Health
Canada

Supporting document 1

Safety assessment – Application A1216

Food derived from Herbicide-tolerant Canola Line MON 94100

Executive summary

Background

A genetically modified (GM) canola line with OECD Unique Identifier MON-941∅∅-2, hereafter referred to as MON 94100, has been developed to tolerate the following herbicides: dicamba (3,6-dichloro-2-methoxybenzoic acid). This line has been developed by Monsanto Company.

MON 94100 contains a demethylase gene (*dmo* gene) from *Stenotrophomonas maltophilia* resulting in the expression of dicamba mon-oxygenase (DMO), a protein to confer tolerance to the herbicide dicamba. Neither the gene introduced into MON 94100, nor the expressed protein, are new to the food supply, and both have been assessed previously by both Health Canada and FSANZ.

In conducting a safety assessment of food derived from MON 94100, a number of criteria have been addressed including: characterisation of the transferred gene including its origin, function and stability in the canola genome; the nature of the introduced protein and its potential to be either allergenic or toxic in humans; compositional analyses and any resultant changes in the whole food. This approach evaluates the intended and any unintended changes in the plant.

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

- risks related to the environmental release of GM plants used in food production
- risks to animals that may consume feed derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

Food derived from the non-GM (conventional) plant with an accepted history of safe use is used as the benchmark for the comparative analysis.

History of use

Canola is a highly domesticated crop that has a history of safe consumption. Canola is rapeseed (*Brassica napus*, *B. rapa*, or *B. juncea*) which has been conventionally bred to contain less than 2 % erucic acid and less than 30 micromoles of glucosinolates per gram of seed solids, by definition. Rapeseed is the second largest oilseed crop in the world behind soybean.

Canola seeds are processed into two major products, oil and meal. The oil is the major

product for human consumption, being used directly for cooking and as an ingredient in a variety of manufactured food products including salad and cooking oil, margarine, shortening, and a range of prepared foods such as 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. More recently the meal has been identified as a potential alternative source of protein for human consumption.

Molecular characterisation

MON 94100 was generated through Agrobacterium-mediated transformation. Molecular analyses of MON 94100 indicate that a single copy of the expression cassette containing the *dmo* gene, plus regulatory elements, is present at a single insertion site in the genome. The introduced gene is 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

MON 94100 expresses one novel protein, dicamba mono-oxygenase (DMO). DMO protein was detected in all plant tissues analysed. The highest mean DMO level was 5.0 µg/g dry weight (DW) in the root, and the lowest was 0.64 µg/g DW in grain. The grain is the tissue used for food.

The identity of the MON 94100-produced DMO protein was confirmed by Western blot analysis, sequence analysis, liquid chromatography-tandem mass spectrometry, and a specific functional enzyme assay. Indirect evidence also indicated that the DMO protein is not glycosylated in MON 94100.

Bioinformatics analysis on the DMO protein were updated and confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens. Experimental data suggests that the protein is readily digestible in simulated gastric and simulated intestinal fluid thus DMO is expected to be completely digested before absorption in the gastrointestinal tract would occur. The protein's functionality is heat sensitive at temperatures above 55°C and likely inactivated and degraded during processing of canola oil. An acute oral toxicity study in mice failed to demonstrate any signs of clinical toxicity, at the highest dose tested, 140 mg/kg body weight.

Dietary exposure to canola is limited to canola oil which contains very low amounts of protein. The DMO protein comprises only 0.00022% of total protein in the MON 94100 seed. On this basis, the dietary exposure to the DMO protein from MON 94100 is considered negligible. For this reason, a margin of exposure could not be determined.

Based on the available data, canola oil produced from MON 94100 does not pose an additional risk of toxicity or allergenicity relative to conventional canola oil.

