

Supporting document 1

Safety Assessment Report – Application A1118

Food derived from Herbicide-tolerant Corn Line MON87419

Summary and conclusions

Background

A genetically modified (GM) corn line with OECD Unique Identifier MON-87419-8 (referred to as MON87419 in this document) has been developed by Monsanto Company.

MON87419 contains a demethylase gene from *Stenotrophomonas maltophilia* resulting in the expression of dicamba mono-oxygenase (DMO), a protein to confer tolerance to the herbicide dicamba. The corn also contains the phosphinothricin N-acetyltransferase (*pat*) gene from *Streptomyces viridochromogenes* resulting in the expression of the PAT protein, to confer tolerance to glufosinate ammonium herbicide. Neither of the genes introduced into MON87419, nor the expressed proteins, are new to the food supply, and both have been assessed previously.

In conducting a safety assessment of food derived from MON87419, a number of criteria have been addressed including: a characterisation of the transferred gene sequences, their origin, function and stability in the corn genome; the changes at the level of DNA, and protein in the whole food; compositional analyses; and evaluation of intended and unintended changes.

This safety assessment report addresses only food safety and nutritional issues of the GM food *per se*. 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

In terms of food production, corn is the world's dominant cereal crop. It has a long history of safe use in the food supply, dating back thousands of years. Sweet corn is consumed directly while corn-derived products are routinely used in a large number and diverse range of foods (e.g. cornflour, starch products, breakfast cereals and high fructose corn syrup). Corn is also widely used as a livestock feed.

Molecular characterisation

MON87419 was generated through *Agrobacterium*-mediated transformation. Molecular analyses of MON87419 indicate that a single copy of the expression cassette containing the linked *dmo* and *pat* genes, plus regulatory elements, is present at a single insertion site in the genome. The introduced genes are stably inherited from one generation to the next. There are no antibiotic resistance marker genes, nor extraneous plasmid sequences present in this line.

Characterisation and safety assessment of new substances

Newly expressed proteins

MON87419 contains two newly expressed proteins, DMO and PAT. The highest mean DMO level was 26 µg/g dw in leaf, and the lowest was 0.19 µg/g dw in grain. The highest mean PAT level was 11 µg/g dw in leaf, and the lowest was 0.93 µg/g dw in grain. Expression levels were generally low in all plant tissues, but particularly low in grain which is the source of food products for human consumption.

The identity of the MON87419-produced proteins was confirmed by Western blot analysis, sequence analysis, matrix assisted laser desorption/ionization time-of-flight mass spectrometry, liquid chromatography-tandem mass spectrometry, and specific functional enzyme assays. Indirect evidence also indicated that neither of the newly expressed proteins is glycosylated in MON87419.

Bioinformatics studies on each of the proteins were updated and confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens. Laboratory studies demonstrated the DMO and PAT proteins are susceptible to the action of digestive enzymes and would be thoroughly degraded before they could be absorbed during passage through the gastrointestinal tract. The proteins are also susceptible to heat denaturation at the high temperatures typically used in food processing. Taken together, the evidence supports the conclusion that neither DMO nor PAT is toxic or allergenic in humans.

Compositional analyses

Detailed analyses were carried out, according to guidance provided in OECD consensus documents, on grain harvested from MON87419 (treated in the field with dicamba and glufosinate) and the conventional counterpart to establish the nutritional adequacy of food derived from MON87419 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients characteristic of corn. In all, 53 components were statistically analysed. No biologically significant differences were found between MON87419 and the conventional control; all analytes fell within the range of natural variation published in the literature. Grain from MON87419 is therefore 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 line MON87419. On the basis of data provided in accordance with the FSANZ Handbook, and other available information, food derived from MON87419 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
BLAST	Basic Local Alignment Search Tool
<i>bar</i>	Coding sequence of phosphinothricin N-acetyltransferase gene from <i>Streptomyces hygroscopicus</i> encoding PAT
bp	base pairs
CTP	Chloroplast transit peptide
<i>dmo</i>	Coding sequence of dicamba mono-oxygenase gene from <i>Stenotrophomonas maltophilia</i>
DMO	Dicamba mono-oxygenase protein
DNA	deoxyribonucleic acid
T-DNA	transfer DNA
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FARRP	Food Allergy Research and Resource Program
FASTA	Fast Alignment Search Tool - All
fw	fresh weight of tissue
GM	genetically modified
ILSI-CCDB	International Life Sciences Institute – Crop Composition Database
JSA	Junction Sequence Analysis
JSC	Junction Sequence Class
kDa	kilo Dalton
LB	Left Border of T-DNA (<i>Agrobacterium tumefaciens</i>)
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI-TOF MS	matrix-assisted laser desorption/ionisation–time of flight mass spectrometry
NCBI	National Centre for Biotechnology Information
NDF	neutral detergent fibre
NGS	Next Generation Sequencing
OECD	Organisation for Economic Co-operation and Development
OGTR	Australian Government Office of the Gene Technology Regulator
ORF	open reading frame
<i>pat</i>	Coding sequence of phosphinothricin N-acetyltransferase (PAT) gene from <i>Streptomyces viridochromogenes</i>
PAT	Phosphinothricin N-acetyltransferase protein
PCR	polymerase chain reaction
RB	Right Border of T-DNA (<i>Agrobacterium tumefaciens</i>)
RNA	ribonucleic acid
mRNA	messenger RNA
SAS	Statistical Analysis Software
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	standard error of mean

1 Introduction

Monsanto Australia Limited has submitted an application to FSANZ to vary Schedule 26 in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) corn with OECD Unique Identifier MON-87419-8 (herein referred to as MON87419). This corn has been genetically modified for tolerance to two herbicides: dicamba and glufosinate ammonium. Dicamba is used to control annual and biennial weed species and suppress the growth of perennial broadleaf weeds and woody plant species. Glufosinate ammonium-based herbicides provide nonselective control of approximately 120 broadleaf and grass weeds from emergence to early bloom growth stage. The use of both herbicides together on corn crops is anticipated to provide more effective weed management, including the control of weeds tolerant to another herbicide, glyphosate.

Tolerance to dicamba is achieved through expression of a dicamba mono-oxygenase (DMO) protein encoded by a gene from *Stenotrophomonas maltophilia*. The DMO protein rapidly demethylates dicamba to the herbicidally inactive metabolite 3,6-dichlorosalicylic acid (DCSA). Expression of the enzyme phosphinothricin N-acetyltransferase (PAT), encoded by the *pat* gene from *Streptomyces viridochromogenes*, confers tolerance to glufosinate herbicides. The PAT protein acetylates the free amino group of glufosinate to produce the herbicidally inactive metabolite, 2-acetamido-4-methylphosphinico-butanoic acid (N-acetyl glufosinate). Both DMO and PAT have been assessed by FSANZ previously as newly expressed proteins in several GM crops.

It is likely that MON87419 will be combined with other approved corn lines, including glyphosate-tolerant lines, using traditional plant breeding methods. The rationale for combining multiple herbicide tolerance traits is to improve control of a greater variety of weeds, minimise or delay the development of herbicide resistance in weedy species, and improve crop yield with the use of herbicides supported by evidence-based safety records.

Corn line MON87419 was primarily developed for agriculture in North America, and therefore approval for cultivation in Australia or New Zealand is not being sought. If approved, food derived from this line may enter the Australian and New Zealand food supply as imported food products. A commercial trade name for MON87419 has not yet been determined.

2 History of use

2.1 Host organism

Corn (*Zea mays*) is also referred to as maize and has been cultivated for human consumption and other uses for thousands of years. It has been studied extensively due to its economic importance in many industrialised countries of the world.

Mature corn plants consist of 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 agronomically undesirable, since the random nature of the crossing leads to 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. The resulting hybrid varieties are known to be superior to open-pollinated varieties in terms of their consistently higher yields and other performance characteristics.

In terms of production, corn is the world's dominant cereal crop (2015 forecast = 1,007 MT¹) ahead of wheat (723 MT) and rice (499 MT) and is grown in over 160 countries (FAOSTAT3 2015). In 2013, worldwide production of corn was over 1 billion tonnes, with the United States and China being the major producers (~353 and 217 million tonnes, respectively) (FAOSTAT3 2015). Corn is not a major crop in Australia or New Zealand and in 2013, production was approximately 506,000 and 201,000 tonnes respectively (FAOSTAT3 2015). In the U.S. it is estimated that approximately 93% of all corn planted is GM² while in Canada, the estimate of GM corn is approximately 80% of the total corn³. No GM corn is currently grown commercially in Australia or New Zealand.

Limited domestic production is supplemented by importing corn grain and corn-based products. Imports to Australia and New Zealand included 6,050 and 2,096 tonnes respectively of corn flour and 3,455 and 13 tonnes respectively of corn oil (FAOSTAT3 2015). Corn is a major source of crystalline fructose and high fructose corn syrup, both of which are processed from corn starch. Approximately 3,000 tonnes of crystalline fructose, but negligible high fructose corn syrup, were imported into Australia in 2011 (Green Pool 2012); neither Australia nor New Zealand currently produce fructose (either crystalline or as high fructose corn syrup). Corn products are used widely in processed foods.

Dent corn is the most commonly grown for grain and silage and is the predominant type grown in the U.S. (OGTR 2008). The conventional parent line LH244 used to produce MON87419, is a patented hybrid corn line assigned to Holden's Foundation Seeds LLC in 2001 (Armstrong 2001). It is a medium season, yellow dent corn line that is adapted to the central regions of the U.S. corn-belt.

Dent corn is processed by one of two main production methods (White and Pollak 1995):

- Dry milling gives rise to food by-products such as corn meal, flour and hominy grits.
- Wet milling (see Figure 1), which involves steeping the grain, coarse and fine grinding, centrifugation and evaporating the steep, to yield food by-products such as starch (for corn starch, corn syrup and individual sweeteners such as dextrose and fructose) and germ (for oil) (CRA 2006).

¹ FAO Cereal Supply & Demand Brief, <http://www.fao.org/worldfoodsituation/csdb/en/>

² USDA, Economic Research Service, - <http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us.aspx>

³ USDA Gain Report, CA14062, 2014 - http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Ottawa_Canada_7-14-2014.pdf

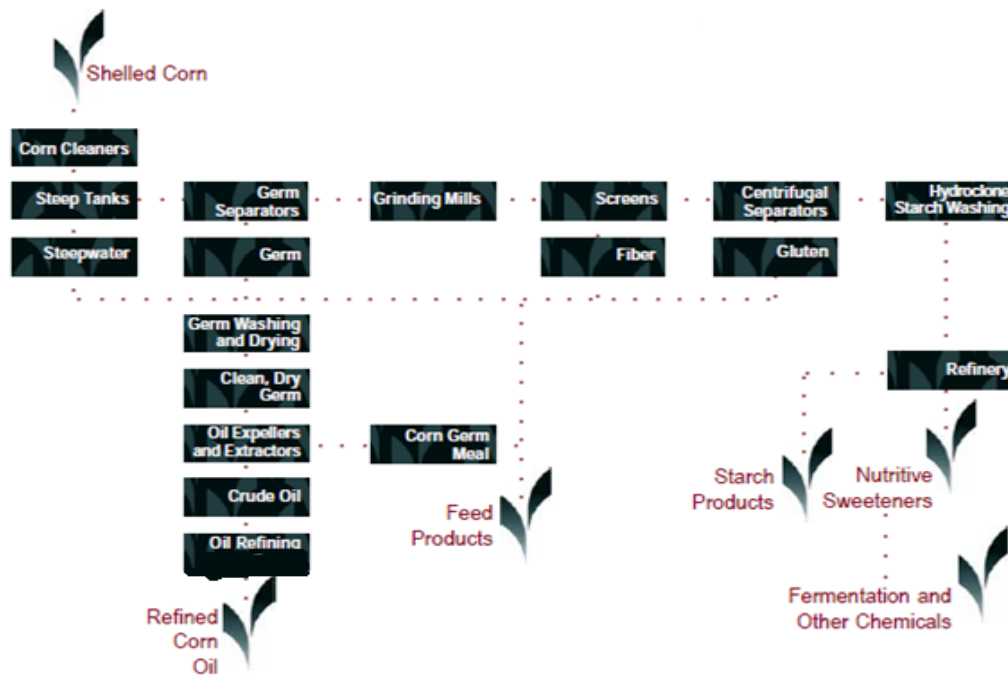


Figure 1: The corn wet milling process [diagram from CRA (2006)]

2.2 Donor organisms

2.2.1 *Stenotrophomonas maltophilia*

The *dmo* gene is derived from the bacterium *Stenotrophomonas maltophilia* strain DI-6, isolated from soil at a dicamba manufacturing plant (Krueger et al, 1989). This bacterium has been assessed as a donor organism previously under Applications A1063: food derived from soybean line MON87708, and A1080: food derived from cotton line MON88701. The organism was originally named *Pseudomonas maltophilia*, but was subsequently changed to *Xanthomonas maltophilia*, before it was given its own genus (Palleroni and Bradbury, 1993).

S. maltophilia is an aerobic, gram negative bacterium commonly present in aquatic environments and soil. It is also commonly associated with plants (Ryan et al., 2009) and has been isolated from the rhizosphere of wheat, maize, grasses, beet, cucumber, potato, strawberry, sugarcane and rapeseed, and has also been isolated from cottonseed, bean pods and coffee. *S. maltophilia* is widespread in moist sites in domestic houses, particularly in bathrooms and kitchens, as well as a variety of foods such as fruits, vegetables, frozen fish, milk and poultry (Denton et al., 1998).

