]			[0.038, 2.24]
Stachyose	4.59 (0.63)	4.93 (0.63)	-0.34 (0.22)	-0.83, 0.16	0.156	(1.97 - 5.55)
	[1.83 - 6.42]	[2.27 - 6.65]	[-1.00 - 0.73]			[0.99, 7.93]
Trypsin Inhibitor (TIU/mg DW)	26.06 (1.24)	28.57 (1.24)	-2.51 (0.96)	-4.48, -0.54	0.014	(20.84 - 37.24)
	[61.08 - 228.79]	[61.28 - 227.25]	[-49.56 - 88.71]			[13.58, 46.02]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval. [2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero. Statistically significant differences between MON 87701 and the control are highlighted in green.

5.3.6 *Vitamins*

Vitamin analysis was done only for Vitamin E (see Table 12). Levels of Vitamin E were significantly higher in seed of soybean MON 87701 than in the control (7.69 mg/100g DW. P=<0.001). This level was within the commercial tolerance interval of 0 – 11.09 mg/100g DW. Vitamin E was also significantly higher in four sites: site AR (6.88 mg/100g DW, p=<0.001; site GA (9.16 mg/100g DW, p=0.011; site IL (6.72 mg/100g DW, p=<0.001); site NC (7.83 mg/100g DW, p=0.017). All these levels are within the commercial tolerance interval.

Table 12: summary of combined-site soybean seed vitamin E content for MON 87701 vs the conventional control (A5547)

Component (Units)[1]	MON 87701 Mean (S.E.) [Range]	A5547 Mean (S.E.) [Range]	Difference (Test minus Control)			Commercial (Range) [99% Tolerance Interval[2]]
			Mean (S.E.) [Range]	95% CI (Lower, Upper)	p-Value	
Vitamin E (mg/100g DW)						
Vitamin E	7.69 (0.52)	6.24 (0.52)	1.45 (0.27)	0.81, 2.09	<0.001	(1.65 - 8.08)
	6.36 - 9.62]	[4.88 - 7.94]	[0.57 - 2.25]			[0, 11.09]

[1]DW = dry weight; FW = fresh weight; FA = fatty acid; S.E. = standard error; CI = Confidence Interval. [2]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero. Statistically significant differences between MON 87701 and the control are highlighted in green.

5.3.7 *Further studies*

Since submission of the application to FSANZ, the compositional analyses presented above were published in a peer-reviewed journal, the Journal of Agricultural and Food Chemistry (Berman *et al*., 2009). In addition to the analysis of forage and seed, Berman *et al* analysed the composition of several additional products relevant for human consumption: processed fractions; toasted, defatted meal; refined, bleached and defatted oil, protein isolate and lecithin.

MON 87701 and the control were planted in the 2007 growing season in the USA (as described above) as well as in three replicated sites in Argentina (Tacuari, Gahan and Berdier) during the 2007/2008 growing season.

5.3.7.1 Processed fractions

In the analysis of processed fractions from US-grown soybeans, 3 out of 47 comparisons showed significant differences from the control. These were tyrosine, 16:0 palmitic acid and 20:1 eicosenoic acid. All three values fell within the range given in the literature (International Life Sciences Institute 2003). No statistically significant difference between MON 87701 and the control soybean were observed in the Argentinean trial. These data suggest that the processed fractions of soybean MON 87701 are substantially equivalent to processed fraction of conventional soybean.

5.3.7.2 Toasted, defatted meal

Acid detergent fibre (ADF), ash and four amino acids were found to be significantly different from the control. ADF was also outside the range given in the literature (mean= 4.25% DW, literature range 5.2%-6.7%), although this difference is relatively small (15.7%). There was

no significant difference in neutral detergent fibre (NDF) levels between MON 87701 and the control, despite both being outside the literature range.

Of the four amino acids that showed significant differences from the control, none was outside the range given in the literature. These data suggest that toasted, defatted meal from soybean MON 87701 is substantially equivalent to meal from conventional soybean.

5.3.7.3 Refined, bleached and defatted (RBD) oil

Of the thirteen parameters tested in MON 87701 RBD oil, three were significantly different from the control. These were 20:1 eicosenoic acid, 22:0 behenic acid and vitamin E. All three parameters were within the range provided by the literature (Codex, 2001). These data suggest that the RBD oil from MON 87701 is substantially equivalent to RBD oil from conventional soybean.

5.3.7.4 Protein isolate

No statistically significant differences were observed between MON 87701 and the control for the 19 tested parameters. Interestingly, all but three of the components measured fell outside of the range in the literature. However, because of the similarity in the parameters between MON 87701 and the control, the deviation from the literature must have been a result of the growing conditions and cannot be attributed to the genetic modification. As such, protein isolate from MON 87701 is substantially equivalent to protein isolate from conventional soybean.

5.3.7.5 Lecithin

There were no significant differences between MON 87701 and the control in terms of phosphatides. Two of the four phosphatides fell outside the range in the literature. As all phosphatides were present in similar levels in MON 87701 and the conventional control, the deviation from the levels presented in the literature must result from factors other than the genetic modification. Therefore lecithin from MON 87701 is substantially equivalent to lecithin from conventional soybean.

5.4 Assessment of human IgE binding to MON 87701, control and conventional soybean extracts

Soybean is a food that is known to be allergenic to some people. In order to determine if MON 87701 has endogenous allergens at levels at, above or below those of conventional soybean, a study into IgE binding was carried out.

