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

Supporting document 1

Safety assessment – Application A1071 (Approval)

Food derived from Herbicide-tolerant Canola Line MON88302

Executive summary

Background

Monsanto Company (Monsanto) has developed a genetically modified (GM) canola line known as MON88302 (OECD Unique identifier MON-88302-9) that is tolerant to the herbicide glyphosate. A gene cassette has been incorporated into the line that contains the *cp4epsps* gene from *Agrobacterium* sp. under the control of genetic elements that drive expression in all tissue.

In conducting a safety assessment of food derived from herbicide-tolerant canola line MON88302, a number of criteria have been addressed including: a characterisation of the transferred gene, its origin, function and stability in the canola genome; the changes at the level of DNA, protein and in the whole food; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed protein to be either allergenic or toxic in humans.

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

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

History of Use

Canola is the name used for rapeseed (*Brassica napus, Brassica rapa* or *Brassica juncea*) crops that have less than 2% erucic acid and less than 30 micromoles of glucosinolates per gram of seed solids. Rapeseed is the second largest oilseed crop in the world behind soybean, although annual production is around 25% of that of soybean.

Canola seeds are processed into two major products, oil and meal. The oil is the major product for human consumption, being used in a variety of manufactured food products including salad and cooking oil, margarine, shortening, mayonnaise, sandwich spreads, creamers and coffee whiteners. Canola oil is the third largest source of vegetable oil in the world after soybean oil and palm oil. Whole canola seeds are being used increasingly in products such as breads.

Molecular Characterisation

Canola line MON88302 was generated through Agrobacterium-mediated transformation. The line contains the *cp4 epsps* gene that encodes the enzyme 5-enolpyruvyl-3-shikimatephosphate synthase (CP4 EPSPS), conferring tolerance to the herbicide glyphosate.

Comprehensive molecular analyses of canola line MON88302 indicate that there is a single insertion site comprising a single, complete copy of the *cp4 epsps* expression cassette. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and plasmid backbone analysis shows no plasmid backbone has been incorporated into the transgenic locus.

Characterisation of Novel Protein

Canola line MON88302 expresses one novel protein, CP4 EPSPS. The level of CP4 EPSPS is lowest in the pollen (approximately 9 µg/g dry weight) and highest in leaves at the stage where stem elongation (bolting) begins (approximately 230 µg/g dry weight). The level in the mature seed is approximately 27 µg/g dry weight.

The identity of MON88302-derived CP4 EPSPS was confirmed by a number of analytical techniques, namely recognition by anti-CP4 EPSPS antibody, MALDI-TOF analysis, N-terminal sequencing and enzymatic activity.

Bioinformatic studies have confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that CP4 EPSPS would be completely digested before absorption in the gastrointestinal tract would occur. The protein also loses enzyme activity with heating.

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

Herbicide Metabolites

Residue data derived from field trials indicate the residue levels in MON88302 seed are low. In the absence of any significant exposure to either glyphosate or its major metabolite, the risk to public health and safety is negligible.

Compositional Analyses

Detailed compositional analyses were done to establish the nutritional adequacy of seed from MON88302 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamin E and five anti-nutrients. The levels were compared to levels in a) the non-GM parental line, ‘Ebony’ b) a tolerance range compiled from results taken for seven non-GM commercial lines grown under the same conditions and c) levels recorded in the literature.

A total of 69 analytes were measured of which 18 fatty acids and sodium had 50% of their values below the assay limit of quantitation and were therefore not used in the statistical analysis. Of the remaining 51 analytes, only nine in MON88302 deviated from the ‘Ebony’ control in a statistically significant manner. However, all analytes except oleic acid fell within both the tolerance interval and the historical range from the literature. For oleic acid, both the MON88302 and ‘Ebony’ mean levels were higher than found in the literature range but were within the tolerance interval.

It is concluded that seed from MON88302 is compositionally equivalent to seed from conventional canola varieties.

Conclusion

No potential public health and safety concerns have been identified in the assessment of herbicide-tolerant canola MON88302. On the basis of the data provided in the present Application, and other available information, food derived from canola line MON88302 is considered to be as safe for human consumption as food derived from conventional canola varieties.

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List of Abbreviations

ADF	acid detergent fibre
a.e.	acid equivalent
AMPA	aminomethylphosphonic acid
BBCH	Bayer, BASF, Ciba-Geigy & Hoechst Cereal Grain Growth
BLAST	Basic Local Alignment Search Tool
BLOSUM	Blocks Substitution Matrix; used to score similarities between pairs of distantly related protein or nucleotide sequences
bp	base pairs
bw	body weight
CCI	Confidential Commercial Information
DNA	deoxyribonucleic acid
T-DNA	transferred DNA
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FARRP	Food Allergy Research and Resource Program
FASTA	Fast Alignment Search Tool - All
FMV	Figwort mosaic virus
FSANZ	Food Standards Australia New Zealand
GM	genetically modified
IgE	immunoglobulin E
kDa	kilo Dalton
LC/MS	liquid chromatography mass spectrometry
LC/MS/MS	liquid chromatography/tandem mass spectrometry
LLMV	lower limit of method validation
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI-TOF	matrix-assisted laser desorption/ionisation–time of flight
MRL	maximum residue limit
NDF	neutral detergent fibre
ORF	open reading frame
PCR	polymerase chain reaction
ppm	parts per million
PVDF	polyvinylidene difluoride
P-value	probability value
RAC	raw agricultural commodity
RBD	refined, bleached and deodorised
mRNA	messenger ribonucleic acid
SAS	Statistical Analysis Software
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
U.S.	United States of America

1. Introduction

Monsanto Australia Limited has submitted an application to FSANZ to vary Standard 1.5.2 – Food produced using Gene Technology – in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) canola line MON88302 (OECD Unique Identifier MON-88302-9). The canola has been modified such that all plant tissue is tolerant to the herbicide glyphosate.

Tolerance to glyphosate is achieved through expression of the enzyme 5-enolpyruvyl-3-shikimatephosphate synthase (CP4 EPSPS) encoded by the *cp4epsps* gene derived from the common soil bacterium *Agrobacterium* sp. The CP4 EPSPS protein has previously been assessed by FSANZ in a range of crops including canola. In comparison with a previous GM canola line developed by the Applicant and assessed by FSANZ (FSANZ 2000), MON88302 has tolerance to higher rates of glyphosate and shows greater flexibility in the timing for glyphosate herbicide application.

Initially MON88302 canola will be grown in North America, but it is likely the Applicant will apply at some future date for a licence to grow the crop commercially in Australia. Therefore, if approved, food from this line may enter the Australian and New Zealand food supply both as imported and locally-produced food products.

Canola is the most commonly grown oilseed crop in Australia with an estimated area of 1.8 million hectares planted in the 2011/2012 season of which approximately 45% was grown in Western Australia (AOF 2011). In 2011, canola seed was Australia's 8th largest commodity export in monetary value (FAOSTAT 2011) and, worldwide, Australia was the fourth largest exporter of canola seed behind Canada, Ukraine and France. Approximately 8% of the canola planted in Australia is GM (DPI Vic 2012).

Production of canola in New Zealand is very small by world standards and there is a focus on using canola for biodiesel oil. Currently no GM canola is grown in New Zealand.

2. History of use

2.1 Host organism

Canola (an acronym of 'Canadian oil low acid') is the name used for rapeseed (*Brassica napus, Brassica rapa* or *Brassica juncea*) crops that have less than 2% erucic acid (a fatty acid) and less than 30 micromoles of glucosinolates per gram of seed solids (OECD 2001). Canola varieties were first developed in Canada in the 1950s, using traditional breeding techniques, in response to a demand for food-grade rapeseed products. Rapeseed-derived products that do not meet the compositional standard cannot use the trademarked term, canola. However, it is not uncommon for the generic term rapeseed to be used to describe canola.

Rapeseed is the second largest oilseed crop in the world behind soybean, although annual production is around 25% of that of soybean. In 2010, worldwide production of rapeseed was over 59 million tonnes, with China, Canada and India being the major producers (~13, 11 and 6 million tonnes, respectively) (FAOSTAT 2011). In the case of China and India, a significant amount of non-canola quality rapeseed, is included in the term 'rapeseed'. All of Australia's 2 million tonnes produced in 2010 was canola.

Canola seeds are processed into two major products, oil and meal. The oil is the major product for human consumption, being used in a variety of manufactured food products including salad and cooking oil, margarine, shortening, mayonnaise, sandwich spreads,

creamers and coffee whiteners. The meal provides a good protein source in stock feed for a variety of animals (Bonnardeaux 2007). Canola oil is the third largest source of vegetable oil in the world after soybean oil and palm oil (ACIL Tasman 2007; USDA-ERS 2010). Whole canola seeds are being used increasingly in products such as breads.

Very briefly, the processes involved in preparation of the oil and meal (CCC 2012a) involve seed cleaning, seed pre-conditioning and flaking, seed cooking, pressing the flake to mechanically remove a portion of the oil, solvent extraction of the press-cake to remove the remainder of the oil, and desolventising and toasting of the meal (see Figure 1).