Infections in humans caused by *S. maltophilia* are rare; it is found in healthy individuals without any adverse associations. Although not regarded as virulent, *S. maltophilia* can be an opportunistic pathogen when conditions are more favourable, for example in immunocompromised hospital patients (Looney, 2009), particularly those with ventilator tubes or catheters inserted for prolonged periods of time.

2.2.2 *Streptomyces viridochromogenes*

The source of the *pat* gene is the bacterial species *Streptomyces viridochromogenes*. The *Streptomycetaceae* bacteria were first described in the early 1900's and are widespread in the environment. These organisms are generally soil-borne, although they may also be isolated from water. They are not typically pathogenic to animals including humans, and few species have been shown to be phytopathogenic (Kützner, 1981).

The *bar* gene from the closely related species *S. hygroscopicus* produces a protein that is structurally and functionally equivalent to the protein encoded by the *pat* gene (Wehrmann *et al.*, 1996). Although *S. viridochromogenes* and *S. hygroscopicus* are not used in the food industry directly, the *pat* and *bar* genes have been used to confer tolerance to glufosinate ammonium herbicides in food producing crops for almost two decades.

2.2.3 Other organisms

Genetic elements from several other organisms, mainly plants such as wheat and rice, have been used in the expression cassette used to construct MON87419 (refer to Table 1). These non-coding sequences have various gene regulatory functions within the cassette. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic element derived from the caulimovirus Peanut Chlorotic Streak Virus (PCISV), a plant pathogen, is not pathogenic in humans, nor does it cause pathogenic symptoms in MON87419.

3 Molecular characterisation

Molecular characterisation provides 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 requires consideration of:

- 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:

2014. Amended Report for MSL0025438: Molecular Characterization of Herbicide Tolerant Corn MON 87419. **MSL0025902**. Monsanto Company (unpublished)
 2014. Segregation Analysis of the T-DNA Insert in Herbicide Tolerant Corn MON 87419 Across Three Generations. **MSL0025519** Monsanto Company (unpublished)
 2014. Bioinformatics Evaluation of DNA Sequences Flanking the 5' and 3' Junctions of Inserted DNA in MON 87419: Assessment of Putative Polypeptides. **MSL0025920**. Monsanto Company (unpublished)

3.1 Method used in the genetic modification

Immature embryos excised from maize line LH244 (parent line) were transformed by co-culturing with *Agrobacterium tumefaciens* carrying the plasmid vector PV-ZMHT507801 (see Figure 2), following the method of Sidorov and Duncan (2009). The plasmid contained two unlinked T-DNA regions, one containing the *cp4epsps* gene which was used as a selectable marker for successfully transformed cells, the second containing the two genes conferring resistance to dicamba and glufosinate herbicides. The embryos were subsequently placed on selection medium containing glyphosate and carbenicillin, to inhibit the growth of untransformed plant cells and excess *Agrobacterium* respectively, and to permit the development of callus tissue. Resulting callus was then placed in a medium that supported shoot regeneration and root development.

Rooted plants (generation R₀) with normal phenotype and containing both the glyphosate (*cp4epsps*) expression cassette (T-DNA II) and the DMO/ PAT expression cassette (T-DNA I) were self-pollinated to produce R1 seed. Plants that grew from this seed were then further selected using a polymerase chain reaction (PCR) method to identify those that were positive for only the DMO/PAT cassette (T-DNA I).

Plants that were homozygous for T-DNA I and not containing the *cp4epsps* cassette were then selected for further assessment and development (see Section 3.3). Many thousands of transformation events were evaluated in the laboratory and greenhouse before MON87419 was ultimately chosen as the lead event, based on superior agronomic, phenotypic and molecular characteristics.

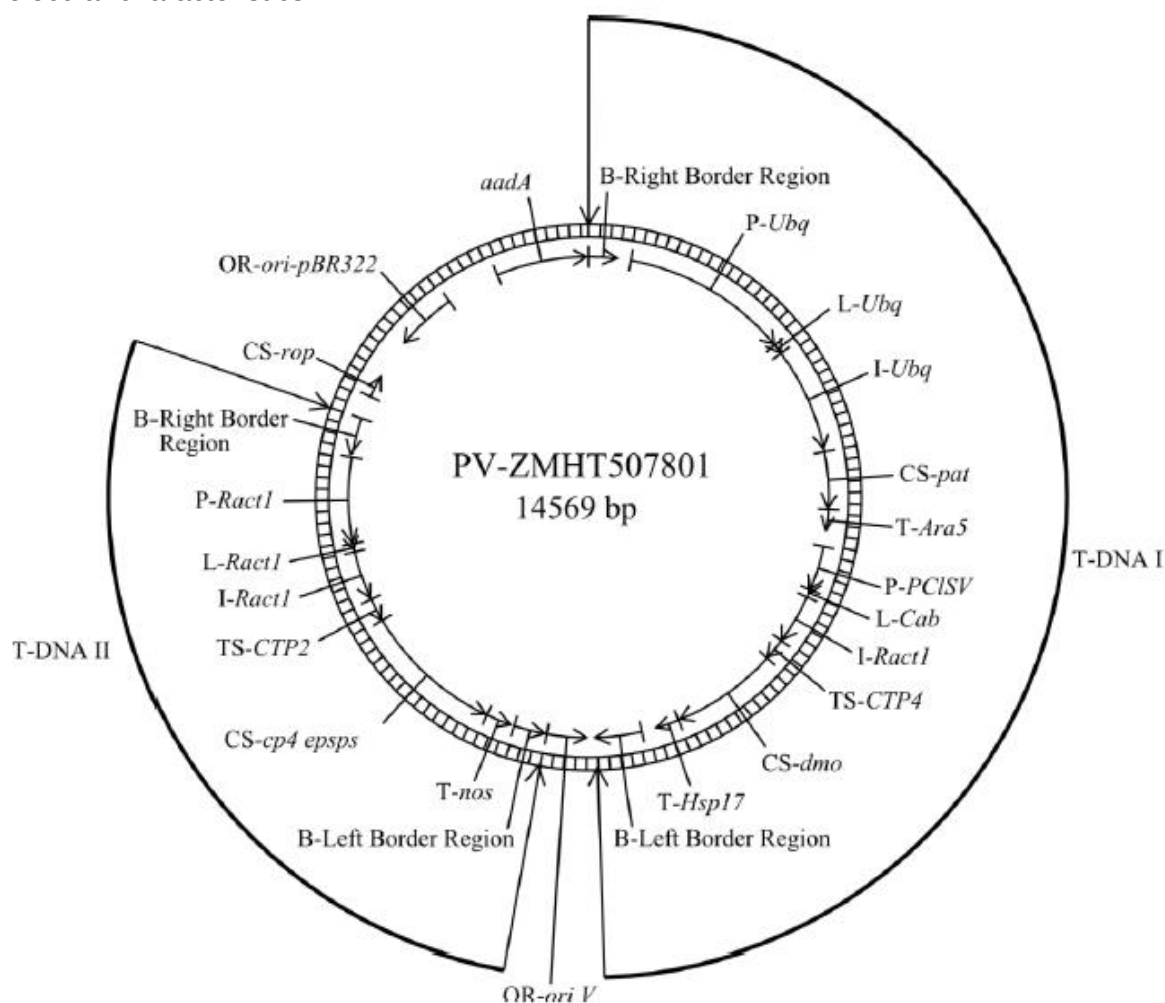


Figure 2: Map of plasmid PV-ZMHT507801 showing genes in T-DNA I and T-DNA II

3.2 Function and regulation of introduced genes

Information on the genetic elements in the two separate T-DNA regions of the transforming plasmid PV-ZMHT507801 is summarised in Tables 1 and 2. T-DNA I is comprised of two linked gene expression cassettes, one encoding the PAT protein (glufosinate resistance) and the other encoding the DMO protein (dicamba resistance). T-DNA II contains a single expression cassette encoding the EPSPS protein. Expression of this protein confers tolerance to glyphosate which was used only as a selectable marker immediately after transformation. Subsequent crossing of transformants gave rise to progeny that did not contain T-DNA II. The complete plasmid is 14,569 bp in size.

Table 1: Description of the genetic elements in T-DNA I of PV-ZMHT507801

Genetic element	Relative nt location on plasmid	Source	Description & Function	References
T-DNA I				
RIGHT BORDER	1 - 285	<i>A. tumefaciens</i>		
Intervening sequence	286 - 410			
P-Ubq	411 - 2054	<i>Andropogon gerardii</i> (big bluestem grass)	Promoter for a ubiquitin gene (<i>Ubq</i>) • Initiates and directs transcription of the <i>pat</i> gene	Joung and Kamo, 2006
L-Ubq	2055 - 2153	<i>Andropogon gerardii</i> (big bluestem grass)	5' UTR leader sequence of the ubiquitin gene <i>Ubq</i> • Regulates expression of the <i>pat</i> gene	Joung and Kamo, 2006
I-Ubq	2154 - 3195	<i>Andropogon gerardii</i> (big bluestem grass)	Intron sequence of the ubiquitin gene <i>Ubq</i> • Regulates expression of the <i>pat</i> gene	Joung and Kamo, 2006
Intervening sequence	3196 - 3200			
CS - <i>pat</i>	3201 - 3752	<i>Streptomyces viridochromogenes</i>	Gene coding sequence of phosphinothricin N-acetyltransferase (PAT) protein • Confers tolerance to glufosinate-ammonium herbicides	Wehrmann et al., 1996; Wehrmann et al., 1988
Intervening sequence	3753 - 3760			
T-Ara5	3761 - 3973	<i>Oryza sativa</i> (rice)	3' UTR sequence of the RA5B precursor gene, encoding an alpha-amylase/trypsin inhibitor (<i>Ara5</i>) • Directs polyadenylation of mRNA	Hunt, 1994
Intervening sequence	3974 - 4120			
P-PCISV	4121 - 4553	Peanut Chlorotic Streak caulimovirus (PCISV)	Promoter for the full length transcript of PCISV • Directs transcription in plant cells.	Maiti & Shepherd, 1998
Intervening sequence	4554 - 4558			
L-Cab	4559 - 4619	<i>Triticum aestivum</i> (wheat)	5' UTR leader sequence from chlorophyll a/b-binding (CAB) protein • Regulates expression of the <i>dmo</i> gene	Lamppa et al. 1985
Intervening sequence	4620 - 4635			
I-Ract1	4636 - 5115	<i>Oryza sativa</i> (rice)	Intron and flanking UTR sequence of the <i>act1</i> gene, encoding rice Actin 1 protein • Regulates expression of the <i>dmo</i> gene	McElroy et al. 1990
Intervening sequence	5116 - 5124			
TS ⁷ -CTP4	5125 - 5340	<i>Petunia hybrida</i>	Targeting and 5' UTR leader sequence of the <i>ShkG</i> gene, encoding the EPSPS transit peptide region • Directs protein to the chloroplast	Gasser et al 1988; Herrmann, 1995

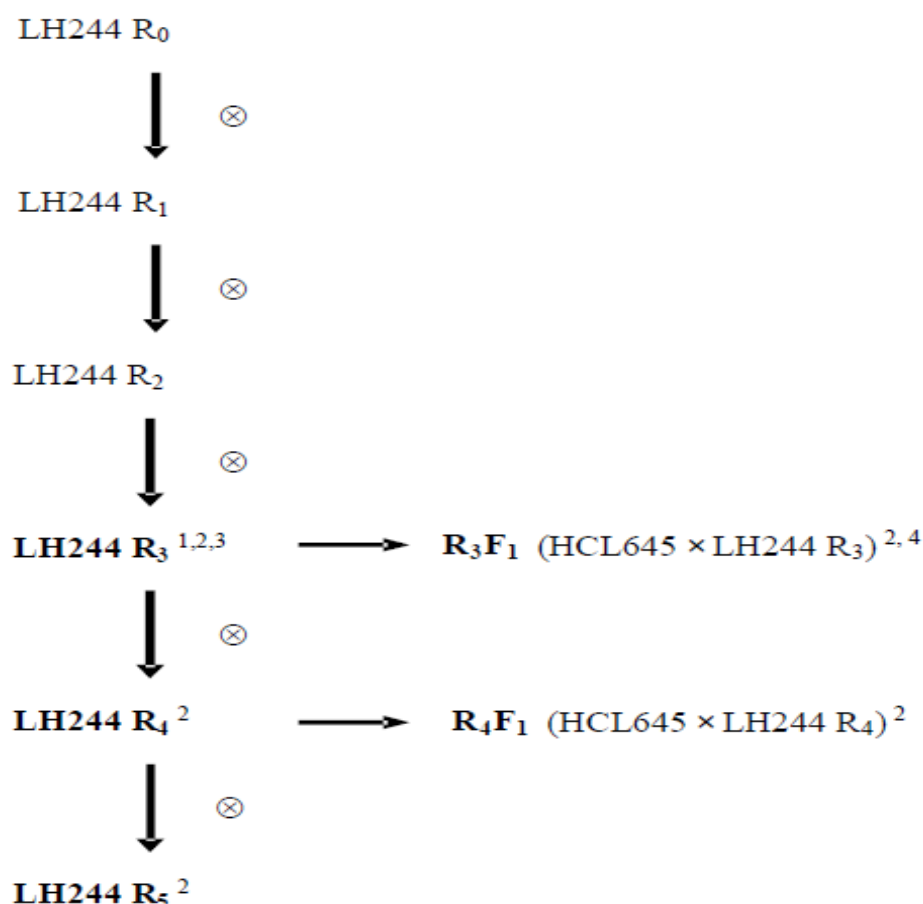
Genetic element	Relative nt location on plasmid	Source	Description & Function	References
T-DNA I				
CS-dmo	5341 - 6363	<i>Stenotrophomonas maltophilia</i>	Coding sequence for the dicamba mono-oxygenase (DMO) protein (codon optimized) Expression of DMO confers resistance to dicamba	Herman et al, 2005; Wang et al, 1997
Intervening sequence	6364 - 6393			
T-Hsp17	6394 - 6603	<i>Triticum aestivum</i> (wheat)	3' UTR sequence from a heat shock protein Hsp17 Directs polyadenylation of the mRNA	McElwain & Spiker, 1989
Intervening sequence	6604 - 6765			
LEFT BORDER	6766 - 7207	<i>Agrobacterium tumefaciens</i>		

