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

Supporting Document 1

Safety Assessment Report

Application A1066

Food derived from Herbicide-tolerant Corn Line MON87427

Summary and Conclusions

Background

Monsanto Company (Monsanto) has developed a genetically modified (GM) corn line known as MON87427 that has tissue-selective tolerance to the herbicide glyphosate. A gene cassette has been incorporated into the line that contains the *cp4epsps* gene from *Agrobacterium* sp. under the control of genetic elements that drive expression in all tissue but pollen. This means that pollen development can be prevented by application of glyphosate, thus allowing glyphosate-treated MON87427 lines to serve as a male sterile female parent in the production of hybrid seed.

In conducting a safety assessment of food derived from herbicide-tolerant corn line MON87427, a number of criteria have been addressed including: a characterisation of the transferred gene, its 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 protein to be either allergenic or toxic in humans.

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

- environmental risks related to the environmental release of GM plants used in food production
- the safety of animal feed or animals fed with feed derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

History of Use

Corn is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries. Corn-derived products are routinely used in a large number and diverse range of foods and have a long history of safe use. Products derived from MON87427 corn may include flour, starch products, breakfast cereals and high fructose corn syrup. Corn is also widely used as a feed for domestic livestock.

Molecular Characterisation

Corn line MON87427 was generated through *Agrobacterium*-mediated transformation. The line contains the *cp4 epsps* gene that encodes a protein conferring tolerance to the herbicide glyphosate. Due to a specific promoter/intron interaction, tolerance to glyphosate is expressed in all plant tissues except pollen.
Comprehensive molecular analyses of corn line MON87427 indicate that there is a single insertion site comprising a single, complete copy of the *cp4 epsps* expression cassette. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and plasmid backbone analysis shows no plasmid backbone has been incorporated into the transgenic locus.

Characterisation of Novel Protein

Corn line MON87427 expresses one novel protein, CP4 EPSPS. As expected, the level of CP4 EPSPS in pollen was either very low or below the LOD. Of the remaining tissues tested, the level of CP4 EPSPS was lowest in the grain (approximately 4 µg/g dry weight) and highest in young leaves (approximately 680 µg/g dry weight).

The identity of MON87427-derived CP4 EPSPS was confirmed by a number of analytical techniques, namely recognition by anti-CP4 EPSPSA antibody, MALDI-TOF analysis, N-terminal sequencing and enzymatic activity.

Bioinformatic studies have confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that CP4 EPSPS would be completely digested before absorption in the gastrointestinal tract would occur. The protein exhibits heat stability however, given its digestive lability combined with the lack of similarity to known protein toxins or allergens and the loss of enzyme activity with heating, this does not raise any safety concerns.

Taken together, the evidence indicates the CP4 EPSPS protein is unlikely to be toxic or allergenic to humans.

Herbicide Metabolites

Residue data derived from supervised trials indicate the residue levels in grain are low. In the absence of any significant exposure to either glyphosate or its major metabolite, the risk to public health and safety is negligible.

Compositional Analyses

Detailed compositional analyses were done to establish the nutritional adequacy of grain from MON87427 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients. The levels were compared to levels in a) an appropriate non-GM hybrid line b) a tolerance range compiled from results taken for 12 non-GM hybrid lines grown under the same conditions and c) levels recorded in the literature. Only five of the 53 measured analytes deviated from the control in a statistically significant manner. However, all analytes fell within both the tolerance range and the historical range from the literature. It can therefore be concluded that grain from MON87427 is compositionally equivalent to grain from conventional corn varieties.

Conclusion

No potential public health and safety concerns have been identified in the assessment of herbicide-tolerant corn MON87427. On the basis of the data provided in the present Application, and other available information, food derived from corn line MON87427 is considered to be as safe for human consumption as food derived from conventional corn varieties.

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List of Abbreviations

ADF	acid detergent fibre
AMPA	aminomethylphosphonic acid
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CCI	Confidential Commercial Information
EPSPS	5-enolpyruvyl-3-shikimatephosphate synthase
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FARRP	Food Allergy Research and Resource Program
FASTA	Fast Alignment Search Tool - All
FSANZ	Food Standards Australia New Zealand
GM	genetically modified
IgE	Immunoglobulin E
kDa	kilo Dalton
LC/MS	liquid chromatography mass spectrometry
LC/MS/MS	liquid chromatography/tandem mass spectrometry
LLMV	lower limit of method validation
LOD	Level of detection
LOQ	Level of quantitation
MALDI-TOF	matrix-assisted laser desorption/ionisation–time of flight
NDF	neutral detergent fibre
ORF	open reading frame
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
P-value	probability value
SAS	Statistical Analysis Software
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
U.S.	United States of America

1. Introduction

Monsanto Australia Limited has submitted an application to FSANZ to vary Standard 1.5.2 – Food produced using Gene Technology – in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) corn line MON87427. The corn has been modified such that all plant tissue except pollen is tolerant to the herbicide glyphosate. This means that pollen development can be prevented by application of glyphosate at the appropriate developmental stage, thus allowing glyphosate-treated lines containing MON87427 to serve as a male-sterile female parent in the production of hybrid corn seed.

Tolerance to glyphosate is achieved through expression of the enzyme 5-enolpyruvyl-3-shikimatephosphate synthase (CP4 EPSPS) encoded by the *cp4epsps* gene derived from the common soil bacterium *Agrobacterium* sp. The CP4 EPSPS protein has previously been assessed by FSANZ in a range of crops including corn.

Corn is not a major crop in Australia or New Zealand. Domestic production is supplemented by the import of a small amount of corn-based products, largely as high-fructose corn syrup, which is not currently manufactured in either Australia or New Zealand. Such products are processed into breakfast cereals, baking products, extruded confectionery and food coatings. In 2009, Australia imported 856 tonnes of corn, 4734 tonnes of corn flour and 1520 tonnes of corn oil; no forage or silage were imported (FAOSTAT 2011).

MON87427 corn will be grown in North America and is not intended for cultivation in Australia or New Zealand. Therefore, if approved, food from this line may enter the Australian and New Zealand food supply as imported food products.

2. History of use

2.1 Host organism

A hybrid resulting from a cross between the inbred lines LH198 and Hi-II was used as the parent for the genetic modification described in this application. LH198 was released in 1992 by Holden's Foundation Seeds Inc., Iowa and is itself a hybrid. Hi-II is also a hybrid and was specifically developed for use in corn transformation (Armstrong et al. 1991).

Corn is the world's third leading cereal crop, behind wheat and rice, and is grown in over 25 countries (OECD 2002). In 2009, worldwide production of corn was over 818 million tonnes, with the United States and China being the major producers (~333 and 164 million tonnes, respectively) (FAOSTAT, 2011).

The majority of grain and forage derived from corn is used as animal feed, however corn also has a long history of safe use as food for human consumption. There are five main types of corn grown for food (flour, flint, dent, sweet and pop) of which dent corn is the most commonly grown for grain and silage and is the predominant type grown in the U.S. (OGTR 2008). MON87427 is a dent corn.

Two main grain processing routes are followed for dent corn (White and Pollak 1995):

- Dry milling that gives rise to food by-products such as flour and hominy grits.
- Wet milling (CRA 2006), that involves steeping the grain, coarse and fine grinding, centrifugation and evaporating the steep, to yield food by-products such as starch (for cornstarch, corn syrup and individual sweeteners such as dextrose and fructose) and germ (for oil) – see Figure 1. Corn products are used widely in processed foods.