Compositional analyses

In order to evaluate if there were any unanticipated consequences of the genetic modifications to MON 94100, the nutritional and anti-nutritional components of the MON 94100 grain were analysed and compared to a non-GM (conventional) control. This is consistent with guidance provided in the OECD document on Low Erucic Acid Rapeseed (Canola) issued in 2011 (OECD, 2011). The compositional analytes measured in MON 94100 and the control were: carbohydrates (by calculation), protein, total fat, acid detergent

fibre, neutral detergent fibre, ash, minerals (calcium and phosphorous), vitamins (vitamin E and vitamin K_1), fatty acids (21), amino acids (18), total glucosinolates, total alkyl glucosinolates, total indolyl glucosinolates, sinapine, phytic acid, and tannins. Of the analytes measured, only sinapine showed a statistically significant treatment effect in the MON 94100 compared to the control but the level remained within the ILSI Crop Composition Database range. The remaining analytes were not statistically significantly different in MON 94100 compared to control. MON 94100 canola grain has similar nutritional composition to its control.

Conclusion

No potential public health and safety concerns have been identified in the assessment of herbicide tolerant canola line MON 94100. On the basis of data provided in accordance with the Health Canada *Guidelines for the Safety Assessment of Novel Foods*, and other available information, food derived from MON 94100 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
bp	base pairs
CTP	Chloroplast transit peptide
dmo	Coding sequence of dicamba mono-oxygenase gene from *Stenotrophomonas maltophilia*
DMO	Dicamba mono-oxygenase protein
DNA	deoxyribonucleic acid
T-DNA	transfer DNA
DW	dry weight
ELISA	enzyme linked immunosorbent assay
GM	genetically modified
ILSI-CCDB	International Life Sciences Institute – Crop Composition Database
JSA	Junction Sequence Analysis
LB	Left Border of T-DNA (*Agrobacterium tumefaciens*)
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOQ	Limit of quantitation
MALDI-TOF MS	matrix-assisted laser desorption/ionisation–time of flight mass spectrometry
NDF	neutral detergent fibre
NGS	Next Generation Sequencing
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
PCR	polymerase chain reaction
RB	Right Border of T-DNA (*Agrobacterium tumefaciens*)
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

Health Canada has received an application from Bayer CropScience on behalf of Monsanto Canada ULC to conduct a pre-market safety assessment for food derived from the genetically modified (GM) herbicide-tolerant canola line MON 94100 (hereafter referred to as MON 94100), with the OECD Unique Identifier MON-941∅∅-2. This line has herbicide tolerance to dicamba (3,6-dichloro-2-methoxybenzoic acid). Dicamba is used to control annual and biennial weed species and suppress the growth of perennial broadleaf weeds and woody plant species.

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).

It is likely that MON 94100 will be combined with other approved canola lines through traditional breeding methods to create commercial products with tolerance to multiple herbicides, insect protection, or other traits offering broader grower choice, continued weed control durability, crop protection, and increased yield.

Canola line MON 94100 was primarily developed for agriculture in North America. At the time of assessment, approval for commercialisation in Australia or New Zealand had not yet been 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 MON 94100 has not yet been determined.

2 History of use

2.1 Host organism

Canola (*Brassica napus* L.) is Canada's most valuable crop and the second largest crop grown in Canada in terms of acreage planted. In 2018, canola was harvested on 22.7 M acres, with an average yield of over 0.903 tonnes/acre (https://www150.statcan.gc.ca/n1/dailyquotidien/180629/dq180629b-eng.htm). In 2018, the Canola Council of Canada estimated that canola contributed approximately $26.7 billion to the Canadian economy each year through revenue to growers, processors and exporters (https://www.canolacouncil.org/). See Section 1 of the safety assessment supplement for Australia and New Zealand specific information about history of use of canola (SD2).

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 by FSANZ: food derived from corn line MON 87419, food derived from soybean line MON 87708, and food derived from cotton line MON 88701. The organism was originally named *Pseudomonas maltophilia*, but was subsequently re-named to *Xanthomonas maltophilia*, before it was given its own genus (Palleroni and Bradbury, 1993).

S. maltophilia is ubiquitously present in the environment (Mukherjee and Roy, 2016), including in water and dairy products (An and Berg, 2018; Okuno, et al., 2018; Todaro et al., 2011). This bacterium has been used as an effective biocontrol agent in plant and animal

pathogenesis (Mukherjee and Roy, 2016), and has antibacterial activity against both gram-positive and gram-negative bacteria (Dong et al., 2015). These bacteria can form biofilms that become resistant to antibiotics (Berg and Martinez, 2015; Brooke et al., 2017). Several alternative compounds have been shown to be effective against *S. maltophilia* antibiotic resistance, such as immunoglobulin, epigallocatechine-3-gallate from green tea, and essential oils (Mukherjee and Roy, 2016). *S. maltophilia* has been found in healthy individuals without any hazard to human health (Heller et al., 2016; Lira et al., 2017). The opportunistic pathogenicity of *S. maltophilia* is mainly associated with hosts with compromised immune systems rather than with any specific virulence genes of these bacteria. Documented occurrences of *S. maltophilia* infections have been limited to immunocompromised individuals in hospital settings (Lira et al., 2017).