Table 2: Description of the genetic elements in T-DNA II of PV-ZMHT507801

Genetic element	Relative nt location on plasmid	Source	Description & Function	References
T-DNA II				
LEFT BORDER	7697 - 8015	<i>A. tumefaciens</i>		
Intervening sequence	8016 - 8045			
T-nos	8046 - 8298	<i>Agrobacterium tumefaciens</i>	3' UTR sequence of the nopaline synthase (nos) gene. • Directs polyadenylation of the mRNA	Bevan et al, 1983; Fraley et al, 1983
Intervening sequence	8299 - 8313			
CS-cp4 epsps	8314 - 9681	<i>Agrobacterium</i> sp strain CP4	Coding sequence of the <i>aroA</i> gene encoding CP4 EPSPS protein. • Directs polyadenylation of the mRNA	McElwain & Spiker, 1989
TS-CTP2	9682 - 9909	<i>Arabidopsis thaliana</i>	Targeting sequence of the <i>ShkG</i> gene encoding the transit peptide. • Directs transport of the EPSPS to the chloroplast.	Herrmann, 1995; Klee et al 1987
Intervening sequence	9910 - 9918			
I-Ract1	9919 - 10396	<i>Oryza sativa</i> (rice)	Intron and flanking UTR sequence of the <i>act1</i> gene, encoding rice Actin 1 protein • Regulates expression of the <i>cp4-epsps</i> gene	McElroy et al. 1990
L-Ract1	10397 - 10476	<i>Oryza sativa</i> (rice)	Leader sequence of the <i>act1</i> gene, encoding rice Actin 1 protein • Regulates expression of the <i>cp4 epsps</i> gene.	McElroy et al. 1990
P-Ract1	10477 - 11317	<i>Oryza sativa</i> (rice)	Promoter sequence of the <i>act1</i> gene, encoding rice Actin 1 protein • Directs transcription in plant cells.	McElroy et al. 1990
Intervening sequence	11318 - 11343			
RIGHT BORDER REGION	11344 - 11700	<i>Agrobacterium tumefaciens</i>		

3.3 Breeding of MON87419

The breeding pedigree for the various generations is given in Figure 3. From a single R_0 plant, several rounds of self-pollination and seed bulking occurred in order to produce specific generations of plants that were used in characterisation and analysis, as indicated in Table 3. At the R_3 and R_4 generations, plants were crossed with conventional proprietary hybrid lines and the progeny were used to generate information on insert stability.



R_0 corresponds to the transformed plant, $F\#$ is the filial generation, $\otimes$ designates self pollination.

¹ Generation used for molecular characterization

² Generations used to confirm insert stability

³ Generation used for commercial development of MON 87419

⁴ Generation used for agronomic/phenotypic and compositional analysis studies

Figure 3: Breeding diagram for MON87419

Table 3: MON87419 generations used for various analyses

Analysis	MON87419 generation used	Control(s) used
Molecular characterisation (Section 3.4)	R ₃ (LH244)	LH244
Genetic stability (Section 3.5.1)	R ₃ (LH244) R ₃ F ₁ (HCL645 x LH244) R ₄ (LH244) R ₄ F ₁ (HCL645 x LH244) R ₅ (LH244)	LH244, HCL645 x LH244
Mendelian inheritance (Section 3.5.2)	BC ₁ F ₁ (A9600Z x LH244) BC ₂ F ₁ (A9600Z x LH244) BC ₂ F ₂ (A9600Z x LH244) (see Fig 6)	N/A
Protein characterisation (Section 4.1.3)	Plant-produced protein was compared to <i>E. coli</i> -produced protein used for safety studies; plant used was HCL645 x LH244 at R ₃ F ₁	Protein from <i>E. coli</i>
Protein expression levels in plant parts (Section 4.1.4)	All tissues except grain: R ₃ F ₁ (HCL645 x LH244) Grain: R ₃ F ₂ (HCL645 x LH244)	N/A
Compositional analysis (Section 5)	Grain: R ₃ F ₂ (HCL645 x LH244) Forage: R ₃ F ₁ (HCL645 x LH244) (data on forage not presented in this report)	HCL645 x LH244

3.4 Characterisation of the genetic modification in the plant

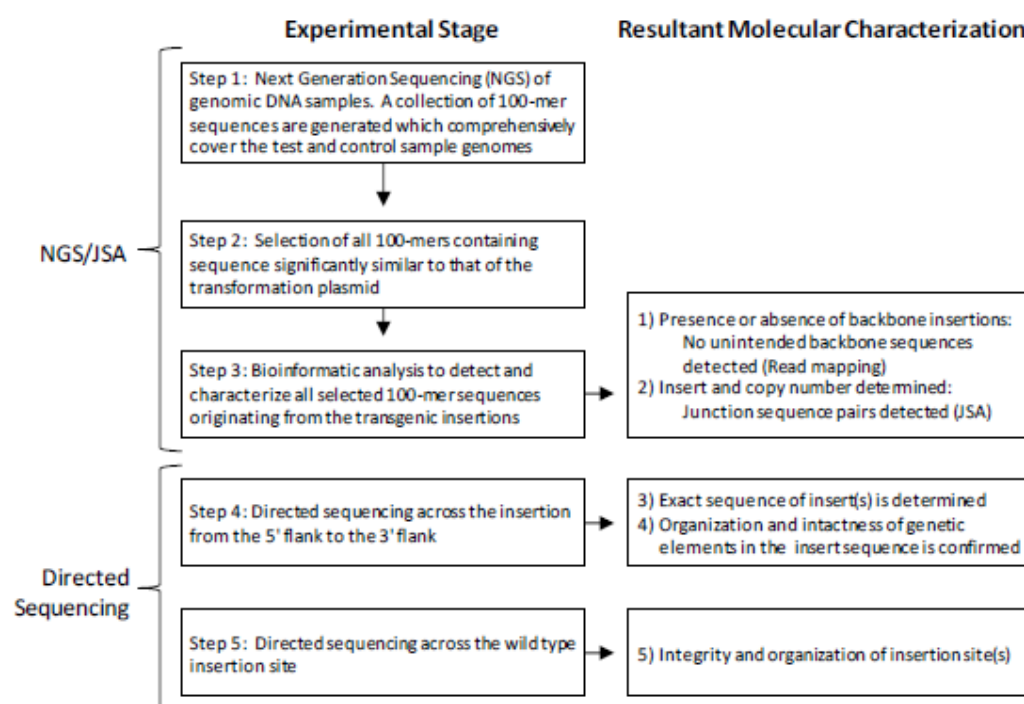
Several methods including DNA sequencing, PCR and bioinformatics were used to fully characterise the genetic modification in MON87419. The analyses focussed on the nature of the inserted genetic elements and whether any unintended genetic re-arrangements had occurred as a consequence of the transformation procedure. The Applicant supplied the flow-diagram shown in Figure 4 to illustrate the technical approach to molecular characterisation.

The approach used to characterise MON87419 is described in Kovalic et al. 2012, and involves a combination of Next Generation Sequencing (NGS) and Junction Sequence Analysis (JSA) together with bioinformatics to determine the number of inserts. This approach allows molecular characterisations equivalent to those achieved in the past with Southern Blot analysis.

The insertion of T-DNA into the plant genome creates two junction regions (i.e. 5' and 3') between the inserted DNA and genomic DNA. The rationale for JSA is that each insertion event will produce two unique junction sequences which are characteristic of that event. By evaluating the number of unique junctions detected, the number of insertion sites can be determined. In addition to this, information can be obtained about the presence or absence of any vector backbone sequences.

Directed sequencing (locus-specific PCR and DNA sequencing analyses) was carried out to complement the information provided by the NGS/JSA. Sequencing of the insert and flanking genomic DNA allowed a direct comparison with the sequence corresponding to T-DNA I in PV-ZMHT507801 to determine insert integrity and further characterise flanking regions.

NGS/JSA methodology was run on all five generations of MON87419 and the conventional controls. The information was used to determine the stability of the insert by evaluating the number and identity of DNA inserts in each generation.



Genomic DNA from MON 87419 and the conventional control was sequenced using Next Generation Sequencing (NGS), which can be used to reach the same conclusions that can be determined using traditional Southern blotting (Kovalic *et al.*, 2012). NGS technology produces a set of short, randomly distributed sequence reads (each approximately 100 bp long) that comprehensively covers both MON 87419 and control genomes (Step 1). Utilizing these genomic sequences, bioinformatics search tools were used to select all sequence reads that were significantly similar to PV-ZMHT507801 (Step 2) for use in read mapping to determine the presence/absence of backbone sequences and T-DNA II and Junction Sequence Analysis (JSA) bioinformatics to determine the insert and copy number (Step 3). Overlapping PCR products are also produced which span any inserts and their wild type loci (Step 4 and Step 5, respectively); these overlapping PCR products are sequenced to allow for detailed characterization of the inserted DNA and insertion site(s).

Figure 4: Steps in the molecular characterisation of MON87419

3.4.1 Insert number and presence of vector

Genomic DNA from seed obtained from five breeding generations of verified MON87419 (R₃, R₄, R₅, R₃F₁ and R₄F₁) and the untransformed parent (LH244) was isolated and analysed using Illumina®⁴ NGS technology. Reference DNA from the plasmid vector PV-ZMHT507801 was also used as a positive control. A sample of conventional control genomic DNA (LH244) was spiked with the plasmid DNA and analysed by NGS and bioinformatics to establish the sensitivity of the method.

The number of insertion sites in MON87419 was assessed by the NGS/JSA method using genomic DNA from the R₃ generation. This method used the entire plasmid vector sequence (T-DNA I, T-DNA II and vector backbone) as a query to determine the number of DNA insertion sites. The analysis identified two unique junction sequence classes (designated A and B). Figure 5 shows a map of the junction sequences (illustrated as stacked bars) that were detected. Both junction sequence classes contain the T-DNA I border sequence joined to genomic flanking sequence, indicating insertion of T-DNA I from PV-ZMHT507801. No junction sequences were found in the DNA from the conventional plant LH244. Mapping the sequence reads obtained from MON87419 and the control to PV-ZMHT507801 sequence also indicated that there were no plasmid backbone sequences present in MON87419.

Complete alignment of the junction sequence classes to the insert/flank sequence confirmed that MON87419 contains a single T-DNA I insert at a single genomic locus.

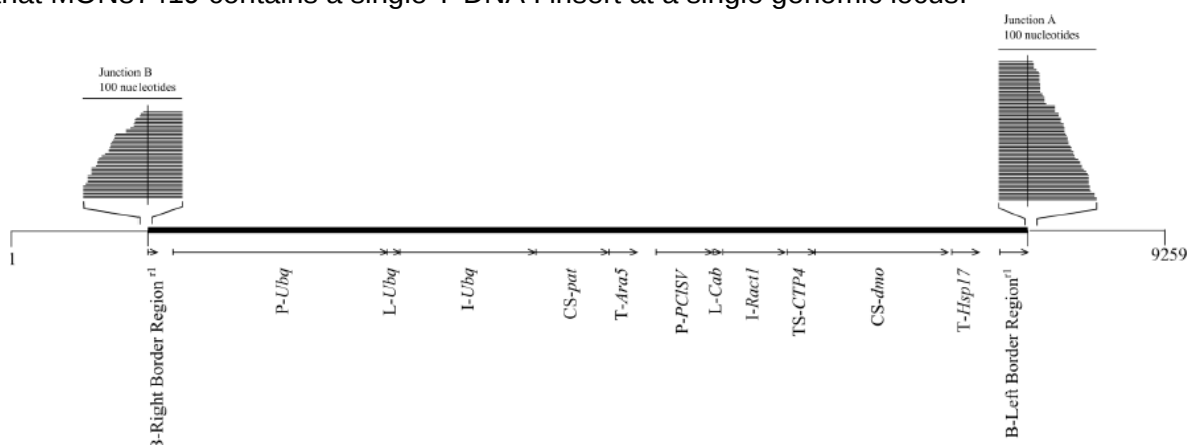


Figure 5: Schematic representation of the junction sequences detected in MON87419

3.4.2 Insert organization and sequence

Directed DNA sequence analysis was used to study the organisation of the elements within the DNA insert and the adjacent plant genomic DNA. PCR primers were designed to amplify six overlapping regions spanning the entire length of the inserted DNA in MON87419 and flanking regions; the amplified PCR products were subjected to DNA sequence analysis. A consensus sequence was generated by compiling sequences from multiple sequencing reactions performed on the PCR products. The consensus sequence was then aligned to the PV-ZMHT507801 sequence to determine the integrity and organisation of the insert and flanking regions.