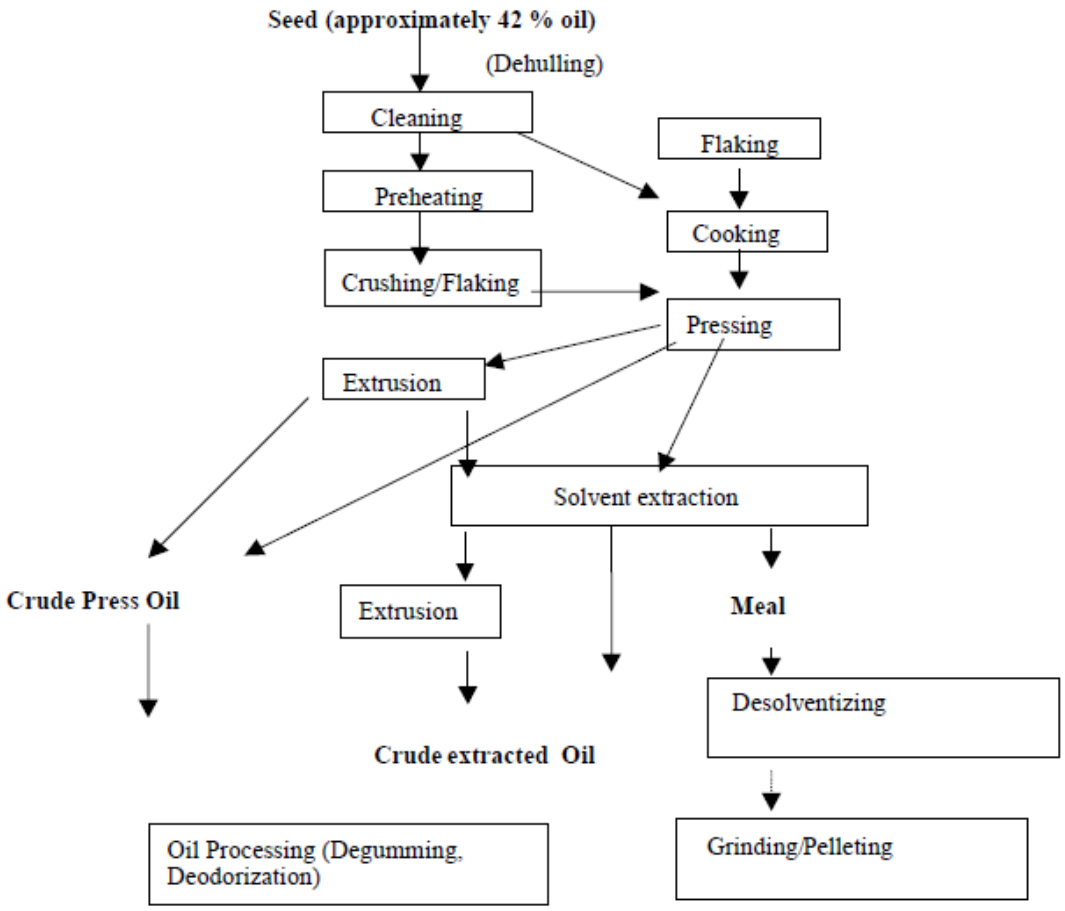

Figure 1: Canola seed processing (diagram taken from OECD (2001))

The canola variety used as the recipient for the DNA insertion to create MON 88302 was 'Ebony' (*Brassica napus* – Argentine Type canola), chosen because of its amenability to *Agrobacterium*-mediated transformation and tissue regeneration. It is a non-GM conventional spring canola variety registered with the Canadian Food Inspection Agency in 1994 by Monsanto Company (CFIA 2012). 'Ebony' originated from a cross of varieties (Bienvenu × Alto) × Cesar, with the selection criteria including yield, oil and protein content, and tolerance to the fungus *Leptosphaeria maculans*, commonly known as blackleg (Government of Alberta 2007).

2.2 Donor organisms

2.2.1 Agrobacterium *sp.*

Agrobacterium sp. strain CP4 produces a naturally glyphosate-tolerant EPSPS enzyme and was therefore chosen as a suitable gene donor for the herbicide tolerance trait (Padgette et al. 1996). The bacterial isolate CP4 was identified in the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are known soil-borne plant pathogens but are not pathogenic to humans or other animals.

2.2.2 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of canola MON88302 (refer to Table 1). These non-coding sequences are used to drive, enhance, target or terminate expression of the novel gene. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans. The genetic elements derived from plant pathogens are not themselves pathogenic and do not cause pathogenic symptoms in canola MON88302.

3. Molecular characterisation

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

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

Studies submitted:

2010. Molecular analysis of glyphosate tolerant Roundup Ready® 2 (RR2) canola MON88302. Study ID# MSL0022523, Monsanto Company (unpublished).
2010. Bioinformatics evaluation of DNA sequences flanking the 5' and 3' junctions of inserted DNA in MON88302: Assessment of putative polypeptides. Study ID# MSL0023088, Monsanto Company (unpublished).
2010. Segregation of the *cp4 epsps* coding sequence in MON88302 in the F_2, F_3 and F_4 populations. Study ID# RPN-10-085, Monsanto Company (unpublished).

3.1 Method used in the genetic modification

Hypocotyl segments from variety 'Ebony' were transformed, using *Agrobacterium tumefaciens,* with the T-DNA from plasmid vector PV-BNHT2672 (see Figure 2) following the method of Radke et al (1992).

After co-culturing with the *Agrobacterium* carrying the vector, the segments were placed on callus growth medium containing antibiotics to inhibit the growth of excess *Agrobacterium*, and then on selection and regeneration media containing glyphosate. Rooted plants (R_0) with normal phenotypic characteristics and tolerance to glyphosate were selected and transferred to soil for growth and further assessment. They were self-pollinated to produce an R_1 generation (see Figure 3) which was also evaluated for tolerance to glyphosate and

screened for the presence of the T-DNA (*cp4 epsps* expression cassette) and absence of plasmid vector backbone.

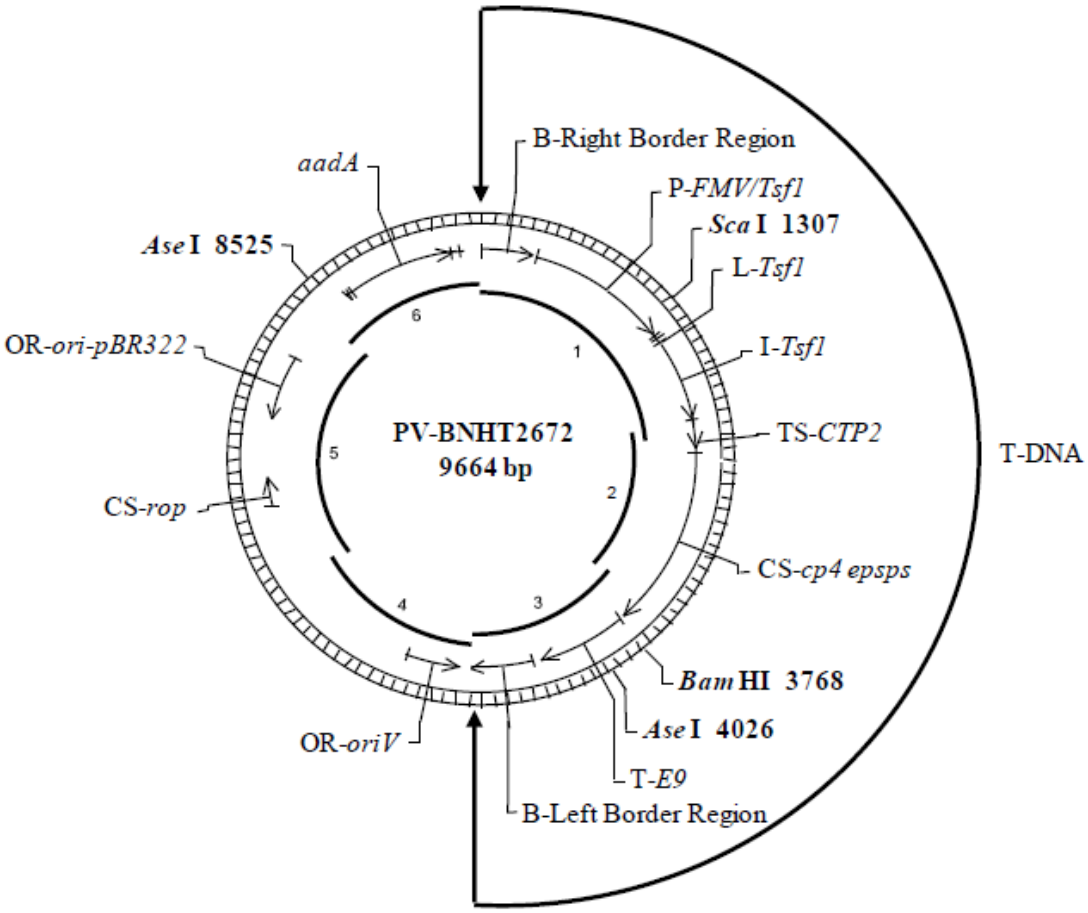

Figure 2: Genes and regulatory elements contained in plasmid PV-BNHT2672. The six probes used for molecular characterisation are indicated in the centre of the diagram.