2.2.2 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of MON 94100 (refer to Table 1 and Table 2). These non-coding sequences are used to regulate the expression of the new genes. None of the sources of these genetic elements is associated with toxic or allergenic responses in humans.

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

3.1 Transformation Method

In order to create MON 94100, plasmid PV-BNHT508701 was transformed into the canola variety 65037, a non-transgenic conventional spring canola inbred line, using *Agrobacterium*-mediated transformation.

The plasmid PV-BNHT508701 is approximately 17.2 kb in size and contains two separate T-DNAs (i.e., T-DNA I and T-DNA II), each delineated by Left and Right Border regions (Figure 1). T-DNA I contains the *dmo* gene expression cassette while the T-DNA II contains a *splA* gene expression cassette and *aadA* selectable marker cassette. Separate Left and Right Borders were used for each T-DNA to achieve separate, unlinked insertion of the T-DNA I and T-DNA II into the recipient genome. The plasmid backbone lacks flanking Left and Right Border regions and is therefore not inserted. After initial selection of transformants using the *aadA* selective marker, conventional breeding followed by molecular analysis were used to select plants containing only the T-DNA I and not the T-DNA II.

Plants that were homozygous for T-DNA I and not containing T-DNA II were then selected for further assessment and development (Section 3.3). Many thousands of transformation events were evaluated in the laboratory and greenhouse before MON 94100 was ultimately chosen as the lead event, based on superior agronomic, phenotypic, and molecular characteristics.