The results showed that the insert is 6,762 bp in length and corresponds exactly with the sequence of T-DNA I of plasmid PV-ZMHT507801, with the exception of the left and right border regions, which both showed small terminal deletions.

⁴ <http://www.illumina.com/technology/next-generation-sequencing/sequencing-technology.ilmn>

It is common for truncations to occur in the LB and RB regions with *Agrobacterium*-mediated transformations, however, these sequences are not part of the expression cassettes and small deletions do not usually affect the function of the genetic elements within the T-DNA. This analysis confirmed the conclusion from the NGS/JSA analysis that a single copy of T-DNA I has been inserted in MON87419 and no vector backbone or sequences from T-DNA II are present.

In addition to the insert, 1,246 bp flanking the 5' end of the insert and 1,251 bp flanking the 3' end of the insert were sequenced. This sequence information was used for further study of the insertion site and for open reading frame (ORF) analysis (see Sections 3.4.3 and 3.4.4).

3.4.3 Insertion site analysis

In order to identify any changes to the plant genomic DNA as a result of the insertion event, PCR and sequence analysis were performed on DNA extracted from the parental control (LH244) using two primers, one specific to the 5' flanking sequence of MON87419 and one specific to the 3' sequence. The genomic DNA sequence of the untransformed parent was then compared with that of the flanking regions at the 5' and 3' ends of the DNA inserted into MON87419. The comparison showed that 602 bp of maize genomic DNA were deleted during integration of T-DNA I. Deletions such as this are relatively common with *Agrobacterium*-mediated transformations. MON87419 plants show a normal phenotype; the changes at the insertion site have had no discernible effect on the growth or productivity of the plants.

3.4.4 Open reading frame analysis

An *in silico* analysis of the flanking regions was done to identify whether any novel ORFs had been created in MON87419 as a result of the T-DNA insertion. Any novel ORFs that corresponded to putative peptides/polypeptides of eight amino acids or greater in length were investigated further to determine whether their amino acid sequence showed similarity with sequences in established databases⁵.

Sequences spanning the 5' and 3' junctions of the MON87419 insert were translated using DNASTar software⁶ from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames. A total of 11 ORFs (six in the 5' junction and five in the 3' junction) were identified that encode putative polypeptides ranging in size from 14 – 97 amino acids. No analysis was done to determine whether any potential regulatory elements were associated with the ORFs. For the T-DNA I insert, the DNA sequences in the sense and anti-sense strands were translated in six reading frames.

The resultant amino acid sequences were subjected to bioinformatics analyses described in more detail in Section 4.1.5.

3.5 Stability of the genetic changes in MON87419

Stability of the genetic changes refers to both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification (as produced in the initial transformation event) over successive generations. Molecular techniques, such as Southern blot analysis or NGS/JSA, are considered the most appropriate techniques for studying genetic stability. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

⁵ AD_2014, TOX_2014 and PRT_2014

⁶ <http://www.dnastar.com/>

It is often quantified by a trait inheritance analysis to determine Mendelian heritability via chemical, molecular or visual assay techniques.

3.5.1 Genetic stability

The genetic stability of MON87419 was evaluated by NGS/JSA (as described in Section 3.4.1) in verified genomic DNA isolated from the grain of plants from five breeding generations (refer to Figure 3 and Table 3). The four successive generations used to analyse stability were compared to the fully characterised R₃ generation. Control genomic DNA was isolated from the non-GM parental line LH244 and a conventional hybrid line (HCL645 x LH244), with similar background genetics to the R₃F₁ and R₄F₁ hybrids, as indicated in Table 3.

No junction sequences were detected in DNA obtained from the control lines (LH244, HCL645 x LH244). Molecular analysis of the MON87419 DNA from all generations showed the presence of the same two junction sequences as described in Section 3.4.1. No other junction sequences were present. The consistency of the JSA results across all generations tested, demonstrates that the single T-DNA I insert is stably maintained in MON87419.

3.5.2 Phenotypic stability

Since it was demonstrated that the insert is present at a single locus in the MON87419 genome, the expectation would be that the genetic elements within it would be inherited according to Mendelian principles.

Chi-square (χ^2) analysis was undertaken over several generations to confirm the segregation and stability of the complete T-DNA I insert in MON87419. The breeding path followed for this analysis (Figure 6) was different from that represented in Figure 3. The inheritance pattern was assessed in the BC₁F₁, BC₂F₁, and BC₂F₂ generations by PCR analysis and by using a glufosinate spray treatment to select for plants containing T-DNA I.

At the BC₁F₁ and BC₂F₁ generations, the MON87419 T-DNA was predicted to segregate at a 1:1 ratio (hemizygous positive:homozygous negative) according to Mendelian principles of inheritance. At the BC₂F₂ generation, the T-DNA was predicted to segregate at a 1:2:1 ratio (homozygous positive: hemizygous positive: homozygous negative) according to Mendelian principles.

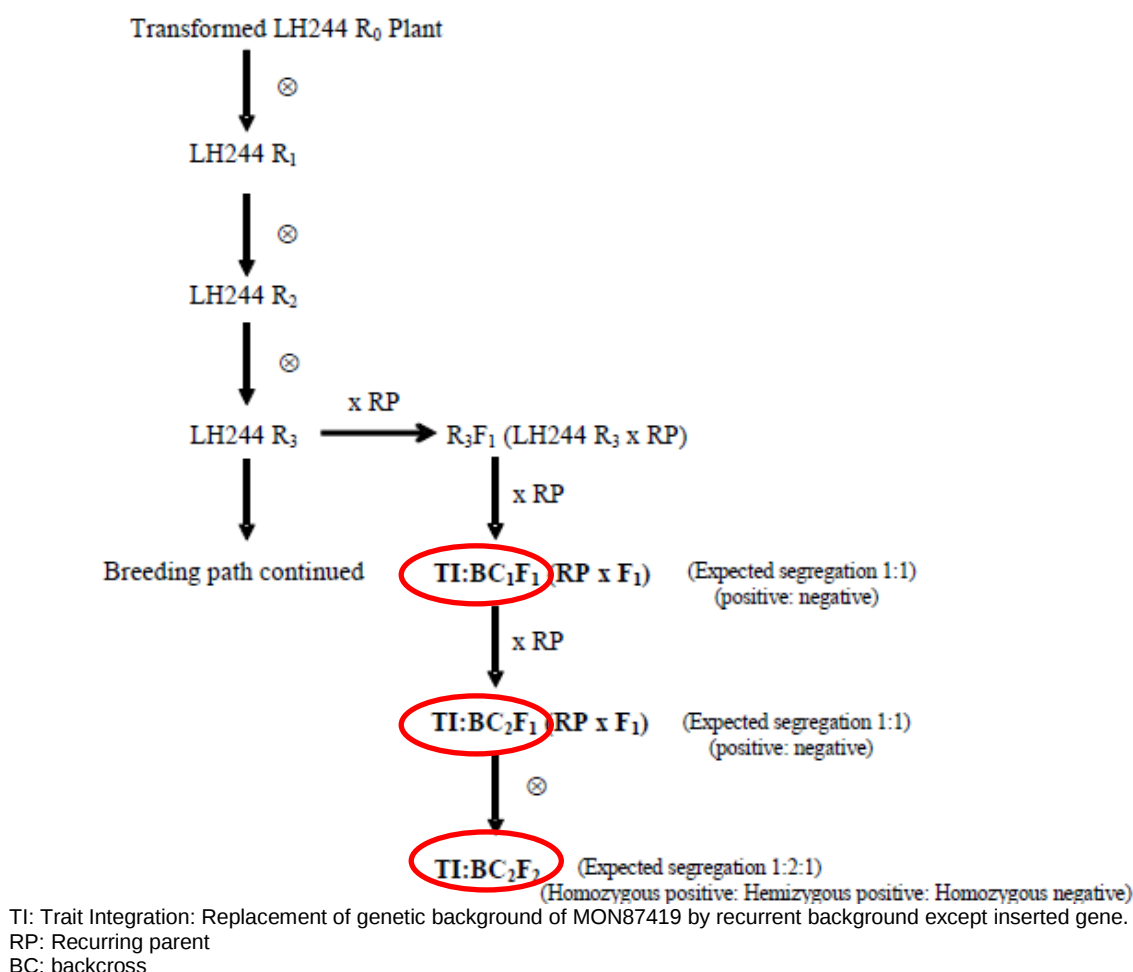


Figure 6: Breeding path for generating segregation data for MON87419

The results presented in Table 4 indicated there were no significant differences between the observed and expected segregation ratios in any of the generations. These data support the conclusion that T-DNA I is present at a single locus in MON87419 and is inherited predictably according to Mendelian principles in subsequent generations.

Table 4: Segregation of the Expression Cassette During Development of MON87419

Generation	Number of Plants	Observed Positives	Observed Negatives	1:1 Segregation			
				Expected Positives	Expected Negatives	χ^2	Probability
BC ₁ F ₁	126	64	62	63	63	0.03	0.859
BC ₂ F ₁	381	192	189	190.5	190.5	0.02	0.878

Generation	Number of Plants	Observed Homozygous Positives	Observed Hemizygous Positives	Observed Homozygous Negatives	1:2:1 Segregation			
					Expected Homozygous Positives	Expected Hemizygous Positives	Expected Homozygous Negatives	Probability
BC ₂ F ₂	164	48	83	33	41	82	41	0.251

3.6 Antibiotic resistance marker genes

No antibiotic resistance marker genes are present in corn line MON87419. The insert sequence analysis (Section 3.4.2) showed no plasmid backbone was integrated into the MON87419 genome during transformation.

The *aadA* gene, which is part of the plasmid backbone, was used as a bacterial selectable marker before the transformation procedure, and is not present in MON87419.

3.7 Conclusion

Comprehensive molecular analyses of MON87419 indicated a single T-DNA I containing intact *dmo* and *pat* expression cassettes was inserted into the corn genome at a single locus. The analyses also showed no T-DNA II or plasmid backbone sequences present in the line. No antibiotic resistance marker genes were transferred to MON87419. The introduced genes were maintained over five generations and were inherited as expected of a single locus according to Mendelian principles.

4 Characterisation and safety assessment of new substances

In considering the safety of newly expressed proteins it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, e.g., because they have anti-nutritional properties, or they can cause allergies in some consumers (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 or allergenic effects.

To effectively identify any potential hazards, knowledge of the characteristics, concentration and localisation of all newly expressed proteins in the organism as well as a detailed understanding of their biochemical function and phenotypic effects is required. It is also important to determine if the newly expressed protein is expressed in the plant as expected, including whether any post-translational modifications have occurred.

This assessment considered safety aspects of:

- The proteins expected to be present in the plant as intended from translation of the introduced genes. A number of different analyses were done to characterise the two newly expressed proteins present in MON87419.
- Possible translation products generated from ORFs as a result of the inserted T-DNA (see Section 3.4.4).

4.1 Newly expressed proteins

4.1.1 The DMO and PAT proteins

4.1.1.1 DMO

Tolerance to dicamba in MON87419 is conferred by the expression in the plant of DMO, a mono-oxygenase enzyme, that catalyses the demethylation of dicamba (3,6-dichloro-2-methoxy benzoic acid) to a non-herbicidal compound DCSA (3,6-dichlorosalicylic acid) and formaldehyde. The active form of the enzyme is a trimer of DMO monomers. This trimeric quaternary structure is the native form of the enzyme observed during crystallisation and is an absolute requirement for electron transfer (oxidation of NADH) and catalysis. Wildtype DMO was initially purified from the *S. maltophilia* strain DI-6, which was isolated from soil at a dicamba manufacturing plant (Krueger *et al* 1989).

The newly expressed DMO protein is active in the chloroplast. To target the protein to the organelle, additional coding sequence (*CTP4* derived from *Petunia*, Table 1) was included in the gene construct to enable translation of a precursor protein with a chloroplast transit peptide of 72 amino acids at the N-terminus of the DMO protein. Although leader (transit) peptides are typically clipped precisely from the precursor protein as uptake occurs, in some cases there is alternative processing resulting in forms of the protein with parts of the transit peptide remaining at the N-terminus.

Alternative processing of the precursor DMO protein has occurred in MON87419, giving rise to two forms of the enzyme. One form has retained 12 amino acids from the *CTP4* and is known as MON87419 DMO+12; the other form, known as MON87419 DMO+7, has retained seven amino acids from *CTP4*. The difference between these two forms is five amino acids of the transit peptide at the N-terminus of the precursor DMO protein. Except for the additional amino acids derived from *CTP4* (7 or 12) and an additional leucine at position two, the mature DMO protein in MON87419 has the same sequence as the wildtype DMO protein from the DI-6 strain of *S. maltophilia* (Herman *et al* 2005).

Similar, alternatively-processed DMO proteins have been assessed previously in Applications A1063 (MON87708 soybean) and A1080 (MON88701 cotton). A comparison of the alternatively-processed DMO proteins in the different plant lines with the wildtype bacterial DMO protein is presented in Figure 7. As indicated, in some forms of the enzyme including those in MON87419, the N-terminal methionine has also been retained. The differences between the wildtype and MON87419 DMO proteins were not expected to result in changes in overall structure, immunoreactivity, enzyme activity or substrate specificity. The N-terminus and position two are sterically distant from the catalytic site of the enzyme (D'Ordine *et al* 2009; Dumitru *et al* 2009).