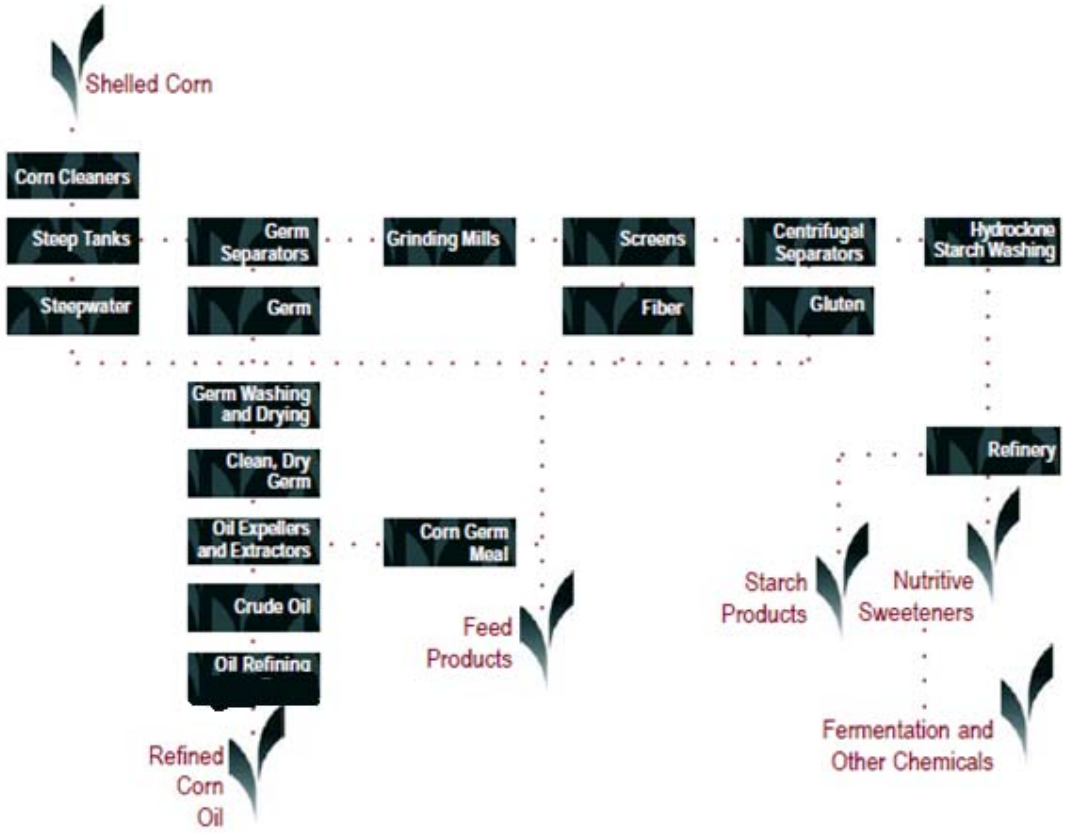

Figure 1: The corn wet milling process (diagram taken from CRA (2006))

Corn plants contain both female and male flowers and usually reproduce sexually by wind-pollination. This provides for both self-pollination and natural out-crossing between plants, both of which are undesirable since the random nature of the crossing leads to the production of grain with properties derived from different lines and which, if planted, could produce lower yields (CFIA 1994). The commercial production of corn now utilises controlled cross-pollination of two inbred lines (using conventional techniques) to combine desired genetic traits and produce hybrid varieties known to be superior to open-pollinated varieties in terms of their agronomic characteristics. The basis for hybrid corn production exploits a phenomenon known as male sterility, where the line used as a female parent is unable to produce pollen, either because the male parts have been physically removed or because the line carries a trait known as cytoplasmic male sterility. Both of these approaches are associated with certain management strategies that are not optimal. Therefore development of other approaches to create male sterile female lines is desirable.

This inbred-hybrid concept and resulting yield response is the basis of the modern corn seed industry and hybrid corn varieties are used in most developed countries for consistency of performance and production.

2.2 Donor organisms

2.2.1 Agrobacterium *sp.*

Agrobacterium sp. strain CP4 produces a naturally glyphosate-tolerant EPSPS enzyme and was therefore chosen as a suitable gene donor for the herbicide tolerance trait (Padgette et al. 1996).

The bacterial isolate CP4 was identified in the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are known soil-borne plant pathogens but are not pathogenic to humans or other animals.

2.2.2 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of corn MON87427 (refer to Table 1). These non-coding sequences are used to drive, enhance, target or terminate expression of the novel gene. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from plant pathogens are not pathogenic in themselves and do not cause pathogenic symptoms in corn MON87427.

3. Molecular characterisation

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

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

Studies submitted:

Arackal, S.M.; Garnaat, C.W.; Lawry, K.R.; Song, Z.; Girault, R.L.; Groat, J.R.; Ralston, L.F.; Masucci, J.D.; Tian, Q. (2010). Molecular characterization of MON87427. Study ID# MSL0021822, Monsanto Company (unpublished).

Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of DNA sequences flanking the 5' and 3' junctions of inserted DNA in MON87427: Assessment of putative polypeptides. Study ID# MSL0022911, Monsanto Company (unpublished).

Groat, J.R.; Tian, Q. (2011). Heritability and stability of coding sequences present in MON87427 across multiple generations. Study ID# RPN-09-275, Monsanto Company (unpublished).

3.1 Method used in the genetic modification

Immature embryos from the cross LH198 x Hi-II were aseptically removed from 10 – 13 day post-pollination ears and transformed, using *Agrobacterium tumefaciens,* with the T-DNA from plasmid vector PV-ZMAP1043 (see Figure 2) following the method of Sidorov and Duncan (2009).

After co-culturing with the *Agrobacterium* carrying the vector, the embryos were placed on selection medium containing glyphosate, and carbenicillin, to inhibit the growth of untransformed plant cells and excess *Agrobacterium*, and to permit the development of callus tissue. Resulting callus was then placed in a medium that supported shoot regeneration. Rooted plants (R_0) with normal phenotypic characteristics and tolerance to glyphosate were selected and transferred to soil for growth and further assessment.

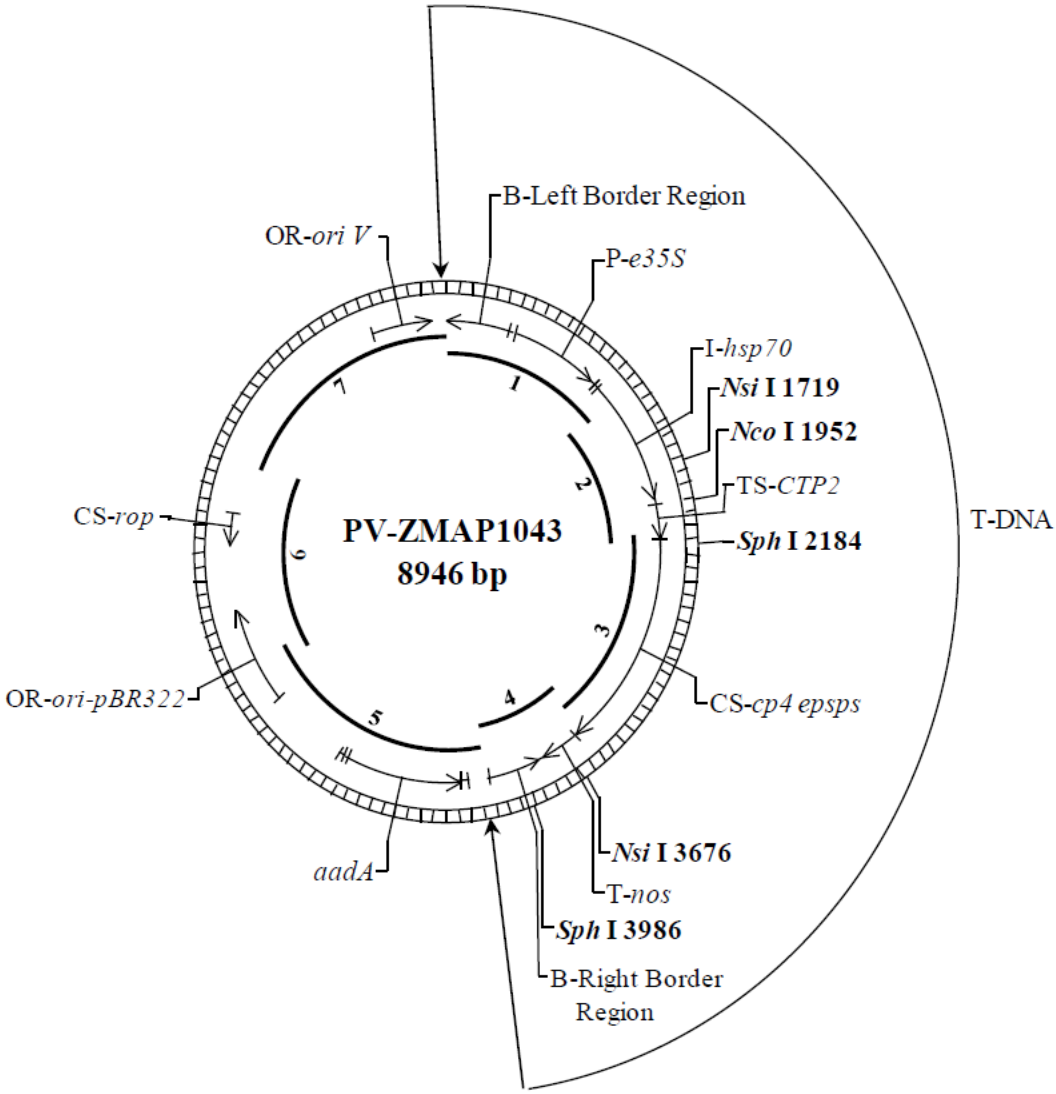

Figure 2: Genes and regulatory elements contained in plasmid PV-ZMAP1043

3.2 Function and regulation of introduced genes

Information on the genetic elements in the T-DNA insert present in MON87427 is summarised in Table 1.