Study submitted:

Scott McClain, Elena Rice, Chen Meng and Gary Bannon. Quantitative ELISA Assessment of Human IgE Binding to MON 87701, Control, and Reference Soybean Using Sera from Soybean-Allergic Subjects. Monsanto Study Report MSL0022043.

Sera from 13 clinically documented, soybean-allergic and five non-allergic subjects were gathered. The soybean-allergic subjects had a documented case history of soybean allergy with anaphylaxis and a positive double-blind, placebo-controlled food challenge response.

Aqueous extracts from ground MON 87701 soybeans, conventional soybeans (the A5547 parental line) and 17 reference variety soybeans were prepared. A validated ELISA was

used to determine soybean-specific IgE binding. The results were compared with a soybean-specific IgE standard curve.

The 17 reference varieties established the lower and upper tolerance limits for 99% tolerance intervals with 95% confidence for each serum. The IgE binding of MON 87701 and the parental conventional soybean A5547 were compared directly (see Figure 6).

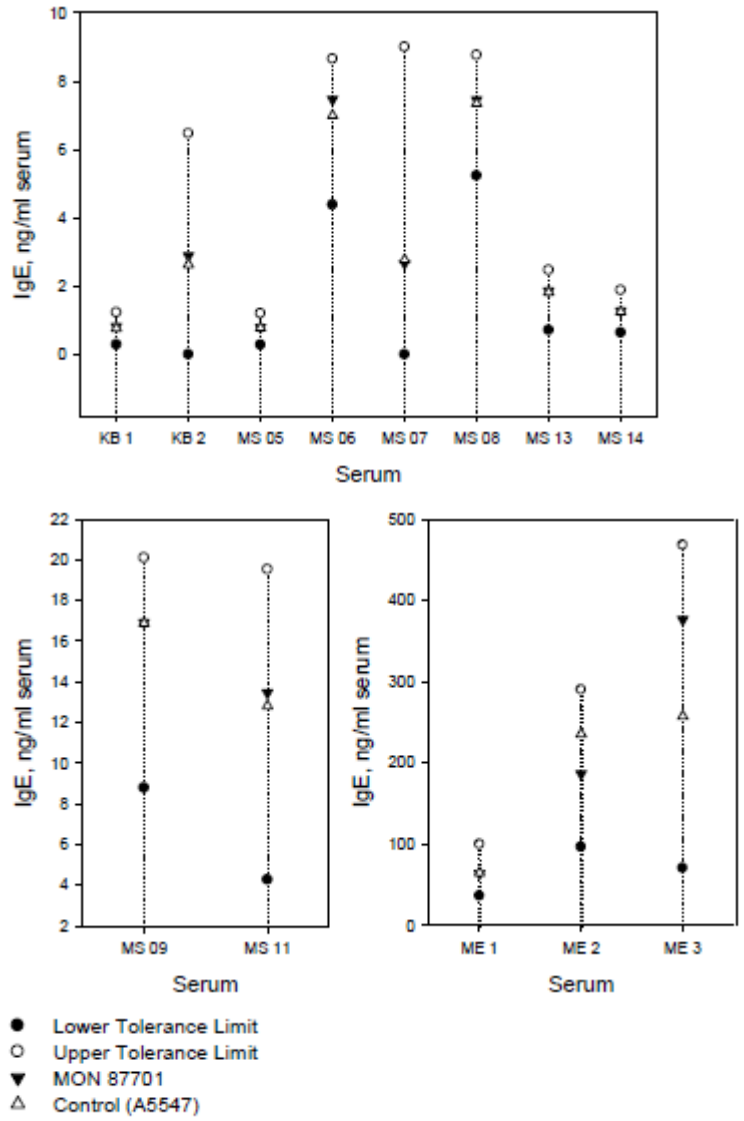

Figure 6: Serum IgE binding values for MON 87701, conventional control (A5547) and the tolerance limits for 17 conventional references

All of the IgE binding to MON 87701 and its parental conventional control were within the reference tolerance limits established for that subject's serum. No binding was seen with any of the non-allergic controls.

These data show that soybean-specific IgE binding to MON 87701 is comparable with that of conventional soybean and within the tolerance limits for 17 soybean varieties.

5.5 Conclusion from compositional studies

Detailed compositional analyses were done to establish the nutritional adequacy of seed from soybean MON 87701 and to characterise any unintended compositional changes. Analyses were done of proximates (ash, carbohydrates, moisture, protein and fat), fibre, amino acids, fatty acids, vitamin E, anti-nutrients and isoflavones. The levels were compared to levels in the non-GM parent and to those in twenty non-GM commercial soybean cultivars. Fifteen analytes in the combined site analysis showed differences between MON 87701 and the parental control. However, each of the mean values generated were within the tolerance interval determined for non-GM commercial cultivars. Because all the analyte levels in soybean MON 87701 fall within the biological range found in non-GM commercial soybeans, it can be concluded that seed from MON 87701 is compositionally equivalent to conventional soybeans.

6. 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.

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).

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. This assessment should include consideration of the bioavailability of the modified nutrient.

In this case, soybean MON 87701 is the result of a simple genetic modification to confer insect protection with no intention to significantly alter nutritional parameters in the food. In addition, extensive compositional analyses have been undertaken of soybean MON 87701 and these indicate it is equivalent in composition to conventional soybean varieties. The introduction of soybean MON 87701 into the food supply would therefore be expected to have little nutritional impact.

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