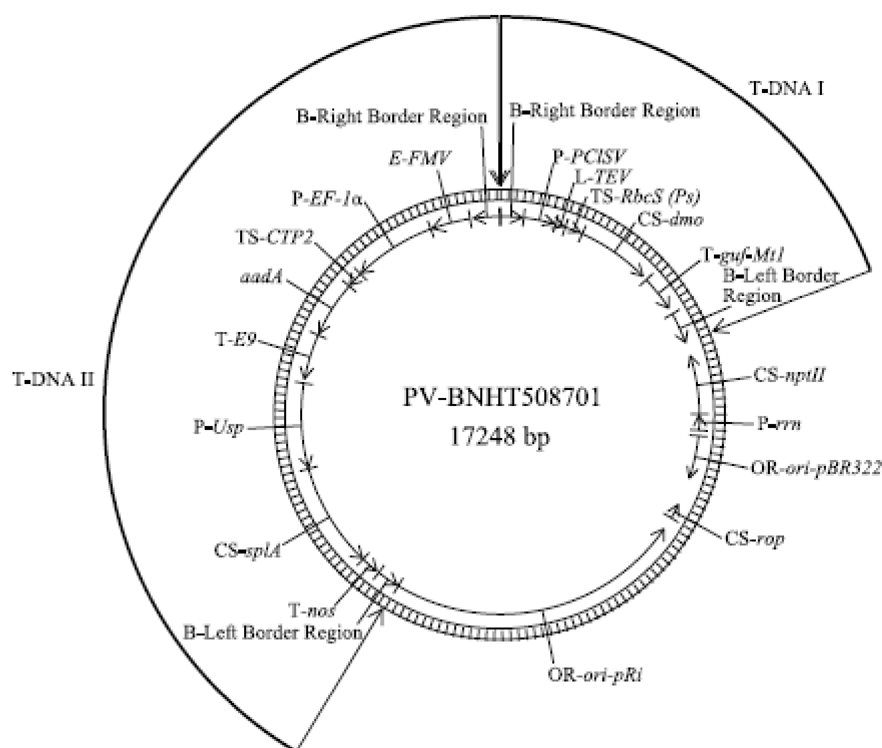


Figure 1: Plasmid map of PV-BNHT508701 showing genes in T-DNA I and T-DNA II

3.2 Detailed description of T-DNA I and T-DNA II

Information on the genetic elements in the T-DNA I and T-DNA II regions of the transforming plasmid PV-BNHT508701 is summarised in Tables 1 and 2. The T-DNA I contains a *dmo* gene expression cassette, encoding a DMO protein (dicamba tolerance). The T-DNA II contains a *splA* gene expression cassette, encoding a sucrose phosphorylase protein, and a *aadA* selectable marker cassette, encoding a bacterial aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase. Expression of this protein confers tolerance to spectinomycin and streptomycin which was used only as a selectable marker immediately after transformation. Subsequent crossing of transformants gave rise to progeny that did not contain the T-DNA II. The complete plasmid is 17,248 bp in size.

Table 1: The genetic elements contained in the T-DNA I region of PV-BNHT508701, used to create MON 94100.

Genetic element	Relative position	Source	Description, Function & Reference
T-DNA I			
B-Right Border Region	1-285	*Agrobacterium tumefaciens*	DNA region from *A. tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening Sequence	286-319		Sequence used in DNA cloning
P-*PClSV*	320-752	*Peanut chlorotic streak caulimovirus (PClSV)*	Promoter for the full-length transcript (FLt) of PClSV that directs transcription in plant cells (Maiti and Shepherd, 1998)
Intervening Sequence	753-772		Sequence used in DNA cloning
L-*TEV*	773-904	*Tobacco etch virus (TEV)*	5′ UTR leader sequence from the RNA of TEV that is involved in regulating gene expression (Niepel and Gallie, 1999)

Genetic element	Relative position	Source	Description, Function & Reference
Intervening Sequence	905-905		Sequence used in DNA cloning
TS-*RbcS (Ps)*	906-1148	*Pisum sativum* (pea)	Targeting sequence and the first 24 amino acids from *P. sativum* (pea) rbcS gene family encoding the small subunit ribulose 1,5-bisphosphate carboxylase protein that is expressed in the chloroplast (Fluhr et al., 1986)
Intervening Sequence	1149-1157		Sequence used in DNA cloning
CS-*dmo*	1158-2180	*Stenotrophomonas maltophilia*	Coding sequence for the dicamba monooxygenase (DMO) protein from *S. maltophilia* (Herman et al., 2005; Wang et al., 1997)
Intervening Sequence	2181-2249		Sequence used in DNA cloning
T-*guf-Mt1*	2250-2749	*Medicago truncatula*	3′ UTR from an expressed gene of *M. truncatula* of unknown function (Genbank Accession MH931406) that directs polyadenylation of mRNA (Hunt, 1994)
Intervening Sequence	2750-2847		Sequence used in DNA cloning
B-Left Border Region	2848-3289	*Agrobacterium tumefaciens*	DNA region from *A. tumefaciens* containing the left border sequence used for transfer of the T-DNA (Barker et al., 1983)

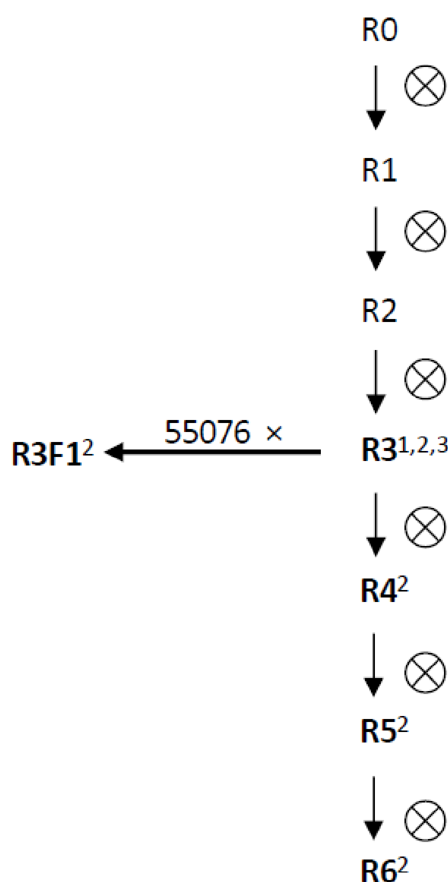
Table 2: The genetic elements contained in the T-DNA II region of PV-BNHT508701, used to create MON 94100.