Table 1: Description of the genetic elements contained in the T-DNA of PV-ZMAP1043

Genetic element	bp location on PV GMHT 4355	Size (bp)	Source	Orient.	Description & Function	References
LEFT BORDER	1 - 442	442				
Intervening sequence	443 - 483	41				
e35S	484 - 1104	621	Cauliflower mosaic virus (CaMV)	clockwise	• Promoter to direct transcription of *cp4epsps* • Also contains a duplicated enhancer region	Odell et al (1985); Kay et al (1987)
Intervening sequence	1105 - 1125	21				
Hsp70	1126 - 1929	804	*Zea mays*	clockwise	• First intron from heat shock protein 70 • Enhances expression in plants	Brown and Santino (1997)
Intervening sequence	1930 - 1953	24				
CTP2	1954 - 2181	228	*Arabidopsis thaliana*	clockwise	• Targeting sequence from the *ShkG* gene that encodes the chloroplast transit peptide. • Directs transport of CP4 EPSPS to the chloroplast	Klee et al (1987); Herrmann (1995)
cp4 epsps	2182 - 3549	1368	*Agrobacterium* sp. strain CP4	clockwise	• Codon optimised codon sequence of the *aroA* gene encoding the CP4 EPSPS protein	Padgette et al (1996); Barry et al (2001)
Intervening sequence	3550 - 3555	6				
nos	3556 - 3808	252	*Agrobacterium tumefaciens*	clockwise	• 3' non-translated region from the *nopaline synthase* gene • Terminates transcription of *cp4 epsps*	Bevan et al (1983)
Intervening sequence	3809 - 3835	27				
RIGHT BORDER	3836 - 4192	357				

3.2.1 cp4 epsps *expression cassette*

The *cp4 epsps* gene was initially isolated and cloned from the bacterium *Agrobacterium* sp. strain CP4. The gene has been optimized for expression in plants (Padgette et al., 1996; Barry et al. 2001) Expression of the gene confers tolerance to the herbicide glyphosate.

The *cp4 epsps* coding region is 1,368 bp in length and is driven by the 35S constitutive promoter from Cauliflower mosaic virus with a duplicated enhancer region. The addition of the first intron from the heat shock protein 70 (Hsp70) of corn enhances expression. This promoter/intron combination also selectively permits expression of the CP4 EPSPS protein in all corn tissue but pollen microspores (which develop into pollen grains) and tapetum cells (that supply nutrients to the pollen).

A transit peptide derived from elements from the *ShkG* gene from *Arabidopsis thaliana* targets the CP4 EPSPS protein to the chloroplasts where chorismate biosynthesis (that in non-GM plants is disrupted by glyphosate) occurs. The 3' *nos* non-translated region from *Agrobacterium tumefaciens* functions to terminate transcription and direct polyadenylation of the mRNA.

3.3 Breeding of corn line MON87427

The breeding pedigree for the various generations is given in Figure 3.

A single R_0 plant was obtained and then backcrossed over four generations with LH198. At each generation, the progeny were evaluated and selected for tolerance to glyphosate. The plants surviving at this stage were then self-pollinated over four generations in order to produce homozygous lines.

Seed from LH198 BC3F4 was used in trait integration and further commercial development through crosses with non-GM commercial lines (RP and LH287). MON87427 was selected as the lead event at [LH198 BC3F7 x LH287] F1 following the 2008 field season.

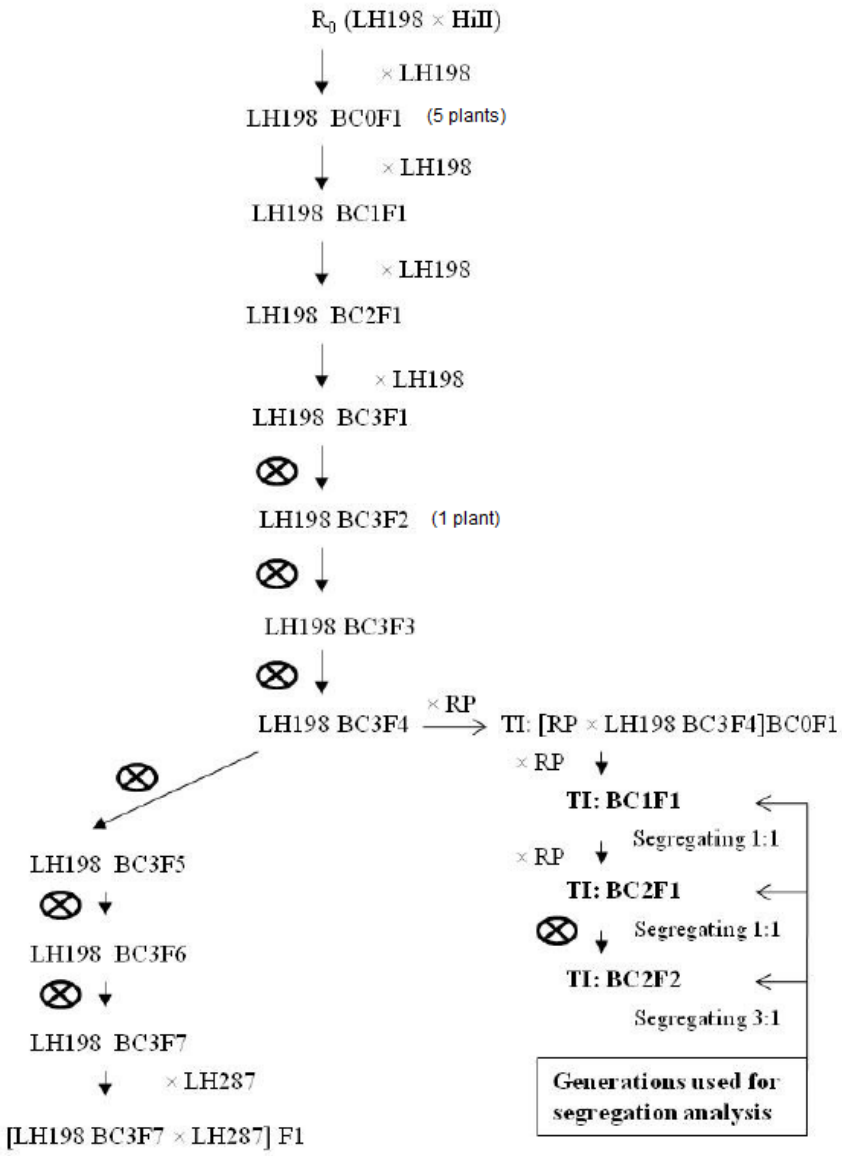

Figure 3: Breeding diagram for MON87427

The hybrid generation [LH198 BC3F7 x LH287] F1 was used for the protein characterisation and compositional analysis sections described in this Safety Assessment because it represents the commercial hybrid form of MON87427 that would be most applicable to food and feed use.

3.4 Characterisation of the genes in the plant

A range of analyses were undertaken in order to characterise the genetic modification in corn line MON87427. These analyses focussed on the nature of the insertion of the introduced genetic elements and whether any unintended genetic re-arrangements may have occurred as a consequence of the transformation procedure.

3.4.1 Transgene copy number and insert characterisation

Total genomic DNA from grain of verified MON87427 (generation LH198 BC3F4) and a negative control (LH198 x Hi-II) was used for Southern blot analysis.

The DNA from both sources was digested with the restriction enzymes *Nco1* or *Nsi1*. The resulting DNA fragments were separated by agarose gel electrophoresis and transferred to a membrane for sequential hybridisation with seven different overlapping radiolabelled probes that, taken together, spanned the insert and adjacent 5' and 3' flanking sequences. A positive control (digested DNA from LH198 x Hi-II spiked with restriction enzyme-digested PV-ZMAP1043) was also included in the Southern blot analyses to demonstrate sensitivity of the Southern blots and to confirm that the probes were recognising the target sequences. Overlapping polymerase chain reaction (PCR) products were generated and used to determine the nucleotide sequence of the insert and flanking regions using BigDye® Terminator chemistry (http://www.appliedbiosystems.com.au/).

The Southern blot analyses indicated that the introduced DNA has been inserted at a single locus that contains one intact copy of the T-DNA. No PV-ZMAP1043 backbone sequences were detected.

Endogenous background hybridization was observed in all digests, including the negative control DNA samples, probed with two of the probes and was considered to represent *Hsp70* sequences in the probes hybridising with endogenous *Hsp70* in both the GM and non-GM genomic DNA samples.

Sequence data were obtained for the insert and flanking regions and confirmed the integrity of the *cp4 epsps* cassette and the sequences of the junction regions. The consensus sequence of the insert is 3,681 bp long beginning at base 191 in the Left Border region (refer to Table 1) and ending at base 3871 in the Right Border region. There is a 1,003 bp sequence flanking the 5' end of the insert and a 1092 bp sequence flanking the 3' end (Figure 4).

A PCR product was generated from the conventional control LH198 x Hi-II and the sequence was compared with the sequences in the 5' and 3' flanking regions. This showed:

- there is a 41 bp insertion at the 5' end of the T-DNA
- there is a 24 bp insertion at the 3' end
- 140 bp from the LH198 x Hi-II 'parent' were deleted during the integration of the T-DNA
- the genomic sequences flanking the insert are native to the corn genome.