3.2 Function and regulation of introduced genes

Information on the genetic elements in the T-DNA insert present in MON88302 is summarised in Table 1.

Table 1: Description of the genetic elements contained in the T-DNA of PV-BNHT2672

Genetic element	bp location on PV GMHT 4355	Size (bp)	Source	Relative Orient.	Description & Function	References
RIGHT BORDER	1 - 357	357				
Intervening sequence	358 - 427	70				
FMV/Tsf1	428-1467	1040	Figwort mosaic virus (FMV)	Anti-clockwise	• Enhancer sequence from the 35S promoter	Richins et al (1987)
			Arabidopsis thaliana		• Promoter (encoding elongation factor EF-1α) driving expression of *cp4 epsps*	Axelos et al (1989)
L - *Tsf1*	1468 - 1513	46	*Arabidopsis thaliana*	Anti-clockwise	• 5' untranslated leader (exon 1) from gene encoding EF-1α	Axelos et al (1989)
I - *Tsf1*	1514 - 2135	622	*Arabidopsis thaliana*	Anti-clockwise	• intron from gene encoding EF-1α	Axelos et al (1989)
Intervening sequence	2136 - 2144	8				
CTP2	2145 - 2372	228	*Arabidopsis thaliana*	Anti-clockwise	• Targeting sequence from the *ShkG* gene that encodes the chloroplast transit peptide. • Directs transport of CP4 EPSPS to the chloroplast	Klee et al (1987); Herrmann (1995)
cp4 epsps	2373 - 3740	1368	*Agrobacterium* sp. strain CP4	Anti-clockwise	• Codon optimised codon sequence of the *aroA* gene encoding the CP4 EPSPS protein	Padgette et al (1996); Barry et al (2001)
Intervening sequence	3741 - 3782	42				
E9	3783 - 4425	643	*Pisum sativum*	Anti-clockwise	• 3' untranslated region from the *rbcS2* gene • Terminates transcription of *cp4 epsps*	Coruzzi et al (1984)
Intervening sequence	4426 - 4468	43				
LEFT BORDER	4469 - 4910	442				

3.2.1 cp4 epsps *expression cassette*

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

The cp4 epsps coding region is 1,368 bp in length and is driven by a chimeric constitutive promoter FMV/Tsf1 derived from Figwort mosaic virus and Arabidopsis thaliana. Tsf1 leader and intron sequences enhance expression of the cp4 epsps gene.

A transit peptide derived from elements from the ShkG gene from *Arabidopsis thaliana* targets the CP4 EPSPS protein to the chloroplasts where chorismate biosynthesis (that in non-GM plants is disrupted by glyphosate) occurs. The 3' rbcS2 non-translated region from Pisum sativa (pea) functions to terminate transcription and direct polyadenylation of the mRNA.

This expression cassette is identical to that used in the transformation of other glyphosate tolerant crops, such as Roundup Ready Flex cotton line MON88913, and Roundup Ready 2 Yield soybean line MON89788 both considered by FSANZ in previous approvals (FSANZ 2006 ; FSANZ 2008 respectively).

3.3 Breeding of canola line MON88302

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

A single R_0 plant was obtained and then self-pollinated over three generations. Homozygous R_2 plants containing only a single T-DNA insertion were identified by a combination of analytical techniques including glyphosate spray, polymerase chain reaction (PCR), and Southern blot analysis, resulting in production of glyphosate-tolerant canola MON 88302 at the R_3 generation. MON 88302 was selected as the lead event based on superior phenotypic characteristics and its comprehensive molecular profile.

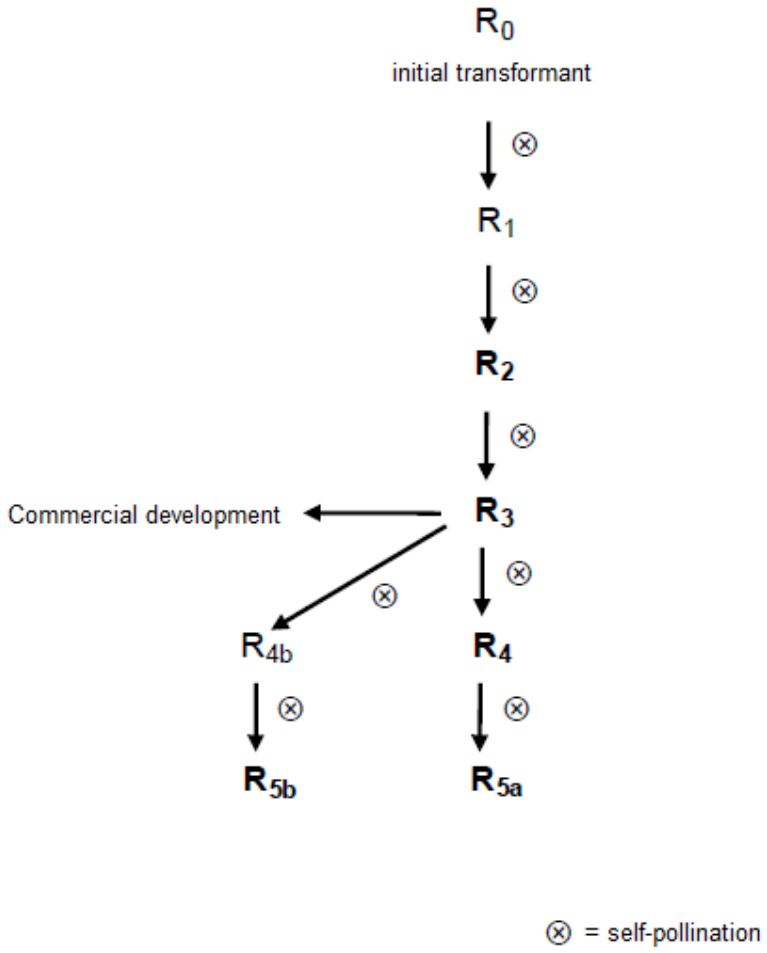

Figure 3: Breeding diagram for MON88302

3.4 Characterisation of the genes in the plant

A range of analyses were undertaken in order to characterise the genetic modification in canola line MON88302. These analyses focussed on the nature of the insertion of the introduced genetic elements and whether any unintended genetic re-arrangements may have occurred as a consequence of the transformation procedure.

3.4.1 Transgene copy number and insert characterisation

Total genomic DNA from leaf tissue of identity-confirmed MON88302 (generationR_3) and a negative control ('Ebony') was used for Southern blot analysis.

The DNA from both sources was digested with restriction enzymes. The resulting DNA fragments were separated by agarose gel electrophoresis and transferred to a membrane for sequential hybridisation with six different overlapping radiolabelled probes (labelled 1 – 6 in the interior of Figure 2) that, taken together, spanned the entire plasmid vector. A positive control (digested DNA from 'Ebony' spiked with restriction enzyme-digested PV-BNHT2672 or with probe templates) was also included in the Southern blot analyses to demonstrate sensitivity of the Southern blots and to confirm that the probes were recognising the target sequences. The negative control was 'Ebony' genomic DNA digested with restriction enzymes.

Southern blot analysis using the three probes that span the T-DNA (see Figure 2) was used to determine the insert/copy number. The three probes spanning the backbone sequences were used in the Southern analysis to detect whether any plasmid backbone sequences had been incorporated into the MON88302 genome.

To determine the integrity and genomic organisation of the insertion site and to investigate whether the DNA sequences flanking the insert are native to the canola genome, polymerase chain reaction (PCR) and DNA sequence analyses were undertaken. PCR primers were designed to amplify two overlapping DNA amplicons that span the insert and flanking regions. Overlapping PCR products were generated and used to determine the nucleotide sequence of the insert and flanking regions using BigDye® Terminator chemistry (http://www.appliedbiosystems.com.au/).

The Southern blot analyses indicated that the introduced DNA has been inserted at a single locus that contains one intact copy of the T-DNA (see Figure 4). No PV-BNHT2672 backbone sequences were detected.