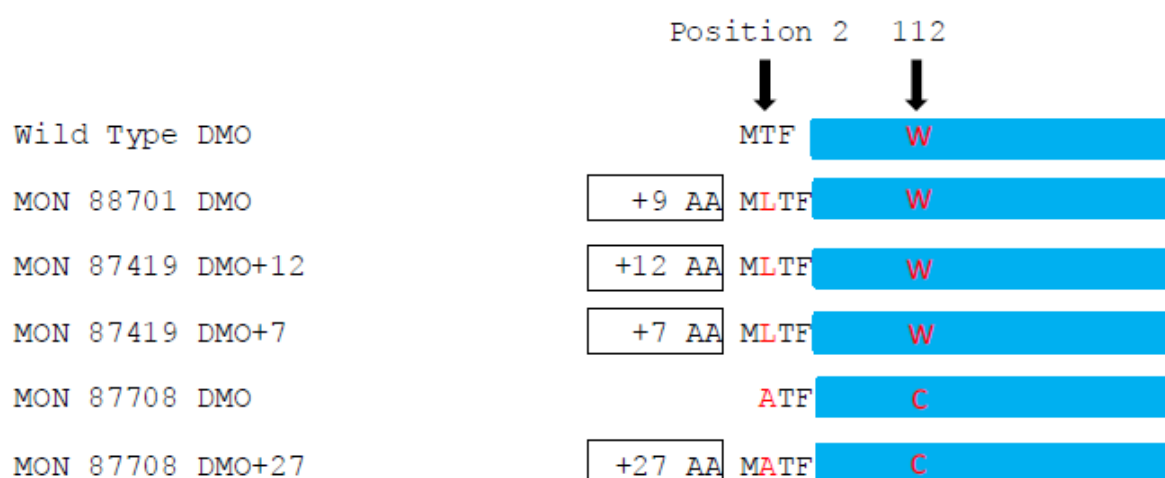


Figure 7: Forms of DMO protein expressed in different GM commodities compared with wildtype DMO derived from *Stenotrophomonas maltophilia* (other lines explained in the text)

4.1.1.2 PAT

The wildtype PAT (phosphinothricin N-acetyltransferase) protein encoded by the *pat* gene from *S. viridochromogenes* consists of 183 amino acids and shares 85% amino acid identity with the PAT protein encoded by the *bar* gene from *S. hygroscopicus*. The PAT proteins (29 amino acids difference) are regarded as equivalent; each exhibits a high degree of enzyme specificity, recognising only one substrate (Wehrmann *et al* 1996). The PAT proteins detoxify phosphinothricin (PPT), the active constituent of glufosinate ammonium herbicides.

PPT inhibits the endogenous plant enzyme, glutamine synthetase, an enzyme involved in amino acid biosynthesis in plant cells. By inhibiting glutamine synthetase, PPT causes rapid accumulation of ammonia in the plant cell, leading to plant death. In glufosinate-tolerant GM plants, the introduced PAT enzyme chemically inactivates PPT by acetylation of the free ammonia group to produce N-acetyl glufosinate, thus allowing plants to continue amino acid biosynthesis in the presence of the herbicide.

The PAT protein in MON87419 is identical to that produced in the source organism *S. viridochromogenes* except that the N-terminal methionine has been removed co-translationally. This results in a protein comprised of 182 amino acids with an apparent molecular weight of approximately 25.2 kDa (see Figure 8).

```

1  MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTPE QTPQEWIDDL
51  ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL
101 GLGSTLYTHL LKSMEAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA
151 AGYKHGGWHD VGFWQDFEL PAPPFVRPV TQI

```

Figure 8: Amino acid sequence of the PAT protein from *S. viridochromogenes*. The methionine at the N-terminus is deleted in MON87419

Several different approaches outlined below (Section 4.1.2) were used to demonstrate that the DMO and PAT proteins expressed in MON87419 are as intended, exhibit the expected enzyme functions, and have the equivalent physicochemical properties to respective bacterially-produced DMO and PAT proteins used as reference material in safety studies.

4.1.2 Characterisation of DMO and PAT proteins in MON87419 and equivalence of bacterially-produced forms

Studies submitted:

2014. Amended Report for MSL0025999: Characterization of the Dicamba Mono-Oxygenase Protein Purified from the Maize Grain of MON87419 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *Escherichia coli* (*E. coli*)-Produced Dicamba Mono-Oxygenase Proteins. **MSL0026361** Monsanto Company (unpublished).

2014. Characterization of the Phosphinothricin N-Acetyltransferase Protein Purified from the Maize Grain of MON87419 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *Escherichia coli* (*E. coli*)-Produced Phosphinothricin N-Acetyltransferase Proteins. **MSL0026031** Monsanto Company (unpublished).

The amount of DMO and PAT proteins expressed in MON87419 plants was insufficient for multiple safety studies. Instead, a microbial expression system in *E. coli* was used to produce larger amounts of protein for evaluation. In order to confirm that the *E. coli*-produced DMO and PAT proteins were equivalent to those expressed in MON87419, a series of analytical techniques were directly employed: (i) determination of the apparent molecular weight, (ii) recognition by specific antibodies (immunoreactivity), (iii) measurement of enzyme activity, and (iv) glycosylation status. The identification of the MON87419-produced DMO and PAT proteins was confirmed by a) N-terminal sequence analysis, b) matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of the protein, and c) Western blot analysis.

The DMO protein was extracted and purified from approximately 10 kg of MON87419 maize flour through a series of extraction and filtration steps, followed by immunoaffinity chromatography using resin cross-linked with mouse monoclonal antibodies raised against *E. coli*-produced DMO protein. The total protein concentration of the MON87419-produced DMO was determined to be 0.115 mg/ml.

The PAT protein was initially extracted from approximately 5 kg of MON87419 maize flour. After sequential chromatography purification steps, the total protein concentration of the final extract was determined by amino acid analysis to be 0.25 mg/ml.

To achieve the same expression products as in the plant, the *E. coli*-produced DMO and PAT proteins were engineered to match the DMO and PAT proteins detected in MON87419 via construction of respective expression plasmids, pMON290351 and pMON282887 (data not shown).

4.1.2.1 Molecular weight and immunoreactivity

The molecular weight of the DMO protein from MON87419 and *E. coli*, and the purity of the protein extract from MON87419 were determined by SDS-PAGE. The plant- and *E. coli*-produced DMO proteins migrated to the same position on the gel with an apparent molecular weight of 39.8 kDa. Based on multiple loadings, the purity of the plant-produced DMO protein was determined to be 98%. Immunoreactivity was detected on Western blots using a polyclonal goat anti-DMO antibody followed by horseradish peroxidase-conjugated horse anti-goat IgG as the secondary antibody. The Western blot showed a single immunoreactive band of the expected mobility, increasing in intensity with protein load, for both MON87419- and *E. coli*-produced DMO. These results confirmed that the samples from both *E. coli* and MON87419 contained a DMO-positive immunoreactive protein of high purity and equivalent molecular weight.

SDS-PAGE and Western blot analyses were also used to determine the molecular weight and immunoreactivity of the PAT protein extracts obtained from MON87419 and *E. coli*. The intact MON87419-produced PAT protein migrated to the same position on the gel as the *E. coli*-produced PAT protein; the apparent molecular weight was determined to be 25.2 kDa. The average purity of the MON87419-produced PAT protein was determined to be 69%. Goat anti-PAT polyclonal antibodies were used on the Western blots. The results showed a single immunoreactive band with the same electrophoretic mobility in all sample lanes loaded with the MON87419-produced or *E. coli*-produced PAT proteins. As expected, the signal intensity was comparable between the samples from both sources and increased with increasing amounts of protein.

4.1.2.2 N-terminal sequence analysis

Fifteen cycles of N-terminal sequencing were carried out on the MON87419-produced DMO protein. The results were compared with the expected sequence deduced from the *dmo* gene present in MON87419. The experimentally determined sequence corresponded exactly with the first eleven amino acids of the deduced DMO sequence beginning at the initial serine residue of DMO+12 (mature DMO with 12 amino acids from the chloroplast transit peptide). The shorter form of the mature protein DMO+7 was also observed.

Fifteen cycles of N-terminal sequencing were also performed on MON87419-produced PAT protein. The experimentally observed amino acid sequence corresponded exactly to the expected sequence of the PAT protein deduced from the *pat* gene present in MON87419, beginning at the second amino acid position. Removal of the N-terminal methionine by methionine aminopeptidase is common and does not alter the protein structure or function.

4.1.2.3 MALDI-TOF mass fingerprint analysis

The identification of proteins by peptide mass fingerprinting is considered reliable if the measured coverage of the sequence is 15% or higher, with a minimum of five peptide matches (Jensen et al. 1997).

An aliquot of the purified MON87419-produced DMO protein was digested with trypsin and the resulting peptide fragments analysed by MALDI-TOF MS. There were 37 unique peptides identified that corresponded to the expected masses deduced from potential trypsin cleavage sites within the DMO amino acid sequence. The experimentally determined coverage of the DMO protein was 77% or 272 of 352 amino acids. A peptide map of the MON87419-produced DMO is shown in Figure 9.

The identity of the MON87419-produced PAT protein was also confirmed by MALDI-TOF MS analysis of peptide fragments produced by the trypsin digestion of the plant-produced PAT protein. The analysis found nine unique peptides that corresponded to the expected masses deduced from potential cleavage sites within the PAT amino acid sequence. The experimentally determined coverage of the PAT protein was 68% or 124 of 182 amino acids. The peptide map of the MON87419-produced PAT protein is shown in Figure 10.

```

1  SFRISASVAT ACMLTFVRNA WYVAALPEEL SEKPLGRTIL DTPLALYRQP
51  DGVVAALLDI CPHRFAPLSD GILVNGHLQC PYHGLEFDGG GQCVHNPHGN
101 GARPASLNVR SFPVVERDAL IWIWPGDPAL ADPGAIPDFG CRVDPAYRQTV
151 GGYGHVDCNY KLLVDNLMDL GHAQYVHRAN AQTDAFDRLE REVIVGDGEI
201 QALMKIPGGT PSVLMKFLR GANTPVDWN DIRWNKVSAM LNFIAVAPEG
251 TPKEQSIHSR GTHILTPETE ASCHYFFGSS RNFGIDDPED DGVLRSWQAQ
301 ALVKEDKVVV EAIERRRAYV EANGIRPAML SCDEAAVRVS REIEKLEQLE
351 AA

```

Figure 9: Tryptic Peptide Map of the MON87419-produced DMO protein. The deduced amino acid sequence of DMO+12 is 352 amino acids as shown. Boxed regions correspond to peptides that were identified from the plant-produced protein sample using MALDI-TOF MS