Genetic element	Relative position	Source	Description, Function & Reference
T-DNA II			
B-Left Border Region	10112-10430	*Agrobacterium tumefaciens*	DNA region from *A. tumefaciens* containing the right border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	10431-10462		Sequence used in DNA cloning
T-*nos*	10463-10715	*Agrobacterium tumefaciens*	3′ UTR sequence of the *nopaline synthase (nos)* gene from *A. tumefaciens* pTi encoding NOS that directs polyadenylation (Bevan et al., 1983; Fraley et al., 1983)
Intervening Sequence	10716-10731		Sequence used in DNA cloning
CS-*splA*	10732-12189	*Agrobacterium tumefaciens*	Coding sequence of the *splA* gene from *A. tumefaciens* strain C58 encoding the sucrose phosphorylase protein that catalyzes the conversion of sucrose to fructose and glucose-1-phosphate (Piper et al., 1999)
Intervening Sequence	12190-12201		Sequence used in DNA cloning
P-*Usp*	12202-13380	*Vicia faba* (broad bean)	5′ UTR leader, promoter, and enhancer sequences of an unknown seed protein gene from *V. faba* (broad bean) encoding an unknown seed protein that is involved in regulating gene expression (Bäumlein et al., 1991)
Intervening Sequence	13381-13431		Sequence used in DNA cloning
T-*E9*	13432-14074	*Pisum sativum* (pea)	3′ UTR sequence from *P. sativum* (pea) rbcS gene family encoding the small subunit of ribulose bisphosphate carboxylase protein (Coruzzi et al., 1984) that directs polyadenylation of the mRNA
Intervening Sequence	14075-14089		Sequence used in DNA cloning
aadA	14090-14881		Bacterial coding sequence for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase, from transposon Tn7 (Fling et el., 1985) that confers spectinomycin and streptomycin tolerance.
TS-*CTP2*	14882-15109	*Arabidopsis thaliana*	Targeting sequence of the *shkG* gene from *A. thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Hermann, 1995; Klee et al., 1987)

Genetic element	Relative position	Source	Description, Function & Reference
Intervening Sequence	15110-15118		Sequence used in DNA cloning
P-*EF-1α*	15119-16266	*Arabidopsis thaliana*	Promoter, leader, and intron sequences of the *EF-1α* gene from *A. thaliana* encoding elongation factor EF-1α (Axelos et al., 1989) that directs transcription in plant cells
Intervening Sequence	16267-16289		Sequence used in DNA cloning
E-*FMV*	16290-16826	*Figwort mosaic virus (FMV)*	Enhancer from the 35S RNA of FMV (Richins et al., 1987) that enhances transcription in most plant cells (Rogers, 2000)
Intervening Sequence	16827-16876		Sequence used in DNA cloning
B-Right Border Region	16877-17233	*Agrobacterium tumefaciens*	DNA region from *A. tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)

3.3 Development of the canola line from original transformation

The breeding pedigree for the various generations is given in Figure 2. 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 generation, plants were crossed with a conventional proprietary hybrid line (55076) and the progeny were used to generate information on insert stability.



[1] Generation used for molecular characterization
[2] Generations used to confirm insert stability
[3] Generation used for commercial development of MON 94100

Figure 2: Breeding diagram for MON 94100

Table 3: MON 94100 generations used for various analyses

Analysis	MON 94100 generation used	Control(s) used
Molecular characterisation (Section 3.4)	R_3 (65037)	65037
Genetic Stability (Section 3.5.1)	R_3 (65037) R_3F_1 (65037×55076) R_4 (65037) R_5 (65037) R_6 (65037)	65037, 65037×55076
Mendelian inheritance (3.5.1)	BC_1F_1 (65037×CP3878) BC_2F_1 (65037×CP3878) BC_3F_1 (65037×CP3878) (see Fig 5)	N/A
Protein characterisation (Section 4.2)	MON 94100-produced DMO compared to MON 87708-produced DMO protein used for safety studies; plant used was 65037×55076 at R_3F_1.	MON 87708-produced DMO protein
Protein expression levels in plant parts (Section 4.3)	All tissues except grain: R_3F_1 (65037×55076) Grain: R_3F_2 (65037×55076)	N/A
Compositional analysis (Section 6)	Grain: R_3F_2 (65037×55076)	65037×55076