Sequence data were obtained for the insert and flanking regions (839 bp sequence immediately flanking the 5' end of the insert; 907 bp sequence immediately flanking the 3' end of the insert). The consensus sequence of the insert is 4,428 bp long and is identical to the corresponding T-DNA except there has been some truncation of the right and left border regions. A PCR product was generated from the 'Ebony' control and the sequence was compared with the sequences in the 5' and 3' flanking regions of the MON88302 insert. This showed that in MON88302:

- there is a 9 bp insertion immediately adjacent to the 3' end of the insert
- there is a single nucleotide difference between the conventional control sequence and the genomic DNA sequence flanking the 3' end of the MON 88302 insert
- the genomic sequences flanking the insert are native to the canola genome but there has been a 29 bp deletion from the 'Ebony' genome in the 3' flanking region

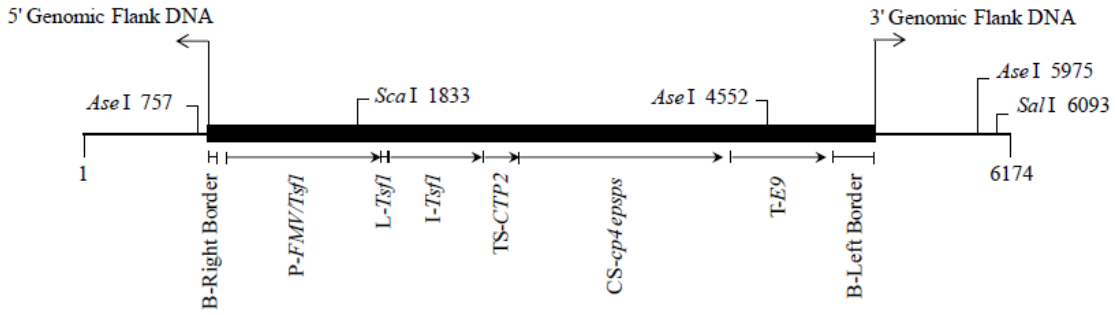

Figure 4: Schematic representation of the insert and flanking regions in MON88302

3.4.2 Open reading frame (ORF) analysis

The transgenic insert has the identical sequence to the T-DNA of the PV-BNHT2672 plasmid (see Section 3.4.1) and therefore has no unexpected ORFs.

Sequences spanning the two junction regions formed as a result of the insertion of the T-DNA were translated using DNAStar software (http://www.dnastar.com/) from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames. A total of 11 ORFs (five spanning the 5' junction and six spanning the 3' junction) were identified that encoded putative polypeptides ranging in size from 13 – 63 amino acids. No analysis was done to determine whether any potential regulatory elements were associated with the polypeptides.

The putative polypeptides encoded by the 11 identified ORFs were then analysed using a bioinformatic strategy to determine whether, in the event they were translated, they would raise any allergenic or toxicity concerns (refer to Section 4.1).

3.5 Stability of the genetic changes in canola line MON88302

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

3.5.1 Genetic stability

The genetic stability of the insert in MON88302 was evaluated by Southern analysis in five leaf tissue samples from plants of generations R_2, R_3, R_4, R_{5a} and R_{5b} (refer to Figure 3)

Genomic DNA obtained from each source was digested with one restriction enzyme and the resulting fragments were hybridised with two of the three T-DNA radio-labelled probes (probes 1 and 3 in Figure 2) outlined in Section 3.4.1. The same positive controls as described in Section 3.4.1 were used.

Analysis of the MON88303 DNA showed the presence of the expected hybridisation fragments in all samples and therefore confirmed the genetic stability of the insert in MON88302 over different generations.

3.5.2 Phenotypic stability

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

Following generation of the R_0 plant, a number of self-pollination rounds were carried out (refer to Figure 3). At each generation, the plants showed the expected ratio of 1:1 (glyphosate tolerant: glyphosate susceptible) using an endpoint Taqman® analysis (see e.g. Gao et al. 2009 for a general description of the technique) and/or a glyphosate spray test

An individual R_3 plant, confirmed by PCR to contain the MON88302 insert, was then crossed with a proprietary canola line to produce hemizygous seed. A plant from the resulting generation was then self-pollinated to produce F_2 seed and an F_2 plant was self-pollinated to produce F_3 seed, and so on to production of F_4 seed (see Figure 5).

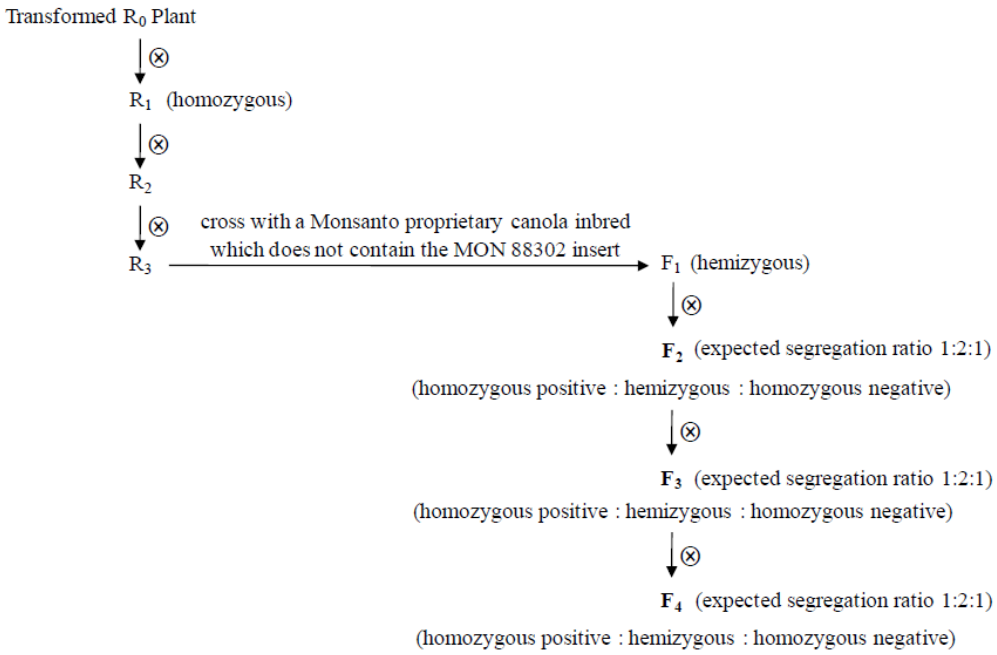

Figure 5: Breeding path for generating segregation data over several generations in MON88302

The copy number of the *cp4 epsps* gene was determined in plants from the F_2, F_3 and F_4 generations using a real time TaqMan® PCR assay. The results of a chi-square analysis (Table 2) showed that there was no significant difference between the expected and observed numbers and therefore, that the *cp4 epsps* expression cassette is stably inherited according to Mendelian principles.

Table 2: Segregation of the cp4 epsps gene in MON88302 over three generations

Generation	Total plants	Observed (Expected) Homozygous +	Observed (Expected) Hemizygous	Observed (Expected) Homozygous -	Probability (P)[1]
F_2	220	51 (55)	122 (110)	47 (55)	0.2511
F_3	166	39 (41.5)	94 (83)	33 (41.5)	0.1874
F_4	198	53 (49.5)	97 (99)	48 (49.5)	0.8465

[1]Statistical significance is when P≤0.05

3.6 Antibiotic resistance marker genes

No antibiotic marker genes are present in canola line MON88302. Plasmid backbone analysis shows that no plasmid backbone has been integrated into the canola genome during transformation, i.e. the *aadA* gene, which was used as a bacterial selectable marker gene, is not present in canola MON88302.

3.7 Conclusion

Canola line MON88302 contains the cp4 epsps gene that encodes a protein conferring tolerance to the herbicide glyphosate.

Comprehensive molecular analyses of canola line MON88302 indicate there is a single insertion site comprising a single, complete copy of the cp4 epsps expression cassette. The introduced genetic elements are stably inherited from one generation to the next. There are no antibiotic resistance marker genes present in the line and plasmid backbone analysis shows no plasmid backbone has been incorporated into the transgenic locus.

4. Characterisation of novel proteins

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

Two types of novel proteins were considered:

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

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

Study submitted:

2010. Bioinformatics evaluation of DNA sequences flanking the 5' and 3' junctions of inserted DNA in MON88302: Assessment of putative polypeptides. Study ID# MSL0023088, Monsanto Company (unpublished).

A bioinformatics analysis was performed to assess the similarity to known allergens and toxins of the putative polypeptides encoded by the 11 sequences obtained from the ORF analysis (refer to Section 3.4.2).

To evaluate the similarity to known allergens of proteins that might potentially be produced from translation of the ORFs, an epitope search was carried out to identify any short sequences of amino acids that might represent an isolated shared allergenic epitope. This search compared the sequences with known allergens in the Allergen, Gliadin and Glutenin sequence database, residing in the FARRP (Food Allergy Research and Resource Program) dataset (Version 10) within AllergenOnline (University of Nebraska; http:www.allergenonline.org/). The FASTA algorithm (Pearson and Lipman 1988), version 3.4t 26, was used to search the database using the BLOSUM50 scoring matrix (Henikoff and Henikoff 1992). No alignments with any of the 11 query sequences generated an E-score[1] of $\leq 1e^{-5}$, no alignment met or exceeded the Codex Alimentarius (Codex 2003) FASTA alignment threshold for potential allergenicity and no alignments of eight or more consecutive identical amino acids (Metcalfe et al. 1996) were found. It was concluded that the 11 putative polypeptides are unlikely to contain any cross-reactive IgE-binding epitopes with known allergens.

The sequences corresponding to the 11 reading frames were also compared with sequences present in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using the FASTA algorithm. No significant similarities of the 11 reading frames to any sequences in the databases (including those of known toxins) were found.

It is concluded that, in the unlikely event transcription and translation of the 11 identified ORFs could occur, the encoded polypeptides do not share any significant similarity with known allergens or toxins.