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1  MSPERRPVEI RPATAADMAA VCDIVNHYIE TSTVNFRTEP QTPQEWIDDL
51  ERLQDRYPWL VAEVEGVVAG IAYAGPWKAR NAYDWTVEST VYVSHRHQRL
101 GLGSTLYTHL LKSMQAQGFK SVVAVIGLPN DPSVRLHEAL GYTARGTLRA
151 AGYKHGGWHD VGEWQRDFEL PAPPRPVRPV TQI

```

Figure 10: Tryptic Peptide Map of the PAT protein encoded by the *pat* gene inserted into MON87419. The methionine residue at the N-terminus is not present in the mature plant-produced protein. The boxed regions correspond to peptides that were identified from the plant-produced protein sample using MALDI-TOF MS

4.1.2.4 Functional activity

The DMO enzyme converts dicamba to DCSA and formaldehyde. The functional activity of the MON87419-produced and *E. coli*-produced DMO proteins was compared by measuring the amount of dicamba that was converted to DCSA *in vitro*, using High Performance Liquid Chromatography (HPLC) and fluorescence detection. Enzyme activity was expressed as specific activity (nmol per minute per mg). In this assay system, the specific activity of the MON87419-produced and *E. coli*-produced DMO proteins was determined to be 232.5 and 240.1 nmol per minute per mg, respectively.

The PAT enzyme catalyses the reaction of phosphinothricin (PPT) with acetyl-CoA to form acetyl-PPT and free CoA. The functional activity of the MON87419-produced and *E. coli*-produced PAT enzymes was assessed and compared using a coenzyme A (CoA) release assay in which reaction products were monitored colorimetrically (Wehrmann et al. 1996). The *E. coli*-produced PAT protein was used as the assay positive control. Specific activity was expressed as $\mu\text{mole per minute per mg}$ of PAT protein. The specific activity of the MON87419-produced and *E. coli*-produced PAT proteins was determined to be 36.6 and 39.2 $\mu\text{mole per minute per mg}$ of PAT protein, respectively.

Based on the results of these enzyme activity assays, the DMO and PAT proteins obtained from *E. coli* expression systems are functionally equivalent to the DMO and PAT proteins produced in MON87419 plants.

4.1.2.5 Glycosylation analysis

Proteins expressed in eukaryotic organisms such as plants, can be modified with carbohydrate moieties after being translated (Rademacher et al. 1988). To test whether the DMO protein was glycosylated when expressed in the grain of MON87419, the plant-produced protein was analysed with a proprietary glycoprotein detection kit. The *E. coli*-produced DMO protein was also analysed as a negative comparator. Transferrin, a glycosylated protein, was used as a positive control. A clear glycosylation signal was observed at the expected molecular weight (approx. 80 kDa) in the transferrin control, whereas no glycosylation signal was observed in the DMO protein samples from either the plant or bacterial source.

Transferrin was also used as the positive control in the analysis of the MON87419-produced and *E. coli*-produced PAT proteins using the same glycoprotein detection method. The results showed a clear glycosylation signal for the positive control at the expected molecular weight. In contrast, no glycosylation signal was detected in the PAT protein samples from either the plant or bacterial source.

4.1.2.6 Conclusion

Analyses of molecular weight, immunoreactivity and functional enzyme activity confirmed that the DMO and PAT proteins produced in the grain of MON87419 plants were as expected from the expression of the inserted genes. The results of N-terminal amino acid sequencing and peptide mass fingerprinting of each protein were further confirmation of their identity. Comparison of the DMO and PAT proteins produced by bacterial fermentation in the laboratory with those produced in MON87419 demonstrated that the plant-produced and *E. coli*-produced forms are equivalent in terms of molecular size, immunoreactivity and specific enzyme activity. Separate analysis of the MON87419-produced DMO and PAT proteins showed that they are not glycosylated. Based on these results, the conclusion is that the *E. coli*-derived DMO and PAT proteins are suitable as substitutes for MON87419-derived DMO and PAT proteins in safety assessment studies.

4.1.3 Expression of DMO and PAT in MON87419 tissues

Study submitted:

2014. Assessment of DMO and PAT Protein Levels in Leaf, Root, Forage and Grain Tissues Collected from Maize MON87419 Produced in United States Field Trials During 2013. **MSL0025758**. Monsanto Company (unpublished)

MON87419 plants⁷ were grown in field trials in the U.S. during 2013. Four replicated plots were planted at each of five trial sites: Iowa, Indiana, Kansas, Nebraska and Pennsylvania. The crop was treated with commercial applications of dicamba and glufosinate herbicides prior to harvest of tissue samples and collection of seed. An *E. coli*-produced DMO protein was used as the reference substance for analysis of DMO protein levels in various plant tissues. Similarly, an *E. coli*-produced PAT protein was used as a reference substance for measurement of PAT protein levels in the same tissues.

Samples from different MON87419 plants were taken at various stages of growth to generate a total of 20 samples for each tissue and time point.

Levels of the DMO and PAT proteins were determined for each sample type using separate validated enzyme linked immunosorbent assays (ELISAs). Detection of the DMO protein utilised a goat polyclonal anti-DMO capture antibody coupled with biotin in conjunction with a commercial streptavidin-horseradish peroxidase conjugate. Plates were analysed and commercial software was used to convert optical density values to protein concentration. The PAT protein was analysed using a commercially available PAT ELISA kit.

The results, averaged over all sites, are given in Tables 5 and 6. The protein levels for nine grain samples were below the limit of quantitation, therefore the data reflect the results from 11 out of the collected 20 samples. Forage refers to the above-ground plant parts used as animal feed.

On a dry weight basis, the mean DMO protein level in MON87419 across all sites was highest in leaf tissue at 26 µg/g dw, and lowest in grain at 0.2 µg/g dw. The mean PAT protein level in MON87419 across all sites was highest in leaf at 11 µg/g dw and lowest in grain at 0.9 µg/g dw.

Table 5: Summary of DMO Protein Levels in Maize Tissues Collected from MON87419 Grown in United States Field Trials in 2013 (Dicamba and Glufosinate Treated)

Tissue Type	Development Stage ¹	Mean (SD) Range (µg/g fw) ²	Mean (SD) Range (µg/g dw) ³	LOQ/LOD (µg/g fw) ⁴
Leaf	V3	3.7 (0.77) 1.9-5.1	26 (6.6) 13-37	0.157/0.027
Root	V3	0.81 (0.16) 0.58-1.1	7.4 (1.4) 5.0-11	0.125/0.038
Forage	R5	1.8 (0.62) 1.0-3.7	6.0 (2.7) 3.1-14	0.157/0.024
Grain	R6	0.17 (0.044) 0.13-0.29	0.19 (0.048) 0.14-0.31	0.125/0.022

¹The crop development stage each tissue was collected.

²DMO protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (number of sites (n)=20 except grain where n=11 due to nine samples having levels <LOQ).

³DMO protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data.

⁴LOQ=limit of quantitation; LOD=limit of detection.

⁷ The identity of the starting seed was verified by event-specific PCR prior to planting.

Table 6: Summary of PAT Protein Levels in Maize Tissues Collected from MON87419 Grown in United States Field Trials in 2013 (Dicamba and Glufosinate Treated)

Tissue Type	Development Stage ¹	Mean (SD) Range (µg/g fw) ²	Mean (SD) Range (µg/g dw) ³	LOQ/LOD (µg/g fw) ⁴
Leaf	V3	1.5 (0.35) 1.1-2.4	11 (2.7) 7.0-17	0.094/0.043
Root	V3	0.84 (0.18) 0.49-1.3	7.7 (1.3) 4.7-11	0.094/0.037
Forage	R5	1.6 (0.50) 0.92-2.3	5.0 (1.6) 2.8-8.5	0.094/0.014
Grain	R6	0.85 (0.25) 0.50-1.4	0.93 (0.27) 0.56-1.6	0.094/0.007

¹The crop development stage each tissue was collected.

²PAT protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20).

³PAT protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data.

⁴LOQ=limit of quantitation; LOD=limit of detection.

4.1.4 Safety of the introduced proteins

Studies submitted:

Digestibility studies (unpublished)

2014. Amended Report for MSL0025997: Assessment of the *in vitro* Digestibility of *Escherichia coli*-produced MON87419 DMO Protein by Pepsin and Pancreatin. MSL0026364 Monsanto Company.

2015. Amended Report for MSL0025998: Assessment of the *in vitro* Digestibility of Phosphinothricin N-Acetyltransferase (PAT) Protein by Pepsin and Pancreatin. MSL0026362 Monsanto Company.

Thermolability studies (unpublished)

2014. Effect of Heat Treatment on the Functional Activity of *Escherichia coli*-Produced MON87419 DMO Protein. MSL0025906 Monsanto Company.

2014. Amended Report for MSL0026186: Effect of Heat Treatment on the Functional Activity of *Escherichia coli*-produced MON87419 Phosphinothricin N-acetyltransferase Protein. MSL0026345 Monsanto Company.

Bioinformatics studies (unpublished)

2014. Bioinformatics Evaluation of the DMO and PAT Proteins in MON87419 Utilizing the AD_2014, TOX_2014 and PRT_2014 Databases. MSL0025907 Monsanto Company.

Acute Toxicity Studies (unpublished)

2012. An Acute Toxicity Study of *E. coli*-produced MON88701 DMO Administered by the Oral Gavage Route to Mice. Charles River Laboratories Study No. 20010948 for Monsanto Company.

2012. An Acute Toxicity Study of *E. coli*-produced Phosphinothricin N-acetyltransferase (PAT/*bar*) Protein Administered by the Oral Gavage Route to Mice. Charles River Laboratories Study No. 20010947 for Monsanto Company.

The results presented in Section 4.1.2 verified the identity of the DMO and PAT proteins as expressed in MON87419. Both proteins have been assessed by FSANZ in previous applications.

The DMO protein was initially considered in Application A1063 – soybean MON87708 (FSANZ, 2012) and again in A1080 – cotton MON88701 (FSANZ, 2013). Results in published literature also support the safety of DMO (see eg. Schmidt & Shaw 2001; Chakraborty et al. 2005; Duke 2005; Behrens et al. 2007; Delaney et al. 2008, EFSA 2011).

The PAT protein, encoded by either the *pat* or *bar* genes (Wehrmann et al. 1996; Hérouet et al. 2005), has now been considered in 21 FSANZ safety assessments (A372, A375, A380, A385, A386, A446, A481, A518, A533, A543, A589, A1028, A1040, A1046, A1073, A1080, A1081, A1087, A1094, A1106 and A1112). These assessments, together with the published literature, firmly establish the safety of PAT and confirm that it does not raise toxicity or food allergenicity concerns in humans (see e.g. Hérouet et al. 2005; Delaney et al. 2008; Hammond et al. 2013).

The relevant FSANZ applications, in which a detailed protein evaluation of either DMO or PAT has been presented⁸, are listed in Table 7. The Applicant has submitted further studies with this application (listed above). The bioinformatics studies, which look for amino acid sequence similarity to known protein toxins and allergens in publicly available databases, were updated and the results do not alter conclusions reached in previous assessments. The Applicant also provided *in vitro* digestibility and thermolability studies for each protein that confirmed conclusions from previous applications as listed in Table 7. The digestibility studies indicate both proteins would be rapidly degraded in the stomach following ingestion, and thermolability studies show the proteins are inactivated by heating.

Taken together, the evidence clearly indicates that neither DMO nor PAT are toxic or allergenic in humans.

Table 7: Previous FSANZ assessments of DMO and PAT

Consideration	Sub-section	DMO	PAT
Potential toxicity	Amino acid sequence similarity to protein toxins	Database search updated in October 2014	Database search updated in October 2014
	<i>In vitro</i> digestibility	A1080 (FSANZ 2013b)	A1080 (FSANZ 2013b)
	Stability to heat	A1080 (FSANZ 2013b)	A1080 (FSANZ 2013b)
	Acute oral toxicity	A1063 (FSANZ 2012)	A1080 (FSANZ 2013b)
Potential allergenicity	Source of the protein	A1063 (FSANZ 2012)	A1087 (FSANZ 2013c)
	Amino acid sequence similarity to allergens	Database search updated in October 2014	Database search updated in October 2014

4.1.5 Bioinformatics analysis of ORFs created by the transformation

Studies submitted:

2014. Amended Report for MSL0025770: Bioinformatics Evaluation of MON 87419 Utilizing the AD_2014, TOX_2014 and PRT_2014 Databases. **MSL0026123**. Monsanto Company (unpublished)

2014. Bioinformatics Evaluation of the DMO and PAT Proteins Utilizing the AD_2014, TOX_2014 and PRT_2014 Databases. **MSL0025907**. Monsanto Company (unpublished)

As described in Section 3.4.4, the putative peptides/polypeptides encoded by the 11 identified ORFs were analysed using a bioinformatics strategy to determine similarity to known protein toxins, allergens, or other proteins with known biological activity.

⁸ Detailed safety assessment reports are available on the FSANZ website.

The allergen analyses considered both $\geq 35\%$ identity over 80 amino acids and an eight amino acid sliding window search.

Using the FASTA algorithm to search for significant alignments with database sequences, no biologically relevant structural similarities were found against the allergen and toxin databases. A search of the protein database (PRT_2014) found that one translated ORF at the 5' end of the insert displayed 95.6% identity with a hypothetical protein encoded by genomic DNA from *Zea mays*. Further inspection revealed that the aligning region was from amino acids 50-95 of the ORF, which was located 66 nucleotides upstream of the T-DNA insertion site. There is no indication of any adverse biological activity associated with this hypothetical corn protein.

Similarly, the FASTA sequence alignment tool was used to assess structural similarity between the DMO protein as expressed in MON87419 (that is with additional 12 amino acids of the CTP) and PAT protein sequences and sequences in the allergen (AD_2014), toxin (TOX_2014) or all proteins (PRT_2014) databases. No biologically relevant similarities with sequences in the public databases were found. This means that, in the event of an unexpected translation product being produced from the T-DNA insert, it would not be structurally related to known allergens or toxins and therefore would not likely be associated with allergenic or toxic effects.

4.1.6 Conclusion