3.4 Characterisation of the inserted DNA and site of insertion

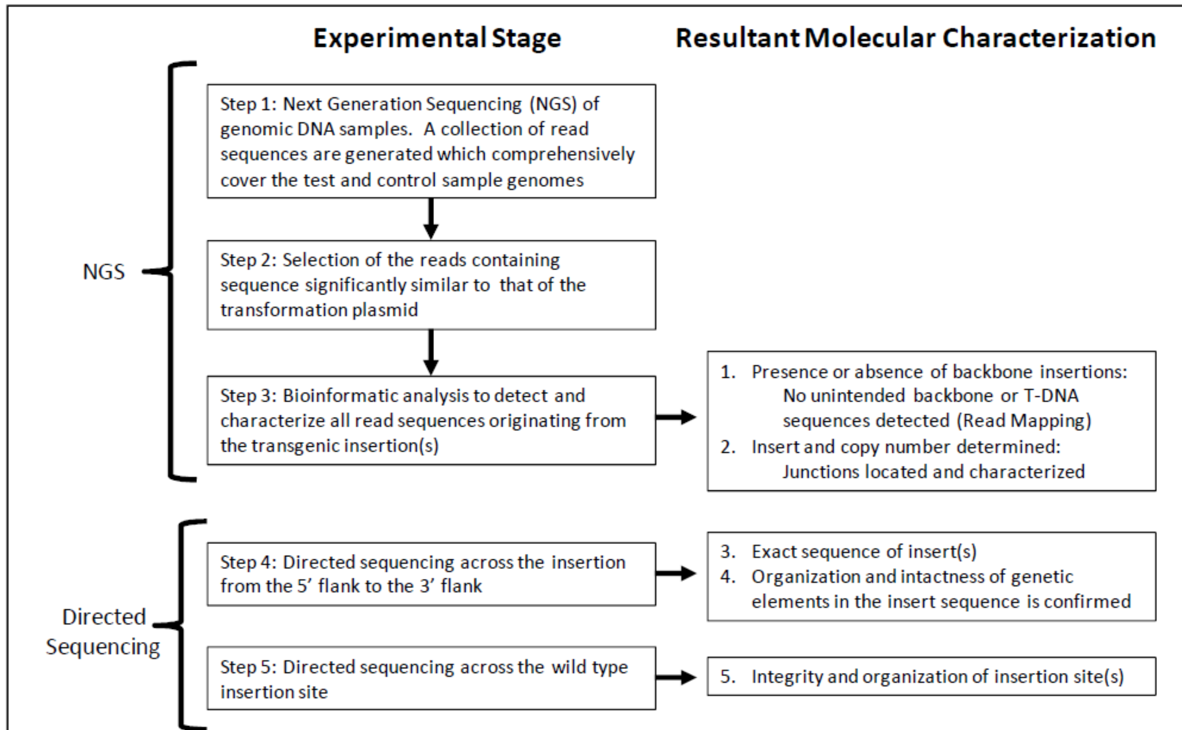
Several methods including DNA sequencing, PCR, and bioinformatics were used to fully characterise the genetic modification in MON 94100. The analyses focussed on the nature of the inserted genetic elements and whether any unintended genetic re-arrangements had occurred as a consequence of the transformation procedure. The applicant supplied the flow-diagram shown in Figure 3 to illustrate the technical approach to molecular characterisation.

The approach used to characterise MON 94100 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 the T-DNA I into the plant genome creates two junction regions (i.e., 5′ and 3′) between the inserted DNA and the 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 the T-DNA I in PV-BNHT508701 to determine insert integrity and further characterise flanking regions. NGS/JSA methodology was run on five breeding generations of MON 94100 and the conventional controls. The information was used to determine the genetic stability of the T-

DNA I insert by evaluating the number and identity of DNA inserts in each generation.