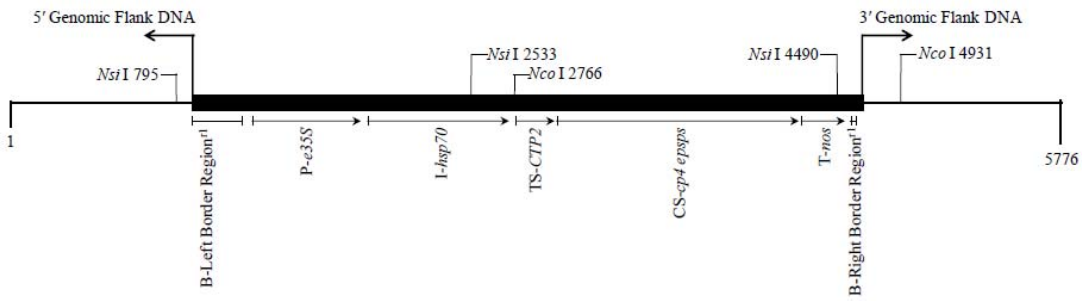

Figure 4: Schematic representation of the insert and flanking regions in MON87427

3.4.2 Open reading frame (ORF) analysis

The transgenic insert has the identical sequence to the T-DNA of the PV-ZMAP1043 plasmid (see Section 3.4.1) and therefore has no unexpected ORFs.

Sequences spanning the two junction regions formed as a result of the insertion of the T-DNA were translated using DNAStar software (http://www.dnastar.com/) from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames. A total of 14 ORFs were identified that encoded putative polypeptides ranging in size from 8 – 97 amino acids. No analysis was done to determine whether any potential regulatory elements were associated with the polypeptides.

The putative polypeptides encoded by the 14 identified ORFs were then analysed using a bioinformatic strategy to determine whether, in the event they were translated, they would raise any allergenic or toxicity concerns (refer to Section 4.1).

3.5 Stability of the genetic changes in corn line MON87427

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

3.5.1 Genetic stability

The genetic stability of event MON87427 was evaluated by Southern analysis in tissue from individual plants of five generations (refer to Figure 3) as given in Table 2; as a reference for MON8742 LH198 BC3F7 x LH287, the analysis included a conventional control LH198 x LH287, while the control LH198 x Hi-II was used as a reference for the other MON87247 generations.

Table 2: Source of tissue used for genetic stability analysis

DNA source	Generation	Tissue type
MON87427	LH198 BC3F3	Leaf
	LH198 BC3F4	Grain
	LH198 BC3F6	Leaf
	LH198 BC3F7	Leaf
	[LH198 BC3F7 x LH287] F1	Grain
LH198 x LH287		Grain
LH198 x Hi-II		Grain

Genomic DNA obtained from each source was digested with one restriction enzyme (*Nsi 1*) and the resulting fragments were hybridised with two probes. In all cases, endogenous background hybridisation was noted (see discussion in Section 3.4.1).

Analysis of the MON87427 DNA showed the presence of the expected hybridisation fragments in all samples and therefore confirmed the genetic stability of the insert in MON87427 over different generations.

3.5.2 Phenotypic stability

Chi square analysis was undertaken over several generations to confirm the segregation and stability of the glyphosate tolerance trait.

Following generation of the R_0 plant, a number of crosses with LH198 were carried out (refer to Figure 3). At each generation, the plants showed the expected ratio of 1:1 (glyphosate tolerant: glyphosate susceptible). Heterozygous plants from generation LH198 BC3F1 were then self-pollinated and the resulting progeny segregated in the expected ratio of 3:1 (glyphosate tolerant: glyphosate susceptible). One of these plants was then selected and two rounds of self-pollination were done from which homozygous plants were identified by endpoint Taqman® analysis (see e.g. Gao et al. 2009 for a general description of the technique)

Seed from LH198 BC3F4 was then used in further commercial development and plants were crossed then backcrossed to a recurrent parent (designated 'RP' in Figure 3) and finally self-pollinated, to obtain a number of generations – T1 BC1F1, T1 BC2F1 and T1 BC2F2. Plants from these three generations were sprayed with glyphosate and results were analysed using chi-square to indicate whether there were significant differences between the observed and expected CP4 EPSPS expression ratios (Table 3). The results showed that there was no significant difference between the expected and observed ratios and therefore, that the *cp4 epsps* expression cassette is stably inherited according to Mendelian principles.

Table 3: Segregation of the cp4 epsps gene over three generations

Generation	Total plants	Ratio[1]		Probability (P)[1]
		Observed	Expected	
T1 BC1F1	238	0.84 : 1	1:1	0.1948
T1 BC2F1	290	1:1	1:1	1.000
T1 BC2F2	1107	2.8 : 1	3:1	0.4768

[1]Ratio is positives:negatives for each of observed and expected
[2]Statistical significance is when P≤0.05

3.6 Antibiotic resistance marker genes

No antibiotic marker genes are present in corn line MON87427. Plasmid backbone analysis shows that no plasmid backbone has been integrated into the corn genome during transformation, i.e. the *aadA* gene, which was used as a bacterial selectable marker gene, is not present in corn MON87427.

3.7 Conclusion

Corn line MON87427 contains the *cp4 epsps* gene that encodes a protein conferring tolerance to the herbicide glyphosate. Because of an interaction between the promoter and the *Hsp70* intron, tolerance to glyphosate is not expressed in pollen.

Comprehensive molecular analyses of corn line MON87427 indicate there is a single insertion site comprising a single, complete copy of the *cp4 epsps* expression cassette. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and plasmid backbone analysis shows no plasmid backbone has been incorporated into the transgenic locus.

4. Characterisation of novel proteins

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

Two types of novel proteins were considered:

- Those that may be potentially translated as a result of the creation of ORFs during the transformation process (see Section 3.4.2).
- Those that were expected to be directly produced as a result of the translation of the introduced genes. A number of different analyses were done to determine the identity, physiochemical properties, *in planta* expression, bioactivity and potential toxicity and allergenicity.

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

Study submitted:

Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of DNA sequences flanking the 5' and 3' junctions of inserted DNA in MON87427: Assessment of putative polypeptides. Study ID# MSL0022911, Monsanto Company (unpublished).

A bioinformatics analysis was performed to assess the similarity to known allergens and toxins of the putative polypeptides encoded by the 14 sequences obtained from the ORF analysis (refer to Section 3.4.2).
To evaluate the similarity to known allergens of proteins that might potentially be produced from translation of the ORFs, an epitope search was carried out to identify any short sequences of amino acids that might represent an isolated shared allergenic epitope. This search compared the sequences with known allergens in the Allergen, Gliadin and Glutenin sequence database, residing in the FARRP (Food Allergy Research and Resource Program) dataset (Version 10) within AllergenOnline (University of Nebraska; http:www.allergenonline.org/). The FASTA algorithm (Pearson and Lipman 1988), version 3.4t 26, was used to search the database using the BLOSUM50 scoring matrix (Henikoff and Henikoff 1992). No alignments with any of the 14 query sequences generated an E-score[1] of ≤1e^{-5}, no alignment met or exceeded the Codex Alimentarius (Codex 2003) FASTA alignment threshold for potential allergenicity and no alignments of eight or more consecutive identical amino acids (Metcalfe et al. 1996) were found. It was concluded that the 14 putative polypeptides are unlikely to contain any cross-reactive IgE binding epitopes with known allergens.

The sequences corresponding to the 14 reading frames were also compared with sequences present in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the FASTA algorithm. No significant similarities of the 14 reading frames to any sequences in the databases (including those of known toxins) were found.

It is concluded that, in the unlikely event transcription and translation of the 14 identified ORFs could occur, the encoded polypeptides do not share any significant similarity with known allergens or toxins.

4.2 Function and phenotypic effects of the CP4 EPSPS protein

Glyphosate acts as a herbicide by inhibiting the EPSPS. This endogenous enzyme is involved in the shikimate pathway for aromatic amino acid biosynthesis which occurs exclusively in plants and microorganisms, including fungi. Inhibition of the wild type EPSPS enzyme by glyphosate leads to deficiencies in aromatic amino acids in plant cells and eventually to the death of the whole plant. The shikimate biochemical pathway is not present in animals.

EPSPS proteins occur ubiquitously in plants and microorganisms and have been extensively studied over a period of more than thirty years. In plants, the EPSPS enzyme is inhibited by glyphosate (Steinrucken and Amrhein 1980), but bacterial EPSPSs, such as the CP4-EPSPS, have a reduced affinity for glyphosate (Padgette et al., 1996; Barry et al., 2001) thereby allowing the continued action of the enzyme in the presence of glyphosate. The CP4 EPSPS protein present in corn MON87427 is functionally the same as the wild type *Agrobacterium* enzyme. It is a 47.6 kDa protein containing 455 amino acids.

The specific promoter/intron combination (*e35S – hsp70*) used to drive CP4 EPSPS expression in MON87427 while conferring tolerance to glyphosate in the leaves, stalk and root tissues and in tissues that develop into grain and silks (i.e. female reproductive tissues), results in limited or no production of CP4 EPSPS in pollen microspores (which develop into

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

pollen grains) and tapetum cells (that supply nutrients to developing pollen grains). There is expression of CP4 EPSPS in other male tissue of the anther but developing pollen tissues in MON87427 are not tolerant to glyphosate.
The corn hybrid LH198 x Hi-II used as the parent for the genetic modification described in this application, contains a wild type EPSPS protein.