4.2 Function and phenotypic effects of the CP4 EPSPS protein

Glyphosate is a herbicide because of its ability to inhibit the catalytic activity of EPSPS. EPSPS is an endogenous enzyme involved in the shikimate pathway for aromatic amino acid biosynthesis which occurs exclusively in plants and microorganisms, including fungi. Inhibition of the wild type EPSPS enzyme by glyphosate leads to deficiencies in aromatic amino acids in plant cells and eventually to the death of the whole plant. The 'Ebony' variety used as the parent for the genetic modification described in this application, contains wild type EPSPS which can be inhibited by glyphosate. The shikimate biochemical pathway is not present in mammals, which includes humans.

EPSPS proteins occur ubiquitously in plants and microorganisms and have been extensively studied over a period of more than thirty years. In plants, the EPSPS enzyme is inhibited by glyphosate (Steinrucken and Amrhein 1980), but bacterial EPSPSs, such as the CP4-EPSPS, have a reduced affinity for glyphosate (Padgette et al. 1996; Barry et al. 2001) thereby allowing the continued action of the enzyme in the presence of glyphosate. The CP4 EPSPS protein present in canola MON88302 is functionally the same as the wild type *Agrobacterium* enzyme. It is a 47.6 kDa protein containing 455 amino acids.

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

4.2.1 CP4 EPSPS protein expression in MON88302 tissues

Studies submitted:

2010. Amended report for MSL0022681: Assessment of CP4 EPSPS protein levels in canola tissues collected from MON88302 produced in United States and Canadian field trials during 2009. Study ID# MSL0023090, Monsanto Company (unpublished).

2011. Assessment of CP4 EPSPS protein levels in canola pollen tissues from MON88302 produced in United States greenhouse trials during 2010. Study ID# MSL0023598, Monsanto Company (unpublished).

Plants of MON88302 (generation R_4) were grown from verified seed lots at three field sites in the U.S[2] and three field sites in Canada[3] during the 2009 growing season. The identity of any subsequent harvested grain from each site was also confirmed using event-specific polymerase chain reaction (PCR). There were four replicated plots at each site. Samples were taken at various stages of growth (Table 3) and the level of CP4 EPSPS protein was determined for each sample type using a validated enzyme linked immunosorbent assay (ELISA) utilising a mouse anti-CP4 EPSPS capture antibody and a commercial detection reagent (Sigma-Aldrich) comprising goat-antiCP4 EPSPS conjugated to horse radish peroxidase. Plates were analysed on a commercial microplate spectrophotometer. Quantification of total CP4 EPSPS protein was accomplished by interpolation on a CP4 EPSPS protein standard curve.

In addition to the above study, a separate 2010 glasshouse study was done to collect data on the level of CP4 EPSPS in pollen. Pollen was collected from three plots of MON88302 plants (generation R_5) identified from Taqman® PCR analysis. The same antibodies as described above were used for the ELISA analysis.

The results are given in Table 3.

Table 3: CP4 EPSPS protein content in MON88302 canola parts at different growth stages (averaged across six sites – except for pollen data which is averaged over three plots)

Tissue	Generation	Growth stage[1]	n	µg/g dry weight		Standard Deviation
				Mean	Range	
Forage[2]	R_4	30 BBCH	20	170	120 - 210	22
Seed	R_5	99 BBCH	16	27	22 - 46	5.6
Over-season leaf -1	R_4	13 – 14 BBCH	16	180	110 - 250	40
Over-season leaf - 2	R_4	17 – 19 BBCH	9	180	120 - 250	41
Over-season leaf - 3	R_4	30 BBCH	20	230	130 - 300	50
Over-season leaf - 4	R_4	60 – 62 BBCH	20	210	110 - 500	80
Root - 1	R_4	30 BBCH	19	82	46 - 100	17
Root - 2	R_4	71 – 73 BBCH	11	38	24 - 62	14
Pollen	R_5	60 – 69 BBCH	3	9	8.2 – 9.6	0.71

[1]The canola growth stages are based on the Bayer, BASF, Ciba-Geigy and Hoechst Cereal Grain Growth Scale (BBCH) see e.g. CCC (2012b)
[2]Forage is the above ground plant parts used for animal feed.

The level of CP4 EPSPS was lowest in the pollen (approximately 9 µg/g dry weight) and highest in leaves at the stage where stem elongation (bolting) begins (approximately 230 µg/g dry weight). The level in the mature seed was approximately 27 µg/g dry weight.

[2] Power County (Idaho); Wilkin County (Minnesota); McHenry County (North Dakota).
[3] Portage la Prairie (Manitoba); Newton (Manitoba); Saskatoon (Saskatchewan).

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

Study submitted:

2010. Characterization of the CP4 EPSPS protein purified from the seed of MON88302 and comparison of the physicochemical and functional properties of the MON88302-produced and E. coli-produced CP4 EPSPS proteins. Study ID# MSL0022841, Monsanto Company (unpublished).

The amount of CP4 EPSPS protein produced in MON88302 was insufficient for safety evaluations. Therefore, the CP4 EPSPS protein for these evaluations was produced in *Escherichia coli.* In order to confirm that the *E. coli*-produced CP4 EPSPS is equivalent to that expressed in MON88302, a range of analytical techniques was employed. These techniques included determining the molecular weight, protein purity, protein identity (through immunoblot analysis, MALDI-TOF mass spectrometry and N-terminal sequencing), glycosylation analysis and functional activity comparison.

The MON88302-derived CP4 EPSPS protein was purified from defatted, ground seed of generation $R_{5.}$ Fractions containing the protein were identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. The concentration of the MON88302 protein in the final desalted sample was 0.26 mg/mL.

4.3.1 Molecular weight and immunoreactivity

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

The SDS-PAGE gel containing the purified plant and bacterial proteins and stained with Brilliant Blue G-Colloidal stain, showed a single band with an average apparent molecular weight of 43.5 kDa. This molecular weight estimate was in good agreement with the value of 47.6 kDa that was calculated from the DNA sequence. Based on a densitometric analysis of SDS-PAGE, the average purity of the MON88302 protein was estimated to be approximately 99%.

Western blot analysis showed a single immunoreactive band, increasing in intensity with protein load, that had co-migrated in separate extracts from MON88302 and *E. coli.* This co-migrating band can be taken as evidence of extensive immunological cross reactivity between the proteins from the two sources.

4.3.2 MALDI-TOF tryptic mass fingerprint and intact mass analyses

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

It was estimated that the peptide mapping of the plant CP4 EPSPS protein identified 85% of the expected protein sequence and this was adequate to provide convincing evidence of the identity of the protein.

The intact mass of the MON88302 protein was also determined by MALDI-TOF based on an average of three separate mass spectral acquisitions and indicated a value of 47.3 kDa.

4.3.3 N-terminal sequence analysis

Automated Edman degradation chemistry was performed on MON88302-derived CP4 EPSPS protein that had been eluted from SDS-PAGE.

The results yielded two possible protein sequences, both of which were consistent with the predicted sequence of CP4 EPSPS and represent a staggered N-terminal sequence that has previously been reported in a other GM crops containing the CP4 EPSPS protein (see Thorp and Silvanovich 2004 and references therein). The occurrence is not unusual since the N-terminal methionine can be processed from proteins in eukaryotes (see e.g. Polevoda and Sherman 2000) and additional N-terminal residues can then be lost due to endogenous protease activity that is released when the plant cells are homogenised.

On the weight of evidence, the amino acid analyses from the N-terminus yielded a 15 amino acid sequence which matched the expected sequence of the mature CP4 EPSPS protein after processing of the chloroplast transit peptide and loss of the N-terminal methionine.

4.3.4 Glycosylation analysis

Many eukaryotic proteins are glycoproteins that have been post-translationally modified by the addition of carbohydrate moieties (glycans) covalently linked to the polypeptide backbone. Glycosylation that occurs on side chains of asparagine residues is termed N-glycosylation. The addition of N-acetylglucosamine to the β-hydroxyl of either serine or threonine residues is known as O-glycosylation. The carbohydrate component may represent from <1% to >80% of the total molecular weight of glycoprotein..

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

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

Carbohydrate detection was performed directly on MON88302-derived CP4 EPSPS bands (transferred from SDS-PAGE to a PVDF membrane) using a commercial glycoprotein detection kit (Amersham ECL Glycoprotein Detection Module). Transferrin, a major serum glycoprotein, was used as a positive control.

Transferrin was detected at the expected molecular weights and in a concentration-dependent manner. No signal was detected at the molecular weight expected for CP4 EPSPS in either the MON88302- or *E.coli*-derived samples.

4.3.5 Enzymatic activity

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

CP4 EPSPS from both plant- and *E. coli*-derived sources was tested for activity and values of 4.93 U/mg and 2.79 U/mg respectively were obtained. Within the accuracy of the method

used, these values were considered to be indicative of equivalent functional activity of CP4 EPSPS from the two sources.

4.3.6 Conclusion

The identity of MON88302-derived CP4 EPSPS was confirmed by a number of analytical techniques, namely recognition by anti-CP4 EPSPS antibody, MALDI-TOF analysis, N-terminal sequencing and enzymatic activity.