The genetic modification in MON87419 corn results in the expression of two new enzymes, DMO and PAT, in a range of plant tissues. The mean levels of both proteins in herbicide-sprayed plants grown in the field are highest in leaves (not consumed by humans), and lowest in the grain. In MON87419 field trials, the mean DMO protein levels were 26 $\mu\text{g/g}$ in leaf and 0.2 $\mu\text{g/g}$ in grain, on a dry weight basis; the mean PAT protein levels in leaf and grain were respectively, 11 $\mu\text{g/g}$ and 0.9 $\mu\text{g/g}$, on a dry weight basis.

The identity of the MON87419-produced DMO and PAT proteins was confirmed by a range of analyses including Western blots, N-terminal sequencing, MALDI-TOF mass spectrometry, and functional enzyme activity assays. Indirect evidence indicated that neither DMO nor PAT is glycosylated in MON87419.

Previous assessments of the safety of the DMO and PAT proteins in food crops have concluded that neither protein is toxic or allergenic in humans. Revised digestibility and thermolability studies on the proteins as produced in MON87419 confirm the proteins would be degraded during digestion, and are susceptible to heat denaturation. Updated bioinformatics searches found no significant similarity between DMO and PAT sequences and known protein toxins or allergens. Taken together, the evidence supports the conclusion that the DMO and PAT proteins present in MON87419 are innocuous and raise no food safety concerns.

4.2 Herbicide metabolites and residues

Studies submitted:

2015. Nature of ^{14}C -Dicamba Residues in Corn Raw Agricultural Commodities Following Preemergence or Postemergence Application to Dicamba-Glufosinate Tolerant Corn. MSL0025703 Monsanto Company (unpublished).

2015. Analytical Method for the Determination of Dicamba and Major Metabolites in Raw Agricultural Commodities by LC-MS/MS. MSL0026344 Monsanto Company (unpublished).

2015. Summary of the Magnitude of Residues of Dicamba in Dicamba Glufosinate Tolerant Maize Grain (MON87419) after Application of Dicamba-Based Formulation. MSL0026819 Monsanto Company (unpublished).

As part of the safety assessment of herbicide-tolerant plants, it is important to establish whether the genetic modification is likely to result in the accumulation of any novel metabolites. If such substances are found to occur in the GM plant, it is important to assess whether they could cause toxicity in humans.

As discussed in Section 4.1.1, the demethylation of dicamba by the DMO enzyme produces the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA) and formaldehyde (Chakroborty *et al.*, 2005). DCSA is produced in soil from the metabolism of dicamba by aerobic microorganisms in the soil. Previous assessment of DCSA found that it is not persistent in the environment, has low potential to leach into ground water and does not present a significant toxicological risk to other organisms in the environment (EFSA 2007a, 2007b).

Formaldehyde is also a metabolite produced in MON87419 plants. Plants can readily metabolise formaldehyde naturally produced from internal processes (Hanson and Roje, 2001). Any additional amount of formaldehyde theoretically produced by dicamba treatment of MON87419 plants would be quickly metabolised in the plant. There are many sources of human exposure to formaldehyde through the atmosphere and from the widespread use of industrial and cosmetic products⁹. Formaldehyde is only emitted from plant foliage under certain conditions and emission rates are low in any case (Cojocariu *et al* 2005; Cojocariu *et al* 2004; Nemecek-Marshall *et al* 1995). Incrementally small increases in the amount of formaldehyde produced in MON87419 plants do not represent a food safety concern.

4.2.1 Dicamba metabolism study

A metabolism study with radiolabelled [¹⁴C] dicamba in dicamba-glufosinate tolerant corn was conducted as part of the herbicide registration requirements in the United States¹⁰, and therefore also considered elsewhere including in Australia. The purpose of using radiolabelled dicamba was to determine the nature of residues found in or on agricultural commodities of dicamba-glufosinate tolerant corn, following pre- or post-emergence treatments with the herbicide.

The major metabolite identified in corn foliage following herbicide treatment was DCSA glucoside, constituting 26.9–53.2% of total radioactive residues. Enzymatic hydrolysis of this major metabolite indicated that the identified glucosylated metabolites were β-linked D-glucose conjugates. In grain, DCSA glucoside was <0.001 mg/kg.

Unchanged dicamba ranged from 6.3 – 8.6% of total radioactive residues in foliage (forage). A range of minor metabolites was identified. Radioactive residues in the hexane extracts of grain were characterised as a mixture of naturally-occurring triglycerides (fatty acids and glycerol) amounting to 4.64 and 5.31% of total radioactive residues (0.002 and 0.003 mg/kg), pre- and post-treatment respectively. Overall, dicamba was metabolised in dicamba-tolerant corn to DCSA, DCGA (2,5-dichloro-3,6-dihydroxybenzoic acid) and 5-hydroxydicamba, as determined previously to occur in soil and other plant species.

4.2.2 Dicamba residues study

Following the identification of the dicamba metabolites, a residue study was conducted in 2013 to determine the levels of unchanged dicamba and principal metabolites in MON87419 grain, after applications of the herbicide.

⁹ USHHS-ATSDR (1999) Toxicological profile for formaldehyde. US Department of Health and Human Services, Agency for Toxic Substances and Disease Registry – Washington, DC.,

¹⁰ Environment Protection Agency (EPA) residue chemistry test guideline US EPA OCSP 860.1300, "Nature of the Residue – Plants, Livestock" and OECD Guideline for Testing of Chemicals No. 501, "Metabolism in Crops".

The data generated in this study are required to register use in the US of dicamba on dicamba-tolerant crops through the US EPA evaluation process.

To ensure adequate representation and coverage of geographical variability, the study was conducted at 22 locations in typical corn-growing regions in the US¹¹. There were four separate treatment regimes at each of the sites, including an untreated control. A validated analytical method was developed and used to quantify dicamba, DCSA, DCGA and 5-hydroxydicamba to facilitate residue analysis. The LOQ for dicamba, 5-OH dicamba, DCSA and DCGA (expressed as dicamba equivalents) was determined to be 0.010 ppm (mg/kg). The results are shown in Table 8; total residues are expressed as dicamba acid equivalents.

Table 8: Summary of Dicamba Residues in MON87419 Grain

Analyte	Treatment 2			Treatment 3		
	Mean (ppm)	Median ^a (ppm)	Range ^b (ppm)	Mean (ppm)	Median ^a (ppm)	Range ^b (ppm)
Dicamba	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100
5-Hydroxydicamba	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100	<0.0100
DCSA	<0.0111	<0.0100	<0.0100-0.0394	<0.0109	<0.0100	<0.0100-0.0332
DCGA	<0.0105	<0.0100	<0.0100-0.0213	<0.0108	<0.0100	<0.0100-0.0229
Total ^{c,d}	<0.0311	<0.0100	<0.0300-0.0594	<0.0309	<0.0100	<0.0300-0.0532

^aMedian of residues across all sites in this treatment

^bRange of residues across all sites in this treatment

^cIndividual analyte residues are expressed as dicamba acid equivalents

^dTotal residues (ppm) = [Dicamba] + [5-hydroxydicamba] + [DCSA] per current U.S. EPA Definition of the Residue in maize grain per 40 CFR §180.227

Dicamba was metabolised mainly to a glucose conjugate of DCSA, with smaller amounts of conjugates of DCGA, and 5-hydroxydicamba. Dicamba itself was present at very low levels. The analytes proposed to the EPA for MON87419 will therefore include DCSA, in addition to dicamba and 5-OH dicamba, which currently constitute the US EPA residue definition¹².

The results of the study also indicate that the existing EPA dicamba MRL of 0.1 ppm in maize grain is sufficient to account for the proposed new use of dicamba on MON87419, with the addition of DCSA to the definition of residues. However, dicamba is not registered for use in or on corn in Australia and dicamba MRLs do not exist for corn commodities coming into Australia. Accordingly, it will be necessary to apply for the establishment of an import MRL of 0.1ppm to cover residues of dicamba on corn grains imported into Australia.

4.2.3 Glufosinate residues

Tolerance to glufosinate ammonium is a trait present in GM crop lines from 21 previous applications to FSANZ (see Section 4.1.4). The enzyme activity of PAT results in the acetylation of the free amino group of glufosinate to produce the non-herbicidal *N*-acetyl glufosinate in the plant. This is a well-known metabolite in glufosinate-tolerant plants and was previously considered in detail by FSANZ in cotton line LL25 (FSANZ 2006).

¹¹ Control and treated plots were established at Pennsylvania (1 trial), Georgia (1 trial), North Carolina (1 trial), Michigan (1 trial), Kansas (2 trials), Iowa (1 trial), Illinois (6 trials), Indiana (1 trial), Missouri (2 trials), Nebraska (3 trials), South Dakota (1 trial), Wisconsin (1 trial) and Texas (1 trial).

¹² U.S. EPA Definition of the Residue as per 40CFR §180.227

4.2.4 Summary of residue information

No new major metabolites are present in MON87419 as a result of treating the crop with dicamba and glufosinate ammonium herbicides. In terms of MON87419 grain, the appropriate use of the herbicides at specific stages of development according to agricultural requirements does not raise food safety concerns.

5 Compositional analysis

The main purpose of compositional analysis is to determine if, as a result of the genetic modification, there have been any unexpected changes introduced into the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional qualities. 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 those constituents most relevant to the safety of the food or that have an impact on the whole diet. Important analytes include the key nutrients, toxicants and anti-nutrients that are characteristic of the food in question, and that have a potential impact in the overall diet. They are typically major constituents (fats, proteins, carbohydrates), enzyme inhibitors such as anti-nutrients, or minor constituents (minerals, vitamins). Key toxicants are compounds of known toxicological significance that are inherently present in some plant based foods at a level that could have an impact on health (e.g. solanine in potatoes).

5.1 Key components

For corn, there are a number of components that are considered important for a compositional analysis (OECD 2002). 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 and secondary metabolites could be determined for new varieties of corn.

Corn contains a number of substances described as anti-nutrients: phytic acid, raffinose, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) and trypsin and chymotrypsin inhibitor.

DIMBOA is present at highly variable levels in corn hybrids and little evidence is available on either its toxicity or anti-nutritional effects; corn contains only low levels of trypsin and chymotrypsin inhibitor, neither of which is considered nutritionally significant. Only phytic acid and raffinose are considered to be biologically relevant for corn (OECD 2002).

Phytic acid is considered an important anti-nutrient for animals, especially non-ruminants, since it can significantly reduce the bioavailability of minerals. Raffinose is a non-digestible oligosaccharide and is considered an anti-nutrient because of its undesirable gastrointestinal effects (flatulence).

Secondary metabolites in plants are defined as those natural products that do not function directly in the primary biochemical reactions that support normal growth, development and reproduction in the organism. Secondary plant metabolites are neither nutrients nor anti-nutrients but are sometimes analysed in a GM crop as further indicators of the absence of unintended effects on plant metabolism as a result of the genetic modification (OECD 2002).

Characteristic secondary metabolites in corn are furfural and the phenolic acids, ferulic acid and p-coumaric acid.

There are no generally recognised anti-nutrients in corn at levels considered to be harmful, but for the purposes of comparative assessment, the OECD has recommended considering analytical data for the content of the anti-nutrients phytic acid and raffinose, and the secondary metabolites furfural, ferulic acid and p-coumaric acid.

5.2 Study design and conduct

Study submitted:

2014. Compositional Analyses of Maize Forage and Grain from Dicamba and Glufosinate Treated MON87419 Grown in the United States during 2013. **MSL0025559**. Monsanto Company (unpublished).

The purpose of the compositional analyses is to compare a targeted range of components/nutrients in the GM crop and the conventional counterpart, to determine whether the genetic modification has resulted in any biologically significant changes.

As depicted in Figure 3, verified (event-specific PCR) MON87419 generation R₃F₁ (HCL645 x LH244 R₃) was used for these analyses. Ideally, the comparator in compositional analyses should be the non-transformed parental line grown concurrently under similar field conditions (OECD 2002). In this case, the hybrid line HCL645 x LH244 (renamed NL6169) has a genetic background similar to MON87419 and has been used as the near-isogenic control.

The test and control lines were planted in a randomized complete block design with four replicates at each of five field sites in the US corn belt¹³ during the 2013 growing season. All plantings at the field sites were grown under normal agricultural field conditions for their respective geographical regions. Separate samples were included for MON87419 treated and not treated with dicamba and glufosinate ammonium herbicides. The control line was not treated with the herbicides. Grain was harvested at physiological maturity and shipped at ambient temperature to laboratories for analysis. Forage was harvested at the R5 growth stage and shipped on dry ice.

As well as the quantitative comparison between MON87419 and control lines, the composition of MON87419 was also evaluated in the context of natural variability defined by published literature values or the ILSI Crop Composition Database (ILSI-CCDB). Natural variation in crop composition due to environmental and germplasm differences is well described in agricultural science. Consideration of the breadth of natural variability is therefore important for interpreting the biological relevance of any statistically significant differences between MON87419 and its comparator.