4.2.1 CP4 EPSPS protein expression in MON87427 tissues

Study submitted:

Beyene, A.; Niemeyer, K. (2010). Assessment of CP4 EPSPS corn tissues collected from MON87427 produced in U.S. field trials during 2008. Study ID# MSL0022370, Monsanto Company (unpublished).

Plants of MON87427 (generation [LH198 Bc3F7 x LH287] F1) and LH198 x LH287 were grown from verified seed lots at five field sites in the U.S[2] during the 2008 growing season. The identity of any subsequent harvested grain from each site was also confirmed using event-specific polymerase chain reaction (PCR). There were three replicated plots at each site. Samples were taken at various stages of growth (Table 4) and the level of CP4 EPSPS protein was determined for each sample type using a validated enzyme linked immunosorbent assay (ELISA) utilising a mouse anti-CP4 EPSPS capture antibody and a commercial detection reagent comprising goat-antiCP4 EPSPS conjugated to horse radish peroxidase. Plates were analysed on a commercial microplate spectrophotometer. Quantification of total CP4 EPSPS protein was accomplished by interpolation on a CP4 EPSPS protein standard curve.

The results, averaged over all sites, are given in Table 4.

Table 4: CP4 EPSPS protein content in MON87427 corn parts at different growth stages (averaged across 5 sites)

Growth stage[1]/tissue	n	µg/g dry weight		Standard Deviation
		Mean	Range	
Leaf V2 – V5	14	680	400 - 940	170
Leaf V6 – V8	14	410	130 - 560	130
Leaf V10 – V12	14	290	210 - 410	74
Leaf VT	14	370	70 - 520	120
Root V2 – V5	14	140	58 - 210	46
Root V6 – V8	14	110	48 - 240	62
Root V10 – V12	14	73	22 - 110	28
Root VT	14	83	23 - 140	36
Forage Root R5	11[2]	72	39 - 100	23
Senescent Root R6	14	72	26 - 130	37
Forage R5[3]	14	120	21 - 200	48
Stover R6[4]	14	43	13 - 98	27
Whole Plant V2 – V5	14	500	310 - 840	190
Whole Plant V6 – V8	14	360	300 - 420	42
Whole Plant V10 – V12	14	380	230 - 500	78
Whole Plant VT	14	240	160 - 340	42
Silk (at pollination)	14	100	90 - 120	12
Pollen (at pollination)	6[5]	0.87	0.25 – 2.2	0.7
Grain R6	14	4.2	2.8 – 6.2	0.89

[1]For information on corn growth stages see e.g. McWilliams et al. (1999)

[2] Jackson County, AR; Jefferson County, IA; Stark County, IL; Parke County, IN; York County, NE.

[2] Samples from one site (AR) were not cleaned properly and were excluded from the analysis.
[3] Forage is the above ground plant parts used for animal feed.
[4] Stover is the dried stalks and leaves of a field crop used as animal fodder after the grain has been harvested
[5] Out of a total of 14 pollen samples, six samples had CP4 EPSPS levels below the level of detection (LOD). The mean levels given are for the six samples that had levels above the limit of quantitation (LOQ). For two samples, two extractions were done with one analysis giving a result below the LOQ and one result above the LOQ. These two results were considered to be inconclusive.

As expected, the level of CP4 EPSPS in pollen was either very low or below the LOD. Of the remaining tissues, the level of CP4 EPSPS was lowest in the grain (approximately 4 µg/g dry weight) and highest in young leaves (approximately 680 µg/g dry weight). Values obtained for tissue from LH198 x LH287 were below the LOD or LOQ in all but 7 samples (approximately 3% of samples). Since the identity of grain was confirmed both prior to planting and after harvest, it is concluded that these readings above the LOQ were due to sample contamination during processing.

4.3 CP4 EPSPS characterisation, and equivalence of the protein produced *in planta* and in a bacterial expression system

Study submitted:

Chandu, D.; Crowley, K.S.; Lee, T.C.; Finnessy, J.J.(2010). Amended report for MSL0022391: Characterization of the CP4 EPSPS protein purified from the grain of MON87427 and comparison of the physicochemical and functional properties of the plant-produced and E. coli-produced CP4 EPSPS proteins. Study ID# MSL0023119, Monsanto Company (unpublished).

The amount of CP4 EPSPS protein produced in MON87427 was insufficient for safety evaluations. Therefore, the CP4 EPSPS protein for these evaluations was produced in *Escherichia coli.* In order to confirm that the *E. coli*-produced CP4 EPSPS is equivalent to that expressed in MON87427, 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.

The MON87427-derived CP4 EPSPS protein was purified from verified grain of generation [LH198 Bc3F7 x LH287] F1. Fractions containing the protein were identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and confirmed by western blot analysis. The concentration of the MON87427 protein was 0.1 mg/mL.

4.3.1 Molecular weight and immunoreactivity

The molecular weights of CP4 EPSPS protein from the two sources were estimated from densitometric analysis of SDS-PAGE. Immunoreactivity was detected on the Western blots using a polyclonal goat anti-CP4 EPSPS primary antibody and a commercial (Thermo Scientific) rabbit-anti-goat horseradish peroxidase linked secondary antibody.

The SDS-PAGE gel containing the purified plant and bacterial proteins showed a single band with an apparent molecular weight of approximately 44 kDa. This molecular weight estimate was in good agreement with the value of 47.6 kDa that was calculated from the DNA sequence. Based on a densitometric analysis of SDS-PAGE, the average purity of the MON87427 protein was estimated to be about 96%.

Western blot analysis showed a single immunoreactive band that had co-migrated in separate extracts from MON87427 and *E. coli.* This co-migrating band can be taken as evidence of immunological identity of the protein from the two sources.

4.3.2 MALDI-TOF tryptic mass fingerprint and intact mass analyses

Mass spectral analysis using matrix-assisted laser desorption/ionisation–time of flight (MALDI–TOF) was performed on trypsin-digested excised bands corresponding to plant-derived CP4 EPSPS obtained by running samples on SDS-PAGE.

It was estimated that the peptide mapping of the plant CP4 EPSPS protein identified 70% of the expected protein sequence and this was adequate to provide convincing evidence of the identity of the protein.

The intact mass of the MON87427 protein was also determined by MALDI-TOF based on an average of three separate mass spectral acquisitions and indicated a value of 47.5 kDa.

4.3.3 N-terminal sequence analysis

Automated Edman degradation chemistry was performed on CP4 EPSPS protein that had been eluted from SDS-PAGE.

The amino acid analyses from the N-terminus yielded a 15 amino acid sequence which matched the expected sequence of the mature CP4 EPSPS protein after processing of the chloroplast transit peptide and loss of the N-terminal methionine. The absence of the methionine is not unexpected since the terminal methionine is routinely cleaved from nascent proteins by methionine aminopeptidase (see e.g. Polevoda and Sherman 2000).

4.3.4 Glycosylation analysis

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

Proteins produced in prokaryotes are not expected to be glycosylated and only a few specific endogenous proteins in *E. coli* have been confirmed to be glycosylated (Sherlock et al. 2006).

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

Carbohydrate detection was performed directly on plant CP4 EPSPS bands (transferred from SDS-PAGE to a PVDF membrane) using a commercial glycoprotein detection kit (Molecular Probes) Transferrin and horseradish peroxidase were used as positive controls.

Transferrin and horseradish peroxidase were detected at the expected molecular weights and in a concentration-dependent manner. Very faint signals were detected at the molecular weight expected for CP4 EPSPS in both the MON87427 and *E.coli* samples. This faint

staining in samples from both sources is unexpected, especially as published reports (e.g. Harrison et al. 1996) as well as studies provided by the applicant in at least eight previous application dossiers to FSANZ, all involving glycosylation analysis of CP4 EPSPS from a plant and *E. coli* source, failed to show staining in samples from either source. The conclusion is that there is no glycosylation in either the plant- or *E. coli*- derived CP4 EPSPS.

4.3.5 Enzymatic activity

EPSPS catalyses the transfer of the enolpyruvyl moiety of phosphoenolpyruvate to the 5-OH hydroxyl group of shikimate 3-phosphate and releases inorganic phosphate. The amount of inorganic phosphate released in the reaction is measured spectrophotometrically (660 nm) using a malachite green dye method and is directly related to the specific activity of the enzyme.

CP4 EPSPS from both plant- and *E. coli*-derived sources was tested for activity and values of 5.41 U/mg and 8.67 U/mg respectively were obtained. Within the accuracy of the method used, these values were considered to be indicative of equivalent functional activity of CP4 EPSPS from the two sources.

4.3.6 Conclusion

The identity of MON87427-derived CP4 EPSPS was confirmed by a number of analytical techniques, namely recognition by anti-CP4 EPSPSA antibody, MALDI-TOF analysis, N-terminal sequencing and enzymatic activity.