CP4 EPSPS produced in MON88302 and in *E. coli* were compared. The proteins from both sources were found to co-migrate in SDS-PAGE, to be recognised by an anti-CP4 EPSPS antibody, to lack glycosylation and to show equivalent activity. Thus the CP4 EPSPS proteins from the two sources can be said to be equivalent and the *E. coli*-derived CP4 EPSPS protein is a suitable surrogate for MON88302-derived CP4 EPSPS in safety assessment studies.

4.4 Potential toxicity of the CP4 EPSPS protein

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

The assessment focuses on: whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food; amino acid sequence similarity with known protein toxins and anti-nutrients; structural properties of the novel protein including whether it is resistant to heat or processing and/or digestion. Appropriate oral toxicity studies in animals may also be considered, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate a concern.

4.4.1 History of human consumption

As outlined in Section 4.2, the CP4 EPSPS protein is found in plants and microorganisms and would therefore be routinely consumed as a normal part of the diet. In addition, the *cp4 epsps* gene has been widely used in the genetic modification of commercialised food crops over the last 10 years and there have been no safety concerns raised with the consumption of the protein (Delaney et al. 2008).

4.4.2 Similarity of CP4 EPSPS with known toxins

Study submitted:

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

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

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

4.4.3 In vitro digestibility

See Section 4.5.3

4.4.4 Thermolability

The thermolability of an enzyme provides an indication of its stability under cooking/processing conditions.

Study submitted:

2010. Amended Report for MSL0022432: Effect of temperature treatment on the functional activity of CP4 EPSPS. Study ID# MSL0023307, Monsanto Company (unpublished).

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

Results of the activity assay indicated that, at temperatures above 37^{o} C activity starts to be reduced after 15 min (approximately 15% activity lost at 15 min), and by 75^{o} C is below the limit of detection (LOD) after 15 min. These findings are in agreement with past EPSPS thermolability studies submitted to FSANZ as part of application dossiers.

4.4.5 Acute toxicity study

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

Study submitted:

1993. Acute oral toxicity study of CP4 EPSPS protein in albino mice Study ID# MSL0013077. Monsanto Company (unpublished).

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

4.5 Potential allergenicity of the CP4 EPSPS protein

The potential allergenicity of the CP4 EPSPS protein was evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination. This is because no single criterion is sufficiently predictive of either allergenicity or non-allergenicity (see eg Thomas et al. 2009). The assessment focuses on:

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

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

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

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

4.5.1 Source of protein

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

4.5.2 Similarity to known allergens

Study submitted:

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

Bioinformatic analysis provides part of a "weight of evidence" approach for assessing potential allergenicity of novel proteins introduced to GM plants. It is a method for comparing the amino acid sequence of the introduced protein with sequences of known allergens in order to indicate potential cross-reactivity between allergenic proteins and the introduced protein. As with the bioinformatic analysis that looked at similarities of the CP4 EPSPS protein with known protein toxins (see Section 4.4.2), the generation of a small *E*-value provides an important indicator of significance of matches (Pearson 2000; Baxevanis 2005).

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

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

4.5.3 In vitro *digestibility*

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

Study submitted:

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

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

4.6 Conclusion

Canola line MON88302 expresses one novel protein, CP4 EPSPS. The level of CP4 EPSPS is lowest in the pollen (approximately 9 µg/g dry weight) and highest in leaves at the stage where stem elongation (bolting) begins (approximately 230 µg/g dry weight). The level in the mature seed is approximately 27 µg/g dry weight.

The identity of MON88302-derived CP4 EPSPS was confirmed by a number of analytical techniques, namely recognition by anti-CP4 EPSPSA antibody, MALDI-TOF analysis, N-terminal sequencing and enzymatic activity.

Bioinformatic studies have confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated CP4 EPSPS would be completely digested before absorption in the gastrointestinal tract would occur. The protein also loses enzyme activity with heating.

Taken together, the evidence discussed in this section (including a safe history of use of the protein in the food supply over the last 10 years) indicates that the CP4 EPSPS protein is unlikely to be toxic or allergenic to humans.

5. Herbicide metabolites

For GM foods derived from crops that are herbicide tolerant, there are two issues that require consideration. The first is dealt with in this safety assessment and involves assessment of any novel metabolites that are produced after the herbicide is applied, to determine whether these are present in the final food and whether their presence raises any toxicological concerns. In particular, the assessment considers whether appropriate health-based guidance values (i.e. Acceptable Daily Intake [ADI] or Acute reference Dose [ARfD]) need to be established.

The second consideration, which is separate from the GM food approval process and therefore not included as part of this safety assessment, relates to the presence of herbicide residues on the food. Any food products (whether derived from GM or non-GM sources) sold in both Australia and New Zealand must not have residue levels greater than the relevant maximum residue limit (MRL). Where necessary, an MRL may have to be set.

5.1 Glyphosate metabolites

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

The ADI for glyphosate is 0.3 mg/kg bw/day[4]. Given the long history of safe use of glyphosate on GM food crops containing the CP4 EPSPS protein, there are no concerns with its use on canola line MON88302. Nevertheless, the Applicant supplied a metabolite study which has been considered by FSANZ.

Study submitted:

2010. Magnitude of glyphosate residues in glyphosate tolerant canola raw agricultural commodities following applications of a glyphosate-based formulation. 2009 U.S. trials. Study ID # MSL0022984, Monsanto Company (unpublished).

Field trials of plants containing three transformation events (all generated using plasmid vector PV-BNHT2672), one of which was MON88302, were conducted in 2009 at eight sites in the U.S. located in areas representative of commercial canola production. At one of the sites, low temperatures led to a problem with seed maturation and the seed was not used in the final analysis. Treated plants were sprayed with Roundup WeatherMAX® with Transorb 2 Technology liquid herbicide (containing 540 g/L glyphosate acid) applied in combinations at pre-emergence, 4 – 6 leaf, late bolting and 1st flower as shown in Table 4. The post-emergent applications were all via foliar broadcast spray. Treatments 2 and 4 represent the proposed use rates for commercial MON88302 crops. Harvested seed (raw agricultural commodity – RAC) was analysed for glyphosate and AMPA by Liquid Chromatography Mass Spectrometry (LC-MS/MS). Samples of mature seed from Treatment 3 at one of the sites were also dried to 10% moisture content and then used to obtain various processed fractions for which glyphosate and AMPA levels were determined.

Table 4: Applications of glyphosate to canola

Treatment	Growth stages/target application rates (kg a.e/ha)[1]			
	Pre-emergence	4-6 leaf	Late bolting	1st flower
1	0	0	0	0
2	4.25	0.9	0	0.9
3	4.25	0.9	0	1.8
4	4.25	0.9	0.9	

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

For the proposed use rates (Treatments 2 and 4), the median glyphosate (GLY) residue remaining in seed was around 1.5 ppm while the AMPA residue was below the lower limit of method validation (LLMV). As expected, the residue levels were higher in Treatment 3 (Table 5). These low levels are consistent with the time interval between the final application of the herbicide and harvest.

[4] ADIs are established by the Office of Chemical Safety within the Department of Health and Ageing. The most recent (December 2011) list can be found at http://www.health.gov.au/internet/main/publishing.nsf/Content/E8F4D2F95D616584CA2573D700770C2A/$File/ADI-dec11.pdf

Table 5: Levels (ppm) of GLY and AMPA remaining in MON88302 seed after spraying with glyphosate

Treatment	PHI[1] (days)	GLY		AMPA		GLY + AMPA (Median)
		Median	Range	Median	Range	
2	58 - 70	1.6	0.23 – 6.71	< LLMV[2]	<LLMV – 0.16	1.67
3	58 - 70	3.7	1.41 – 11.3	0.1	<LLMV – 0.3	3.85
4	65 - 77	1.45	0.08 – 2.51	<LLMV	<LLMV – 0.06	1.48

[1]PHI = Pre-Harvest Interval i.e. days between last application of herbicide and collection of field sample
[2]LLMV = lower limit of method validation

Table 6 shows the concentration factor of GLY and AMPA in various processed fractions compared to the RAC for Treatment 3. The amount of both residues is negligible in oil, being either not detected or at, or below, the LLMV.

Table 6: Concentration factor of GLY and AMPA in processed fractions of MON88302

Processed fraction	Concentration factor[1]
Unprocessed seed	1
Toasted meal	2.4 Gly; 2.6 AMPA
Crude oil	<0.03
Refined oil	<0.03
RBD[2] oil	<0.03

[1]concentration in processed fraction/concentration in seed.
[2]RBD = refined, bleached de-odorised oil.

In the absence of any significant exposure to either parent herbicide or metabolite, the risk to public health and safety is considered to be negligible.

6. Compositional analysis

The main purpose of compositional analysis is to determine if, as a result of the genetic modification, any unexpected changes have occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

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

6.1 Key components

For canola there are a number of components that are considered to be important for compositional analysis (OECD 2011). Since oil is the major food use, the key nutrients of canola seed appropriate for a comparative study include the fatty acids (including the toxicant, erucic acid), vitamins (Vitamin E and Vitamin K_1) and sterols. It is noted that the

OECD recommendations for analysis of Vitamin K_1 and sterol are not emphasised in the previous version of the compositional document (OECD 2001) and that the compositional studies done by the Applicant were based on this previous version. For animal feed (meal), proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids, glucosinolates, minerals, tannins sinapine and phytic acid should also be considered.