Grain samples were analysed for proximates (protein, fat, ash, moisture), carbohydrates by calculation, fibre (acid detergent fibre – ADF; neutral detergent fibre – NDF; total dietary fibre), fatty acids, amino acids, minerals, vitamins, anti-nutrients and secondary metabolites.

Key analyte levels (proximates, carbohydrates, fibre and minerals) were also analysed in forage but the results are not included in this report. It is noted however that, in the combined site analysis, none of the analyte levels in MON87419 differed significantly from those of the control.

¹³ Boone County, Iowa; Clinton County, Indiana; Pawnee County, Kansas; York County, Nebraska; Lehigh County, Pennsylvania.

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 scientific methods.

5.3 Analyses of key components in grain

In total, 69 different components were analysed in grain. Several analytes had more than 50% of the observations below the assay limit of quantitation (LOQ) and were therefore excluded from the statistical analysis. Moisture values for grain (and forage) were measured only for conversion of components to a dry weight basis. Overall, 53 components in grain were statistically analysed for all samples on data combined across all field sites.

The analysis used a mixed model analysis of variance. Measurements were transformed into Statistical Analysis Software¹⁴ (SAS) data sets and analysed using SAS® software (SAS MIXED, Release 9.2). Data were from test substance MON87419 (treated with dicamba and glufosinate ammonium), test untreated, and conventional control substance NL6169. Descriptive statistics (mean and standard error (SE)) were generated and are presented in Tables 7 – 13, representing results only from MON87419 treated with herbicides. 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).

The results for MON87419 and the near-isogenic control have been compared to a combined literature range for each analyte, compiled from published literature for commercially available corn¹⁵. It is noted that information in the published literature is limited and therefore unlikely to fully reflect the natural diversity that occurs within field-grown corn (Harrigan et al. 2010; Zhou et al. 2011; Ridley et al. 2011). Therefore, even if a mean value falls outside the published range, there are unlikely to be any safety concerns.

5.3.1 Protein and amino acids

Typically, maize grain is approximately 10% protein by weight. The level of protein and associated amino acids vary widely with the local growing conditions. The results of the analyses for protein and amino acids (18 components) are shown in Table 9.

There are no significant differences between the mean levels in MON87419 and the conventional control for any of these analytes. All means were also within the published literature range (not shown).

Table 9: Mean ($\pm$ SE) levels of protein and amino acids in grain from MON87419 (treated) and the control, expressed as percentage dry weight (%dw)

Analyte (%dw)	MON87419 (treated) Mean (SE) Range	Control Mean (SE) Range	p-Value
Protein	11.52 (0.55) 9.14 – 14.60	11.07 (0.55) 9.22 – 14.04	0.120
Alanine	0.92 (0.057) 0.68 – 1.23	0.88 (0.057) 0.71 – 1.17	0.151
Arginine	0.46 (0.014) 0.38 – 0.55	0.45 (0.014) 0.39 – 0.52	0.178
Aspartic Acid	0.73 (0.036) 0.58 – 0.94	0.70 (0.036) 0.59 – 0.88	0.127

¹⁴ SAS website - <http://www.sas.com/technologies/analytics/statistics/stat/index.html>

¹⁵ Published literature for corn incorporates references used to compile listings in the ILSI Crop Composition Database Version 4 (ILSI 2014).

Analyte (%dw)	MON87419 (treated) Mean (SE) Range	Control Mean (SE) Range	p-Value
Cysteine	0.22 (0.005) 0.18 – 0.26	0.22 (0.005) 0.18 – 0.26	0.767
Glutamic Acid	2.43 (0.15) 1.80 – 3.26	2.32 (0.15) 1.88 – 3.12	0.160
Glycine	0.41 (0.011) 0.36 – 0.47	0.40 (0.011) 0.36 – 0.45	0.118
Histidine	0.33 (0.011) 0.27 – 0.40	0.32 (0.011) 0.28 – 0.37	0.114
Isoleucine	0.42 (0.024) 0.32 – 0.56	0.40 (0.024) 0.33 – 0.53	0.123
Leucine	1.59 (0.11) 1.15 – 2.18	1.51 (0.11) 1.20 – 2.08	0.144
Lysine	0.28 (0.0061) 0.25 – 0.33	0.28 (0.0061) 0.24 – 0.32	0.431
Methionine	0.23 (0.0074) 0.19 – 0.27	0.23 (0.0074) 0.18 – 0.28	0.788
Phenylalanine	0.63 (0.040) 0.47 – 0.87	0.61 (0.040) 0.48 – 0.79	0.161
Proline	1.08 (0.044) 0.87 – 1.33	1.04 (0.044) 0.89 – 1.27	0.084
Serine	0.59 (0.032) 0.47 – 0.77	0.57 (0.032) 0.48 – 0.72	0.108
Threonine	0.42 (0.018) 0.34 – 0.53	0.41 (0.018) 0.35 – 0.50	0.074
Tryptophan	0.070 (0.0017) 0.058 – 0.083	0.069 (0.0017) 0.055 – 0.083	0.537
Tyrosine	0.31 (0.018) 0.22 – 0.41	0.30 (0.018) 0.24 – 0.39	0.429
Valine	0.54 (0.025) 0.43 – 0.69	0.52 (0.025) 0.44 – 0.65	0.077

5.3.2 Total fat and fatty acids

Maize grain is approximately 4% fat; the levels of total fat and individual fatty acids can vary widely with the field conditions at the time of growing. Total fat and 22 fatty acids were measured in MON87419 and the control grain. Of these, the following had $\geq 50\%$ of observations below the LOQ and were therefore excluded from analysis - C8:0 caprylic, C10:0 capric, C12:0 lauric, C14:0 myristic, C14:1 myristoleic, C15:0 pentadecanoic, C15:1 pentadecenoic, C17:0 heptadecanoic, C17:1 heptadecenoic, C18:3 gamma linolenic, C20:2 eicosadienoic, C20:3 eicosatrienoic, and C20:4 arachidonic acid. These fatty acids are typically present in low amounts in maize grain, if present at all. Results for total fat and the remaining nine fatty acids are given in Table 10. There was no significant difference between the mean level in MON87419 and the control for any fatty acid. All means were also within the literature range.

Table 10: Mean ($\pm$ SE) levels of total fat (%dw) and fatty acids in grain from MON87419 and the conventional control, expressed as a percentage of total fat

Analyte	MON87419 (treated) (%total)	Control (%total)	p-Value
Total fat (%dw)	3.40 (0.081) 2.89 – 3.81	3.49 (0.081) 2.80 – 3.98	0.297
Palmitic acid (C16:0)	14.51 (0.12) 13.62 – 15.25	14.51 (0.12) 13.80 – 15.56	0.963
Palmitoleic acid (C16:1)	0.12 (0.004) 0.097 – 0.13	0.12 (0.004) 0.095 – 0.14	0.318
Stearic acid (C18:0)	1.62 (0.028) 1.45 – 1.77	1.64 (0.028) 1.46 – 1.84	0.366

Analyte	MON87419 (treated) (%total)	Control (%total)	p-Value
Oleic acid (C18:1)	21.86 (0.20) 20.52 – 23.24	22.37 (0.20) 20.83 – 24.72	0.078
Linoleic acid (C18:2)	60.08 (0.27) 58.17 – 62.44	59.52 (0.27) 57.68 – 61.91	0.150
Linolenic acid (C18:3)	1.00 (0.027) 0.83 – 1.18	1.02 (0.027) 0.84 – 1.16	0.397
Arachidic acid (C20:0)	0.40 (0.0079) 0.35 – 0.43	0.41 (0.0079) 0.37 – 0.45	0.211
Eicosenoic acid (C20:1)	0.27 (0.0049) 0.24 – 0.29	0.27 (0.0049) 0.25 – 0.33	0.381
Behenic acid (C22:0)	0.14 (0.007) 0.065 – 0.17	0.15 (0.007) 0.061 – 0.18	0.781

5.3.3 Carbohydrates and fibre

Maize grain is typically composed of approximately 85% carbohydrates by calculation, including fibre [acid detergent fibre (ADF); neutral detergent fibre (NDF); total dietary fibre (TDF)]. As is true for protein and fat levels in maize grain, the amounts of carbohydrate vary widely, depending on local growing conditions. From the results of analyses for fibre shown in Table 11, there were no significant differences in the levels of carbohydrates between MON87419 and the control. The overall amounts of these components correlated closely with literature values.

Table 11: Mean ($\pm$ SE) levels of carbohydrates in grain from MON87419 and the conventional control (expressed as percentage dry weight)

Component % dw	MON87419 (treated) Mean (SE) Range	Control Mean (SE) Range	p-Value
Carbohydrates by calculation ¹	83.57(0.54) 80.87– 86.28	84.04 (0.54) 81.36– 85.93	0.231
Acid detergent fibre	3.97 (0.12) 3.42 – 5.13	4.04 (0.12) 3.20 – 5.20	0.608
Neutral detergent fibre	9.70 (0.11) 9.14 – 10.53	9.42 (0.11) 8.98 – 10.01	0.099
Total dietary fibre	9.18 (0.23) 7.15 – 11.78	8.97 (0.23) 7.21 – 10.64	0.514

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

5.3.4 Ash and minerals

The levels of minerals (constituents of ash) are known to vary widely according to growing conditions. Ash and mineral components (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc) in grain from MON87419 and the control were measured, and the results obtained for these analytes are shown in Table 12. No statistically significant differences were observed for ash, calcium, iron, magnesium, phosphorus, potassium, sodium and zinc. A statistically significant difference between MON87419 and the control was observed for manganese (Table 12). Copper was excluded from statistical analysis because more than half of the samples returned values that were below the assay LOQ.

The difference in mean values for manganese between MON87419 and the control was 0.52 mg/kg dw, which represents a small overall difference (approximately 10%) between the lines in terms of manganese content. The MON87419 mean manganese value was also within the range of literature values for this analyte, and within the range listed in the ILSI-CCDB (1.69 – 14.30 mg/kg dw).

Table 12: Mean ($\pm$ SE) levels of ash and minerals in the grain of MON87419 (treated with herbicides) and control (not treated)

Analyte	Units	MON87419 (treated) Mean (S.E.) Range	Control Mean (S.E.) Range	P-value
Ash	%dw	1.39 (0.021) 1.27 – 1.48	1.38 (0.021) 1.30 – 1.51	0.686
Calcium	% dw	0.0031 (0.00017) 0.002 – 0.004	0.0029 (0.00017) 0.002 – 0.005	0.427
Iron	mg/kg dw	16.83 (0.54) 13.02 – 21.56	16.57 (0.55) 13.39 – 18.71	0.536
Magnesium	%dw	0.13 (0.0019) 0.12 – 0.15	0.12 (0.0019) 0.086 – 0.14	0.092
Manganese	mg/kg dw	6.03 (0.45) 4.81 – 8.72	5.51 (0.45) 4.50 – 7.41	0.019
Phosphorus	%dw	0.36 (0.0059) 0.32 – 0.40	0.35 (0.0059) 0.25 – 0.40	0.204
Potassium	%dw	0.36 (0.0081) 0.32 – 0.41	0.36 (0.0081) 0.33 – 0.40	0.802
Sodium	mg/kg dw	5.45 (1.92) 0.36 – 24.28	5.63 (1.92) 0.36 – 35.05	0.945
Zinc	mg/kg dw	22.10 (1.13) 17.21 – 29.83	21.18 (1.13) 16.40 – 26.70	0.175

5.3.5 Vitamins

The levels of seven vitamins, as shown in Table 13, were measured. As the results indicate, there was no significant difference between the control and MON87419 for any of the vitamin analytes. All means were also within the literature range.

Table 13: Mean ($\pm$ SE) amount of vitamins in grain from MON87419 and the control, expressed as mg/kg dw

Analyte (mg/kg dw)	MON87419 (treated) Mean (S.E.) Range	Control Mean (S.E.) Range	p-Value
Vitamin A (β -Carotene)	5.44 (0.45) 3.67 – 11.11	5.47 (0.45) 3.66 – 8.19	0.950
Vitamin B ₁ (Thiamine HCl)	2.46 (0.12) 1.94 – 3.25	2.48 (0.12) 1.80 – 3.34	0.850
Vitamin B ₂ (Riboflavin)	2.18 (0.13) 1.52 – 3.47	2.16 (0.13) 1.54 – 3.43	0.917
Vitamin B ₃ (Niacin)	10.22 (0.41) 8.06 – 12.18	10.20 (0.41) 8.23 – 11.97	0.952
Vitamin B ₆ (Pyridoxine HCl)	5.42 (0.22) 3.45 – 6.62	5.43 (0.22) 2.82 – 7.61	0.959
Vitamin B ₉ (Folic acid)	0.65 (0.035) 0.41 – 1.03	0.66 (0.035) 0.48 – 0.89	0.859
Vitamin E (α -Tocopherol)	11.56 (0.43) 9.28 – 13.37	11.07 (0.43) 8.65 – 12.76	0.085

5.3.6 Anti-nutrients

Two anti-nutrient compounds (phytic acid and raffinose - Section 5.1) were measured to compare the levels in MON87419 grain with those in control grain. The results in Table 14 show that the levels of phytic acid and raffinose were almost the same in the two maize lines. Both means also fell within the published literature range.

Table 14: Mean ($\pm$ SE) levels of key anti-nutrients in grain from MON87419 (treated with herbicides) and the control (not treated)

Analyte %dw	MON87419 (treated) Mean (S.E.) Range	Control Mean (S.E.) Range	p-Value
Phytic acid	0.99 (0.031) 0.80 – 1.20	0.93 (0.031) 0.71 – 1.37	0.087
Raffinose	0.28 (0.010) 0.23 – 0.34	0.28 (0.010) 0.24 – 0.35	0.591

5.3.7 Secondary metabolites

Three secondary metabolites (furfural, ferulic acid and p-coumaric acid – Section 5.1) were measured to compare the levels in MON87419 grain with those in the control grain. More than half of the samples analysed for furfural were below the limit of quantitation (LOQ) of the assay and were therefore excluded from the statistical analysis. The levels of the other two secondary metabolites are shown in Table 15. The results show no significant difference between the control and MON87419 for either metabolite.

Table 15: Mean levels ($\pm$ SE) of two secondary metabolites in grain from MON87419 and the control

Analyte μ g/g dw	MON87419 (treated) Mean (S.E.) Range	Control Mean (S.E.) Range	p-Value
p-Coumaric acid	196.51 (12.40) 149.01 – 282.91	187.70 (12.40) 132.56 – 254.88	0.194
Ferulic acid	2352.80 (45.66) 2165.31 – 2652.33	2289.17 (45.66) 1882.22 – 2508.79	0.097

5.4 Summary and conclusion from compositional analyses

Detailed compositional analyses were carried out to compare the nutritional adequacy of grain from MON87419 with conventional control grain, and to characterise any unintended compositional changes in MON87419 as a result of the genetic modification. Measurements of key components including proximates, fibre, minerals, amino acids, fatty acids, vitamins, secondary metabolites and anti-nutrients were also compared with literature values or the levels in the ILSI crop composition database for each analyte. Except for manganese, the levels of all analytes reported in Tables 9-15 were not significantly different between the two lines. The difference in mean manganese levels is small and does not indicate a nutritionally important difference between MON87419 and the control, nor does it suggest a safety concern. Grain from MON87419 can therefore be regarded as equivalent in composition to grain from conventional corn.

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.

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 the food 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 weight to the safety assessment and, in general, are not warranted (see e.g. OECD 2003; Bartholomaeus *et al.* 2013; Herman and Ekmay 2014).

MON87419 is the result of a simple genetic modification designed to protect the plants from the action of herbicides used on broadleaf weeds. As such, there was no intention to significantly alter nutritional parameters in the food. In addition, the compositional analyses of grain from MON87419, which were undertaken to demonstrate the absence of unintended nutritional changes, indicate it is equivalent in composition to conventional corn cultivars. The introduction of food from MON87419 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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