CP4 EPSPS produced in MON87427 and in *E. coli* were compared. The proteins from both sources were found to co-migrate in SDS-PAGE, to be recognised by an anti-CP4 EPSPS antibody, to lack glycosylation and to show equivalent activity. Thus the CP4 EPSPS proteins from the two sources can be said to be equivalent and the *E. coli*-derived CP4 EPSPS protein is a suitable surrogate for plant-derived CP4 EPSPS in safety assessment studies.

4.4 Potential toxicity of the CP4 EPSPS 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 4.2, the CP4 EPSPS protein is found in plants and microorganisms and would therefore be routinely consumed as a normal part of the diet. In addition, the *cp4 epsps* gene has been widely used in the genetic modification of commercialised food

crops over the last 10 years and there have been no safety concerns raised with the consumption of the protein (Delaney *et al*., 2008).

4.4.2 Similarity of CP4 EPSPS with known toxins

Study submitted:

Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of the CP4 EPSPS protein utilizing the AD_2010, TOX_2010 and PRT_2010 databases. Study ID # MSL0022522, Monsanto Company (unpublished).

The CP4 EPSPS sequence was compared with sequences present in the GenBank database, release 175.0 (http://www.ncbi.nlm.nih.gov/genbank/) using the FASTA algorithm (Pearson and Lipman, 1988) and BLOSUM50 scoring matrix. See footnote in Section 4.1 for an explanation of alignment significance.

As expected, the query sequence matched with EPSPS proteins from a range of sources. There were no matches with any sequences from known protein toxins.

4.4.3 In vitro digestibility

See Section 4.5.3

4.4.4 Thermolability

The thermolability of a protein provides an indication of the stability of the protein under cooking/processing conditions.

Study submitted:

Hernan, R.; Chen, B.; Bell, E.; Finnessy, J. (2010). Amended Report for MSL0022432: Effect of temperature treatment on the functional activity of CP4 EPSPS. Study ID# MSL0023307, Monsanto Company (unpublished).

CP4 EPSPS protein from *E. coli* was incubated at 25°, 37°, 55°, 75°, or 95° for 15 min or 30 min. CP4 EPSPS protein maintained on wet ice was used as a control. Following treatment, the samples were analysed by SDS-PAGE or were tested for enzyme activity (as described in Section 4.3.5).

Analysis by SDS-PAGE indicated that the CP4 EPSPS protein is stable (i.e. not degraded) at all the temperatures tested. Results of the activity assay indicated that, at temperatures above 37° C activity was rapidly lost, and by 75° C was below the LOD.

It is concluded that the CP4 EPSPS protein is heat-stable up to 95° C for 30 minutes but that activity of the enzyme is destroyed at 75° C or higher. These findings are in agreement with past EPSPS thermolability studies submitted to FSANZ as part of application dossiers.

4.4.5 Acute toxicity study

Although not required, since no toxicity concerns were raised in the data considered in Sections 4.4.1 – 4.4.4, the Applicant supplied an acute oral toxicity study.

Study submitted:

Naylor, M.W. (1993). Acute oral toxicity study of CP4 EPSPS protein in albino mice Study ID# MSL-13077. Monsanto Company (unpublished).

The above study has been submitted by the Applicant in previous applications to FSANZ involving the CP4 EPSPS protein and will not be re-analysed here. No adverse effects associated with bacterially-derived CP4 EPSPS were observed at doses up to 572 mg/kg body weight administered by oral gavage to mice. The results of the study have also been published in the scientific literature (Harrison et al., 1996).

4.5 Potential allergenicity of the CP4 EPSPS protein

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

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

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

The allergenic potential of the CP4 EPSPS protein was assessed by:

- consideration of the *cp4 epsps* gene source and history of use or exposure
- bioinformatic comparison of the amino acid sequence of the CP4 EPSPS protein with known protein allergen sequences
- evaluation of the lability of the microbially-produced CP4 EPSPS using an *in vitro* gastric digestion model.

4.5.1 Source of protein

As discussed in Section 2.2.1, the CP4 EPSPS protein is derived from a common soil bacterium to which humans have been naturally exposed and which may therefore have been inadvertently ingested on fresh produce. There is therefore a prior history of human exposure to the CP4 EPSPS protein. There are no indications that the protein is associated with any known adverse effects in humans.

4.5.2 Similarity to known allergens

Study submitted:

Tu, H.; Silvanovich, A. (2010). Bioinformatics evaluation of the CP4 EPSPS protein utilizing the AD_2010, TOX_2010 and PRT_2010 databases. Study ID # MSL0022522, Monsanto Company (unpublished).

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 the CP4 EPSPS protein with known protein toxins (see Section 4.4), the generation of a small *E*-value provides an important indicator of significance of matches (Pearson 2000; Baxevanis, 2005).

The same approach as described in Section 4.1 was used to undertake a bioinformatic evaluation of the relatedness between the CP4 EPSPS protein and known allergens in the Allergen, Gliadin and Glutenin sequence database.

No alignment generated an E-score of ≤$1e^{-5}$, no alignment met or exceeded the Codex Alimentarius (Codex, 2003) FASTA alignment threshold (35% identity over 80 amino acids) for potential allergenicity and no alignments of eight or more consecutive identical amino acids were found.

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

Study submitted:

Leach, J.N.; Hileman, R.E.; Thorp, J.J.; George, C. Astwood, J.D. (2002). Assessment of the *in vitro* digestibility of purified *E.coli*-produced CP4 EPSPS protein in simulated gastric fluid. Study ID# MSL-17566, Monsanto Company (unpublished).

The above study has been submitted by the Applicant in previous applications to FSANZ involving the CP4 EPSPS protein and will not be re-analysed here. The results from this study as well as conclusions reached in other publications (see e.g. Harrison et al., 1996; Delaney et al., 2008) confirm that the protein is rapidly digested in simulated gastric fluid.

4.6 Conclusion

Corn line MON87427 expresses one novel protein, CP4 EPSPS. As expected, the level of CP4 EPSPS in pollen was either very low or below the LOD. Of the remaining tissues, the level of CP4 EPSPS was lowest in the grain (approximately 4 µg/g dry weight) and highest in young leaves (approximately 680 µg/g dry weight).

The identity of MON87427-derived CP4 EPSPS was confirmed by a number of analytical techniques, namely recognition by anti-CP4 EPSPSA antibody, MALDI-TOF analysis, N-terminal sequencing and enzymatic activity.

Bioinformatic studies have confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated CP4 EPSPS would be completely digested before absorption in the gastrointestinal tract

would occur. The protein exhibits heat stability however, given its digestive lability combined with the lack of similarity to known protein toxins or allergens and the loss of enzyme activity with heating, this does not raise any safety concerns.

Taken together, the evidence indicates that the CP4 EPSPS protein is unlikely to be toxic or allergenic to humans.

5. Herbicide metabolites

Studies in many different plant species (FAO, 2005) have shown that the major metabolite produced by spraying glyphosate on both conventional and glyphosate-tolerant GM crops, is aminomethylphosphonic acid (AMPA). In turn this may be conjugated to form trace amounts of other metabolites.

Given the long history of safe use of glyphosate on GM food crops containing the CP4 EPSPS protein, there are no concerns with its use on corn line MON87427. Nevertheless, the Applicant supplied two metabolite studies (one of which is essentially a sub-set of the other) which have been considered by FSANZ.

Studies submitted:

Mueth, M.G. (2010). Magnitude of glyphosate residues in corn raw agricultural commodities following applications of a glyphosate-based formulation to MON87427. Study ID # MSL0021925, Monsanto Company (unpublished).

Mueth, M.G.; Allan, J.M. (2011). Magnitude of glyphosate residues in corn raw agricultural commodities following applications of a glyphosate-based formulation to corn hybrids possessing RHS trait. U.S. 2008 trials. Study ID # MSL0022294, Monsanto Company (unpublished).

Field trials of plants containing the MON87427 event were conducted in 2008 at six sites in the U.S. located in areas where field corn is commonly grown commercially. Treated plants were sprayed with Roundup WeatherMAX® herbicide applied at pre-emergence, V3 - V4 and V8 (McWilliams et al. 1999). The combination of applications represented the maximum pre-emergence and in-crop applications permitted on corn containing Roundup Ready 2 Technology. Harvested grain was analysed for glyphosate and AMPA by Liquid Chromatography Mass Spectrometry (LC-MS/MS).

Results indicated that the median residue levels in grain were very close to the lower limit of method validation (0.05 ppm). In the absence of any significant exposure to either glyphosate or AMPA, the risk to public health and safety is negligible.

6. Compositional analysis

The main purpose of compositional analysis is to determine if, as a result of the genetic modification, any unexpected changes have occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus 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 the 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 (e.g. solanine in potatoes).