6.2 Study design and conduct for key components

Studies submitted:

2011. Compositional analysis of canola seed collected from MON88302 grown in the United States and Canada during the 2009 growing season. Study ID# MSL0022806, Monsanto Company (unpublished).
2011. Analysis of tannins in canola seed collected from MON88302 grown in the United States and Canada during the 2009 growing season. Study ID# RAR-2011-0237, Monsanto Company (unpublished).

The test (MON88302, seed of the R_5 generation) and control ('Ebony') lines were grown under typical production conditions at five field sites across North America[5] during the 2009 growing season. Additionally, a minimum of two non-GM commercial lines were grown at each site, with one of the lines being grown at all five sites, in order to generate tolerance ranges for each analyte; in total there were seven non-GM lines grown. The identities of MON88302, 'Ebony' and the commercial references were confirmed by verifying the chain of custody documentation and also through PCR of harvested seed from each site (acceptance level was ≤3.05% unintended traits). MON88302 plants were not sprayed or were sprayed at the 5 – 6 leaf stage with glyphosate at a target rate of 1.8 kg a.e./ha. The test, 'Ebony' and commercial reference lines were planted in a randomised block design with four replicates at each site.

Seed was harvested at physiological maturity and samples were analysed for proximates, fibre (acid detergent fibre – ADF; neutral detergent fibre – NDF; total dietary fibre), fatty acids, amino acids, minerals, vitamin E (α-tocopherol), and anti-nutrients (glucosinolates, phytic acid, sinapine, tannins). Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods. A total of 70 analytes were measured. Although supplied as a separate study, the analysis of tannin levels was done using seed from the same trials as described above.

6.3 Analyses of key components in seed

For each analyte 'descriptive statistics' were generated i.e. a mean (least square mean) and standard error averaged over all sites (combined-site analysis). The values thus calculated (comparing glyphosate-treated MON88302 with 'Ebony') are presented in Tables 7 – 12.

Of the 70 analytes considered 18 had more than half of the observations below the assay limit of quantitation. The remaining 52 analytes were analysed using a mixed model analysis of variance. Data were transformed into Statistical Analysis Software[6] (SAS) data sets and analysed using SAS® software (SAS MIXED). Separate analyses were done to compare glyphosate-treated MON88302 with 'Ebony' and non-treated MON88302 with 'Ebony'. The

[5] The five sites were: Wilkin County, MN ; McHenry County, ND; Portage la Prairie, Manitoba; Newton, Manitoba, Saskatoon, Saskatchewan.
[6] SAS website - http://www.sas.com/technologies/analytics/statistics/stat/index.html

four replicated sites were analysed both separately and combined across all sites (combined-site analysis). In assessing the significance of any difference between means, a P-value of 0.05 was used (i.e. a P-value of ≥0.05 was not significant).

Any statistically significant differences between MON88302 and the 'Ebony' control have been compared to the 95% tolerance interval (i.e. 95% confidence that the interval contains 99% of the values expressed in the commercial lines) compiled from the results of the seven commercial reference lines combined across all sites, to assess whether the differences are likely to be biologically meaningful. Additionally, the results for MON88302 and 'Ebony' have been compared to a combined literature range for each analyte, compiled from published literature for commercially available canola[7]. It is noted, however, that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within a crop species (Harrigan et al. 2010). Therefore, even if means fall outside the published range, this is not necessarily a concern.

6.3.1 Proximates and fibre

Results of the proximate and fibre analysis are shown in Table 7. The only analyte mean to show a significant difference was total dietary fibre, which was higher in MON88302 than in 'Ebony'. However, this mean was within the tolerance interval.

Table 7: Mean (±standard error) percentage dry weight (%dw) of proximates and fibre in seed from glyphosate-treated MON88302 and 'Ebony'.

Analyte	MON88302[2] (%dw)	'Ebony' (%dw)	Overall treat effect (P-value)	Tolerance interval (%dw)	Combined literature range (%dw)
Ash	3.96±0.18	3.90±0.18	0.565	3.32 – 4.66	3.36 – 6.02
Protein	23.04±0.7	23.14±0.69	0.847	17.2 – 30.08	17.4 – 44.3
Total Fat	47.06±0.83	46.82±0.83	0.659	39.65 – 51.24	24.0 – 49.5
Carbohydrate[1]	25.96±0.68	26.13±0.68	0.765	23.12 – 30.77	
ADF	15.32±1.36	14.47±1.36	0.082	6.95 – 23.92	11.6 – 26.7
NDF	17.43±1.38	16.7±1.38	0.231	10.07 – 25.94	16.49 – 34.72
Total Dietary Fibre	20.9±0.79	18.37±0.78	0.004	13.97 – 24.85	
Moisture (%fw)	5.35±0.34	5.45±0.34	0.688	4.33 – 6.91	3.17 – 10.0

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

[2] mauve shading represents MON88302 means that are significantly lower than the 'Ebony' means while orange shading represents MON88302 means that are significantly higher.

6.3.2 Fatty Acids

The levels of 29 fatty acids were measured. Of these, 18 fatty acids (including erucic acid) had more than 50% of the observations below the assay limit of quantitation (LOQ) and were excluded from the statistical analysis. Results for the remaining 11 fatty acids are given in Table 8 and can be summarised as follows:

- There was no significant difference between the means of MON88302 and 'Ebony' for palmitic, eicosenoic, behenic acids, lignoceric and nervonic acids.

[7] Published literature for canola includes Wang et al (1999), Pritchard et al (2000), Szmigielska et al (2000), Marwede et al (2004), Barthet & Daun (2005), Brand et al (2007), Seberry et al (2007), Spragg & Mailer (2007), OECD (2011), Dairy One Cooperative (2011)

- The mean levels of palmitoleic, stearic, oleic arachidic and behenic acids were significantly lower in seed of MON88302 compared with seed from the control but fell within both the tolerance ranges and the combined literature ranges, except in the case of oleic acid. For this fatty acid, the means for both MON88302 and 'Ebony' seed were higher than the literature range but fell within the tolerance range.

- The mean levels of linoleic and linolenic acids were significantly higher in seed of MON88302 compared with seed of the control. Both means fell within both the tolerance interval and combined literature range.

Table 8: Mean (±standard error) percentage composition, relative to total fat, of major fatty acids in seed from glyphosate-treated MON88302 and 'Ebony'.

Analyte	MON88302[1] (%total)	'Ebony' (%total)	Overall treat effect (P-value)	Tolerance interval (%total)	Combined literature range (%total)
Palmitic acid (C16:0)	4.23±0.078	4.10±0.077)	0.094	2.84 – 5.26	2.7 – 7.0
Palmitoleic acid (C16:1)	0.22±0.0081	0.24±0.0081	0.008	0.17 – 0.30	ND[2] – 0.6
Stearic acid (C18:0)	1.68±0.044	1.98±0.044	<0.001	0.90 – 3.05	0.8 – 3.0
Oleic acid (C18:1)	62.82±0.62	65.79±0.62	<0.001	56.13 – 70.69	8.0 – 60.0
Linoleic acid (C18:2)	19.26±0.51	17.67±0.51	<0.001	12.6 – 24.49	15.0 – 30.0
Linolenic acid (C18:3)	9.58±0.27	7.98±0.27	<0.001	6.96 – 11.73	5.0 – 13.0
Arachidic acid (C20:0)	0.54±0.011	0.60±0.011	<0.001	0.45 – 0.80	ND – 3.0
Eicosenoic acid (C20:1)	1.13±0.024	1.09±0.024	0.068	0.83 – 1.68	3.0 – 15.0
Behenic acid (C22:0)	0.27±0.0072	0.28±0.0072	0.016	0.19 – 0.43	ND – 2.0
Lignoceric acid (C24:0)	0.16±0.016	0.16±0.015	0.823	0.033 – 0.25	ND – 2.0
Nervonic acid (C24:1)	0.12±0.015	0.11±0.015	0.377	0.041 – 0.18	ND – 3.0

[1] mauve shading represents MON88302 means that are significantly lower than the 'Ebony' means while orange shading represents MON88302 means that are significantly higher.
[2]ND = not detectable

6.3.3 Amino acids

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

The results in Table 9 show there was no significant difference between the control and canola MON88302 for any of the analyte means.

Table 9: Mean (±standard error) percentage dry weight (dw), relative to total dry weight, of amino acids in seed from glyphosate-treated MON88302 and 'Ebony'.