6.1 Key components

For corn there are a number of components that are considered to be important for compositional analysis (OECD, 2002; EuropaBio 2003). As a minimum, the key nutrients of corn grain 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 and raffinose and the secondary metabolites coumaric acid, ferulic acid and furfural should be determined for new varieties of corn. 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, while not considered nutritionally significant in corn, can interfere with digestion of protein.
- raffinose is a low molecular weight carbohydrate (oligosaccharide) that is associated with production of intestinal gas and resulting flatulence when consumed.
- coumaric and ferulic acids are phenolic acids that might influence digestion.
- furfural is a heterocyclic aldehyde that is considered an oral genotoxic carcinogen of low potency

6.2 Study design and conduct for key components

Study submitted:

Breeze, M.L..; Riordan, S.G.; Miller, K.D.; Sorbet, RD.. (2010). Compositional analysis of corn forage and grain of MON87427 treated with glyphosate grown in the United States during the 2008 field season. Study ID # MSL0022340, Monsanto Company (unpublished).

As explained in Section 3.3, the MON87427 hybrid generation [LH198 BC3F7 x LH287] F1 (refer to Figure 3) was used for compositional analysis because it represents the commercial hybrid form of MON87427 that would be most applicable to food and feed use. Ideally, the comparator in compositional analyses should be the near isogenic parental line grown under identical conditions (OECD, 2002). In the case of corn line MON87427, the control was the hybrid line identified as LH198 x LH287 since this represents the closest genetic line to [LH198 BC3F7 x LH287] F1 for the purposes of comparison.

The test and control lines were grown under typical production conditions at three field sites across North America[3] during the 2008 growing season. Additionally, four non-GM hybrid lines were grown at each site in order to generate tolerance ranges for each analyte i.e. there were twelve different reference lines in total. MON87427 plants were sprayed at the V2 – V6 stage with glyphosate at a target rate of 1.13 kg ae/ha[4]. The test, conventional control and conventional reference lines were planted in a randomised block design with three replicates at each site.

[3] The three sites were: Jefferson County, IA ; Stark County, IL; Jackson County, AR

[4] Herbicide application rates are often expressed as acid equivalents (ae).The acid equivalent is the theoretical yield of parent acid from a pesticide active ingredient that has been formulated as a derivative.

Grain was harvested at physiological maturity and samples were analysed for proximates, fibre (acid detergent fibre – ADF; neutral detergent fibre – NDF; total dietary fibre), fatty acids, amino acids, minerals, vitamins, anti-nutrients and secondary metabolites. The identity of harvested grain from the test and control lines was verified by event specific PCR. Key analyte levels (protein, moisture, fat, ash, carbohydrates, ADF, NDF, calcium and phosphorus) for forage (harvested at R5) were also obtained but are not reported here; it is noted, however, that in the combined site analysis there were no differences between the means of any analytes from MON87427 and the control. Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods. A total of 69 analytes were measured in grain.

6.3 Analyses of key components in grain

For each analyte 'descriptive statistics' were generated i.e. a mean (least square mean) and standard error averaged over all sites (combined-site analysis). The values thus calculated are presented in Tables 5 – 11.

Of the 69 analytes measured, 16 had more than half of the observations below the assay limit of quantitation. The remaining 53 analytes (carbohydrate was calculated rather than being measured) were analysed using a mixed model analysis of variance. Data were transformed into Statistical Analysis Software[5] (SAS) data sets and analysed using SAS® software (SAS MIXED, version 9.2). The three replicated sites were analysed both separately and combined across all sites (combined-site analysis). Data presented in Tables 5 – 13 represent combined-site analysis. In assessing the significance of any difference between means, a P-value of 0.05 was used (i.e. a P-value of ≥0.05 was not significant).

Any statistically significant differences between MON87427 and the LH198 x LH287 control have been compared to the 99% tolerance interval compiled from the results of the 12 non-GM reference lines combined across all sites, to assess whether the differences are likely to be biologically meaningful. Additionally, the results for MON87427 and LH198 x LH287 have been compared to a combined literature range for each analyte, compiled from published literature for commercially available corn[6]. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within corn (Harrigan et al. 2010). Therefore, even if means fall outside the published range, this is not necessarily a concern.

6.3.1 Proximates and fibre

Results of the proximate and fibre analysis are shown in Table 5. The only analyte mean to show a significant difference was total fat which was lower in MON87427 than in the control. However, this mean was within both the tolerance range and the literature range.

[5] SAS website - http://www.sas.com/technologies/analytics/statistics/stat/index.html
[6] Published literature for corn incorporates references used to compile listings in the ILSI database (ILSI 2010)

Table 5: Mean (±standard error) percentage dry weight (%dw) of proximates and fibre in grain from MON87427 and LH198 x LH287.

Analyte	MON8742[2] (%dw)	LH198 x LH287 (%dw)	Overall treat effect (P-value)	Tolerance range (%dw)	Combined literature range (%dw)
Ash	1.58±0.036	1.56±0.038	0.765	1.18 – 1.82	0.62 – 6.28
Protein	10.05±0.63	10.26±0.63	0.594	8.07 – 12.13	6.15 – 17.26
Total Fat	3.5±0.13	3.69±0.13	0.036	2.9 – 4.3	1.74 – 5.82
Carbohydrate[1]	84.88±0.56	84.51±0.57	0.305	82.26 – 87.17	77.4 – 89.5
ADF	3.37±0.23	3.19±0.23	0.521	2.29 – 4.27	1.82 – 11.34
NDF	10.0±0.51	10.12±0.51	0.628	7.06 – 10.66	5.59 – 22.64
Total Dietary Fibre	13.0±0.37	13.05±0.37	0.854	10.25 – 14.3	8.82 – 35.31
Moisture (%fw)	11.62±0.46	11.41±0.46	0.337	9.31 – 12.7	6.1 – 40.5

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

[2] mauve shading represents MON87427 means that are significantly lower than the control means while orange shading represents MON87427 means that are significantly higher.

6.3.2 Fatty Acids

The levels of 22 fatty acids were measured. Of these, the following were below the LOQ - C8:0 caprylic, C10:0 capric, C12:0 lauric, C14:0 myristic, C14:1 myristoleic, C15:0 pentadecanoic, C15:1 pentadecenoic, C16:1 palmitoleic , C17:0 heptadecanoic, C17:1 heptadecenoic, C18:3 gamma linolenic, C20:2 eicosadienoic, C20:3 eicosatrienoic, and C20:4 arachidonic. Results for the remaining eight fatty acids are given in Table 6 and can be summarised as follows:

- There was no significant difference between the means of MON87427 and the control for linolenic, eicosenoic and behenic acids.
- The mean level of linoleic acid was significantly lower in grain of MON87427 compared with grain from the control but fell within both the tolerance range and the combined literature range.
- The mean levels of palmitic, stearic, oleic and arachidic acids were significantly higher in grain of MON87427 compared with grain of the control. All means fell within both the tolerance range and combined literature range.

Table 6: Mean (±standard error) percentage composition, relative to total fat, of major fatty acids in grain from MON87427 and LH198 x LH287.

Analyte	MON87427[1] (%total)	LH198 x LH287(%total)	Overall treat effect (P-value)	Tolerance range (%total)	Combined literature range (%total)
Palmitic acid (C16:0)	10.91±0.26	10.54±0.26	<0.001	9.13 – 12.33	7.94 – 20.71
Stearic acid (C18:0)	1.97±0.091	1.9±0.091	0.038	1.54 – 2.38	1.02 – 3.40
Oleic acid (C18:1)	24.28±0.92	23.52±0.92	0.01	21.39 – 34.71	17.4 – 40.2
Linoleic acid	60.84±1.28	62.06±1.28	0.002	49.38 – 63.16	36.2 – 66.5

Analyte	MON87427[1] (%total)	LH198 x LH287(%total)	Overall treat effect (P-value)	Tolerance range (%total)	Combined literature range (%total)
(C18:2)					
Linolenic acid (C18:3)	1.2±0.014	1.2±0.014	0.935	0.97 – 1.35	0.57 – 2.25
Arachidic acid (C20:0)	0.42±0.03	0.41±0.03	0.005	0.32 – 0.53	0.28 – 0.965
Eicosenoic acid (C20:1)	0.21±0.008	0.21±0.008	0.583	0.21 – 0.31	0.17 – 1.917
Behenic acid (C22:0)	0.17±0.018	0.16±0.018	0.167	0.057 – 0.23	0.11 – 0.349

[1] mauve shading represents MON87427 means that are significantly lower than the control means while orange shading represents MON87427 means that are significantly higher.

6.3.3 Amino acids

Levels of 18 amino acids were measured. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine.

The results in Table 7 show there was no significant difference between the control and corn MON87427 for any of the analyte means.

Table 7: Mean percentage dry weight (dw), relative to total dry weight, of amino acids in grain from MON87427 and LH198 x LH287.

Analyte	MON87427 (%dw)	LH198 x LH287 (%dw)	Overall treat effect (P-value)	Tolerance range (%dw)	Combined literature range (%dw)
Alanine	0.75±0.061	0.76±0.061	0.857	0.58 – 0.98	0.439 – 1.393
Arginine	0.48±0.024	0.49±0.025	0.501	0.34 – 0.57	0.119 – 0.639
Aspartate	0.64±0.041	0.64±0.042	0.92	0.52 – 0.78	0.335 – 1.208
Cystine	0.24±0.01	0.24±0.01	0.75	0.18 – 0.26	0.125 – 0.514
Glutamate	1.87±0.15	1.89±0.015	0.801	1.46 – 2.49	0.965 – 3.536
Glycine	0.38±0.018	0.38±0.018	0.906	0.32 – 0.43	0.184 – 0.539
Histidine	0.3±0.013	0.3±0.013	0.867	0.22 – 0.33	0.137 – 0.434
Isoleucine	0.35±0.026	0.36±0.027	0.901	0.27 – 0.46	0.179 – 0.692
Leucine	1.23±0.11	1.25±0.11	0.725	0.93 – 1.69	0.642 – 2.492
Lysine	0.3±0.012	0.3±0.013	0.782	0.26 – 0.34	0.172 – 0.668
Methionine	0.24±0.019	0.24±0.019	0.964	0.17 – 0.25	0.124 – 0.468
Phenylalanine	0.51±0.04	0.52±0.04	0.714	0.39 – 0.66	0.244 – 0.930
Proline	0.9±0.067	0.9±0.067	0.889	0.66 – 1.07	0.462 – 1.632
Serine	0.47±0.033	0.48±0.033	0.625	0.38 – 0.59	0.235 – 0.769
Threonine	0.35±0.02	0.35±0.02	0.871	0.28 – 0.41	0.224 – 0.666
Tryptophan	0.054±0.0032	0.053±0.0033	0.835	0.039 – 0.063	0.0271 – 0.215
Tyrosine	0.29±0.029	0.3±0.029	0.874	0.11 – 0.43	0.103 – 0.642
Valine	0.48±0.029	0.49±0.029	0.93	0.38 – 0.58	0.266 – 0.855

6.3.4 Minerals

The levels of nine minerals in grain from MON87427 and LH198 x LH287 were measured. Sodium was below the LOQ. Results for the remaining analytes are given in Table 8 and show there was no significant difference between the control and corn MON87427 for any of the analyte means.

Table 8: Mean levels of minerals in the grain of MON87427 and LH198 x LH287

Analyte	Unit	MON87427	LH198 x LH287	Overall treat effect (P-value)	Tolerance range (%dw)	Combined literature range (%dw)
Calcium	%dw	0.006± 0.00063	0.0055± 0.00063	0.176	0.0038 – 0.0068	0.00127 – 0.02084
Copper	mg/kg dw	1.63±0.11	1.71±0.12	0.458	1.10 – 2.62	0.73 – 18.5
Iron	mg/kg dw	23.61±0.78	23.03±0.79	0.368	16.55 – 24.10	10.42 – 49.07
Magnesium	%dw	0.13±0.0033	0.13±0.0033	0.952	0.11 – 0.15	0.0594 – 0.194
Manganese	mg/kg dw	7.91±1.06	8.07±1.06	0.567	4.00 – 9.17	1.69 – 14.30
Phosphorus	%dw	0.34±0.0034	0.34±0.0036	0.185	0.28 – 0.37	0.147 – 0.533
Potassium	%dw	0.40±0.0074	0.40±0.0077	0.546	0.33 – 0.46	0.181 – 0.603
Zinc	mg/kg dw	22.67±1.06	23.99±1.07	0.225	17.30 – 25.45	6.5 – 37.2

6.3.5 Vitamins

Levels of the seven vitamins measured are given in Table 9 and show there was no significant difference between the control and corn MON87427 for any of the analyte means.

Table 9: Mean weight (mg/k g dry weight) of vitamins in grain from MON87427 and LH198 x LH287.

Analyte	MON87427 (mg/kg dw)	LH198 x LH287 (mg/kg dw)	Overall treat effect (P-value)	Tolerance range (mg/kg dw)	Combined literature range (mg/kg dw)
Folic acid	0.36±0.025	0.39±0.025	0.347	0.24 – 0.57	0.147 – 1.464
Vitamin A (β-carotene)	1.01±0.05	0.96±0.051	0.186	0.58 – 1.50	0.19 – 46.81
Vitamin B_1 (Thiamine HCl)	2.97±0.19	2.88±0.20	0.606	2.41 – 3.48	1.26 – 40.00
Vitamin B_2 (Riboflavin)	2.09±0.37	1.93±0.37	0.63	1.28 – 3.29	0.50 – 2.36
Vitamin B_3 (Niacin)	27.22±2.15	27.71±2.18	0.722	20.63 – 43.08	10.37 – 46.94
Vitamin B_6 (Pyridoxine HCl)	7.48±0.60	7.71±0.60	0.589	5.24 – 10.29	3.68 – 11.32
Vitamin E (α-tocopherol)	13.14±2.09	13.46±2.10	0.718	6.67 – 17.34	1.5 – 68.7

6.3.6 Anti-nutrients

Levels of two key anti-nutrients were measured. Results in Table 10 show that mean raffinose levels did not differ significantly between MON87427 and the control. The mean

level of phytic acid was significantly lower in grain of MON87427 compared with grain from the control but the mean fell within both the tolerance range and combined literature range.

Table 10: Mean percentage dry weight (dw), relative to total dry weight, of anti-nutrients in grain from MON87427 and LH198 x LH287.

Analyte	MON87427[1] (%dw)	LH198 x LH287 (%dw)	Overall treat effect (P-value)	Tolerance range (%dw)	Combined literature range (%dw)
Phytic acid	0.96±0.031	1.02±0.031	0.008	0.82 – 1.07	0.111 – 1.570
Raffinose	0.14±0.028	0.15±0.029	0.524	0.092 – 0.21	0.020 – 0.320

[1] mauve shading represents MON87427 means that are significantly lower than the control means while orange shading represents MON87427 means that are significantly higher.

6.3.7 Secondary metabolites

Two key secondary metabolites were measured (see Table 11). There was no significant difference between the control and corn MON87427 for either of the analyte means.

Table 11: Mean weight (µg/g dry weight) of vitamins in grain from MON87427 and LH198 x LH287.

Analyte	MON87427 (µg/g dw)	LH198 x LH287 (µg/g dw)	Overall treat effect (P-value)	Tolerance range (µg/g dw)	Combined literature range (µg/g dw)
Ferulic acid	2348.63±58.17	2387.92±60.24	0.640	1588.35 – 2630.98	291.9 – 3885.8
p-coumaric acid	204.94±17.45	205.00±17.54	0.994	124.16 – 250.30	53.4 – 576.2

6.3.8 Summary of analysis of key components

Statistically significant differences in the analyte levels found between grain of MON87427 and LH198 x LH287 are summarised in Table 12. These differences do not raise safety concerns for a number of reasons.

Firstly, for all analytes the MON87427 means fall within both the combined literature range and the tolerance range. Secondly, it is noted that the percentage differences between the lowest and highest levels in the tolerance range obtained from the reference non-GM lines are higher than the percentage differences between MON87427 and the control means for any analyte. Finally, there are no trends in the results.

Table 12: Summary of analyte levels found in grain of corn MON87427 that are significantly (P < 0.05) different from those found in grain of the control LH198 x LH287.

Analyte	Unit of measure.	MON87427	LH198 x LH287	% difference between means	MON87427 within tolerance range?	MON87427 within literature range?
Total Fat	% dw	3.5	3.69	5.4	yes	yes
Palmitic acid	% total	10.91	10.54	3.4	yes	yes
Stearic acid	% total	1.97	1.9	3.5	yes	yes
Oleic acid	% total	24.28	23.52	3.1	yes	yes
Linoleic acid	% total	60.84	62.06	2	yes	yes
Arachidic acid	% total	0.42	0.41	2.4	yes	yes
Phytic acid	% dw	0.96	1.02	6.2	yes	yes

6.4 Conclusion from compositional analysis

Detailed compositional analyses were done to establish the nutritional adequacy of grain from MON87427 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients. The levels were compared to levels in a) an appropriate non-GM hybrid line b) a tolerance range compiled from results taken for 12 non-GM hybrid lines grown under the same conditions and c) levels recorded in the literature. Only five of the 53 measured analytes deviated from the control in a statistically significant manner. However, all analytes fell within both the tolerance range and the historical range from the literature. It can therefore be concluded that grain from MON87427 is compositionally equivalent to grain from conventional corn varieties.

7. Nutritional impact

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

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

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (OECD 2003; EFSA 2008). Corn line MON87427 is the result of a simple genetic modification to confer herbicide tolerance with no intention to significantly alter nutritional parameters in the food. In addition, the extensive compositional analyses of grain that have been undertaken to demonstrate the nutritional adequacy of MON87427 indicate it is equivalent in composition to conventional corn cultivars. The introduction of food from corn line MON87427 into the food supply is therefore expected to have little nutritional impact and as such no additional studies, including animal feeding studies, are required.

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