Analyte	MON88302 (%dw)	'Ebony' (%dw)	Overall treat effect (P-value)	Tolerance interval (%dw)	Combined literature range (%dw)
Alanine	1.02±0.025	1.04±0.025	0.502	0.77 – 1.34	0.71 – 1.38
Arginine	1.45±0.054	1.51±0.054	0.052	1.10 – 1.93	0.93 – 2.46
Aspartate	1.65±0.067	1.71±0.067	0.238	1.33 – 2.12	1.20 – 2.03
Cystine	0.57±0.027	0.58±0.027	0.781	0.38 – 0.83	0.32 – 0.52
Glutamate	4.06±0.18	4.24±0.017	0.103	2.73 – 5.89	3.23 – 4.71
Glycine	1.14±0.040	1.19±0.040	0.142	0.96 – 1.47	0.82 – 2.22
Histidine	00.63±0.40	0.65±0.23	0.181	0.47 – 0.86	0.41 – 0.82
Isoleucine	0.93±0.028	0.96±0.028	0.299	0.70 -1.22	0.62 – 1.02
Leucine	1.64±0.049	1.68±0.049	0.308	01.21 – 2.18	1.07 – 1.99
Lysine	1.39±0.041	1.41±0.041	0.410	1.02 – 1.90	0.96 – 1.85
Methionine	0.46±0.015	0.46±0.015	0.847	0.30 – 0.65	0.27 – 0.52
Phenylalanine	0.98±0.029	1.00±0.028	0.348	0.77 – 1.26	0.64 – 1.07
Proline	1.40±0.054	1.42±0.054	0.335	0.9 – 2.01	0.85 – 3.74
Serine	1.02±0.030	1.05±0.030	0.105	0.81 – 1.32	0.69 – 1.55
Threonine	0.98±0.030	1.00±0.030	0.192	0.82 – 1.20	0.74 – 1.30
Tryptophan	0.23±0.010	0.24±0.010	0.172	0.13 – 0.35	0.20 – 0.37
Tyrosine	0.67±0.019	0.69±0.019	0.249	0.57 – 0.81	0.51 – 0.92
Valine	1.20±0.035	1.22±0.035	0.352	0.92 – 1.55	0.8 – 1.55

6.3.4 Minerals

The levels of nine minerals in seed from MON88302 and 'Ebony' were measured. Sodium was below the LOQ. Results for the remaining analytes are given in Table 10 and show there was no significant difference between the control and MON88302 for any of the analyte means.

Table 10: Mean (±standard error) levels of minerals in the seed of glyphosate-sprayed MON88302 and 'Ebony'.

Analyte	Unit	MON88302	'Ebony'	Overall treat effect (P-value)	Tolerance interval	Combined literature range
Calcium	% dw	0.41±0.030	0.40± 0.030	0.210	0.16 – 0.61	0.361 – 0.728
Copper	ppm dw	3.78±0.17	3.65±0.17	0.361	2.0 – 4.43	1.57 – 5.39
Iron	ppm dw	48.73±4.28	54.01±4.24	0.102	23.39 – 86.23	ND – 900.59
Magnesium	% dw	0.37±0.014	0.36±0.014	0.508	0.32 – 0.43	0.277 – 0.427
Manganese	ppm dw	41.44±2.02	40.34±1.99	0.551	14.85 – 61.05	33.95 – 65.20
Phosphorus	% dw	0.72±0.042	0.72±0.041	0.692	0.38 – 1.06	0.54 – 0.89
Potassium	% dw	0.64±0.053	0.64±0.052	0.951	0.39 – 0.96	0.702 – 1.02
Zinc	ppm dw	35.58±1.78	33.01±1.76	0.198	20.19 – 48.23	ND – 122.362

6.3.5 Vitamin E

The level of Vitamin E is given in Table 11 and shows there was no significant difference between the control and canola MON88302 for any of the analyte means.

Table 11: Mean (±standard error) weight (mg/k g dry weight) of vitamin E (α-tocopherol) in seed from glyphosate-sprayed MON88302 and 'Ebony'.

Analyte	MON88302 (mg/kg dw)	'Ebony' (mg/kg dw)	Overall treat effect (P-value)	Tolerance interval (mg/kg dw)	Combined literature range (mg/kg dw)
Vitamin E (α-tocopherol)	11.06±2.08	8.85±2.08	0.218	3.88 – 17.28	71.1 – 108.4

6.3.6 Anti-nutrients

Levels of five key anti-nutrients were measured. Results in Table 12 show that the mean level of alkyl glucosinolate was significantly lower in seed of MON88302 compared with seed from 'Ebony' but the mean fell within the tolerance interval. There was no difference between MON88302 and 'Ebony' seed in terms of total glucosinolates which fell well below the level of 30 µmole/g dw that is permitted if a rapeseed species can be referred to as canola (refer to Section 2.1).

Table 12: Mean (±standard error) percentage dry weight (dw), relative to total dry weight, of anti-nutrients in seed from glyphosate-sprayed MON88302 and 'Ebony'.

Analyte	Unit	MON88302[1]	'Ebony'	Overall treat effect (P-value)	Tolerance interval	Combined literature range
Phytic acid	% dw	1.95±0.18	2.11± 0.18	0.064	0.70 – 3.52	
Sinapic acid	% dw	0.86±0.12	0.88±0.12	0.837	0.57 – 1.13	0.7 – 1.1
Total tannins	% dw	0.70±0.11	0.69±0.11	0.966	ND – 1.37	
Alkyl glucosinolate	µmole/g dw	3.68±0.43	5.08±0.42	0.035	ND – 29.02	
Indolyl glucosinolate	µmole/g dw	3.50±0.51	3.89±0.50	0.408	1.37 – 6.62	
Total glucosinolate	µmole/g dw	7.35±0.87	9.08±0.86	0.127	ND – 32.2	1 - 28

[1] mauve shading represents MON88302 means that are significantly lower than the 'Ebony' means while orange shading represents MON88302 means that are significantly higher.

6.3.7 Summary of analysis of key components

Statistically significant differences in the analyte levels found between seed of MON88302 and 'Ebony' are summarised in Table 13. These differences do not raise safety concerns for a number of reasons.

Firstly, for all analytes the MON88302 means fall within both the combined literature range and the tolerance interval (except for oleic acid – as discussed in Section 6.3.2). Secondly, it is noted that the percentage differences between the lowest and highest levels in the tolerance interval obtained from the reference non-GM lines are higher than the percentage differences between MON88302 and the control means for any analyte. Finally, there are no trends in the results.

Table 13:Summary of analyte levels found in seed of glyphosate-treated canola MON88302 that are significantly (P < 0.05) different from those found in seed of the control line 'Ebony'

Analyte	Unit of measure.	MON88302[1]	'Ebony'	% difference between means	MON88302 within tolerance interval?	MON88302 within literature range?
Total dietary fibre	% dw	20.9	18.37	13.8	yes	N/A
Palmitoleic acid (C16:1)	% total	0.22	0.24	7.6	yes	yes
Stearic acid (C18:0)	% total	1.68	1.98	15	yes	yes
Oleic acid (C18:1)	% total	62.82	65.7	4.5	yes	No – but 'Ebony' also higher than lit. range.
Linoleic acid (C18:2)	% total	19.26	17.67	9	yes	yes
Linolenic acid (C18:3)	% total	9.58	7.98	20	yes	yes
Arachidic acid (C20:0)	% total	0.54	0.60	10.7	yes	yes
Behenic acid (C22:0)	% total	0.27	0.28	6	yes	yes
Alkyl glucosinolate	µmole/g dw	3.68	5.08	27.5	yes	N/A

[1] mauve shading represents MON88302 means that are significantly lower than the 'Ebony' means while orange shading represents MON88302 means that are significantly higher.

6.4 Conclusion from compositional analysis

Detailed compositional analyses were done to establish the nutritional adequacy of seed from MON88302 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, minerals, amino acids, fatty acids, vitamin E and five anti-nutrients. The levels were compared to levels in a) the non-GM parental line, 'Ebony' b) a tolerance range compiled from results taken for seven non-GM commercial lines grown under the same conditions and c) levels recorded in the literature.

A total of 70 analytes were measured of which 18 fatty acids and sodium had 50% of their values below the assay limit of quantitation and were therefore not used in the statistical analysis. Of the remaining 52 analytes, only nine in MON88302 deviated from the 'Ebony' control in a statistically significant manner. However, all analytes except oleic acid fell within both the tolerance interval and the historical range from the literature. For oleic acid, both the MON88302 and 'Ebony' mean levels were higher than found in the literature range but within the tolerance interval.

It is concluded that seed from MON88302 is compositionally equivalent to seed from conventional canola varieties.

7. Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can

be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

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

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (OECD 2003; EFSA 2008). Canola line MON88302 is the result of a simple genetic modification to confer herbicide tolerance with no intention to significantly alter nutritional parameters in the food. In addition, the extensive compositional analyses of seed that have been undertaken to demonstrate the nutritional adequacy of MON88302 indicate it is equivalent in composition to conventional canola cultivars. The introduction of food from canola line MON88302 into the food supply is therefore expected to have little nutritional impact and as such no additional studies, including animal feeding studies, are required.

However, the Applicant supplied a broiler feeding study which has been evaluated by FSANZ.

Study submitted:

2011. Comparison of broiler performance and carcass parameters when fed diets containing canola meal produced from MON88302, control, or reference canola. Study ID # CQR-10-323, Monsanto Company (unpublished).

The analysis did not show any significant difference in growth performance and general health between broilers fed a diet containing MON88302 and those fed a diet containing the control canola ('Ebony') or four non-GM reference varieties. This was consistent with the findings from the compositional analysis.

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