Genomic DNA from the test and the conventional control was sequenced using technology that produces a set of short, randomly distributed sequence reads that comprehensively cover test and control genomes (Step 1). Utilizing these genomic sequence reads, bioinformatics searches are conducted to identify all sequence reads that are significantly similar to the transformation plasmid (Step 2). These captured reads are then mapped and analyzed to determine the presence/absence of transformation plasmid backbone sequences, identify insert junctions, and to determine the insert and copy number (Step 3). Overlapping PCR products are also produced which span any insert and their wild type locus (Step 4 and Step 5 respectively); these overlapping PCR products are sequenced to allow for detailed characterization of the inserted DNA and insertion site.

Figure 3: Steps in the molecular characterisation of MON 94100

3.4.1 Insert number and presence of vector

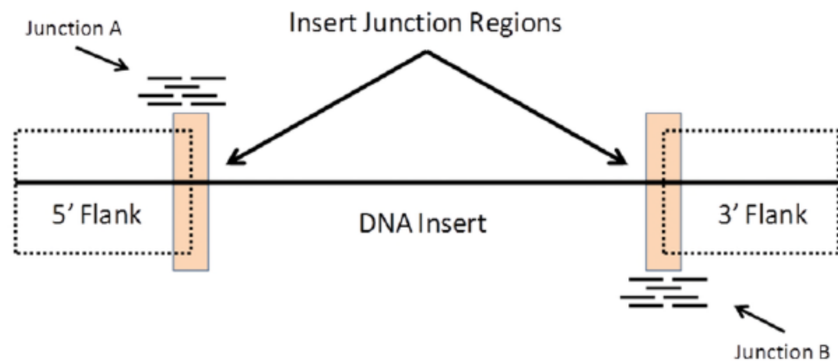
Genomic DNA from seed obtained from five breeding generations of verified MON 94100 (R_3, R_3F_1, R_4, R_5, R_6), the untransformed parent (65037), and the R_3F_1 background control (65037×55076) was isolated and analysed using Illumina®[1] NGS technology. Reference DNA from the plasmid PV-BNHT508701 was also used as a positive control. A sample of conventional control genomic DNA (65037) 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 MON 94100 was assessed by the NGS/JSA method using genomic DNA from the R_3 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 Junction A and Junction B). Figure 4 shows a schematic representation of a single DNA insertion within the genome showing the inserted DNA, the 5′ and 3′ flanking sequences, and the two distinct junction regions. Both junction sequence classes contain the T-DNA I border sequence joined to genomic flanking sequence, indicating insertion of the T-DNA I from PV-

[1] http://www.illumina.com/technology/next-generation-sequencing/sequencing-technology.ilmn

BNHT508701. No junction sequences were found in the DNA from the conventional plant 65037. Mapping the sequence reads obtained from MON 94100 and the control to PV-BNHT508701 sequence also indicated that there were no plasmid backbone sequences present in MON 94100.

Complete alignment of the junction sequence classes to the insert/flank sequence confirmed that MON 94100 contains a single T-DNA I insert at a single genomic locus.



A schematic representation of a single DNA insertion within the genome showing the inserted DNA, the 5′ and 3′ flanks (depicted as areas bounded by dotted lines), and the two distinct junction regions between the inserted DNA and flanking DNA (shaded boxes). The group of sequences of which are derived partially from the DNA insert and partially from the adjacent flanking DNA are the junction sequences. When a single T-DNA inserts into the chromosome, two junctions are created, which results in two sets of unique chimeric T-DNA/genomic reads. In this example, the two distinct junctions are classified as Junction A at the 5′ end and Junction B at the 3′ end.

Figure 4: Schematic representation of the two unique junction sequences produced by the insertion of a single plasmid region

3.4.2 Insert organization and sequence

Directed DNA sequence analysis was used to study the organisation of the elements within the T-DNA I insert and the adjacent plant genomic DNA. PCR primers were designed to amplify two overlapping regions spanning the entire length of the T-DNA I insert in MON 94100 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-BNHT508701 sequence to determine the integrity and organisation of the insert and flanking regions.

The results showed that the T-DNA I insert is 2,913 bp in length and corresponds to the PV-BNHT508701 sequence beginning at base 216 in the Right Border region and ending at base 3130 in the Left Border Region of the plasmid. 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 cassette 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 the T-DNA I has been inserted in MON 94100 and no vector backbone or sequences from the T-DNA II are present.

In addition to the T-DNA I insert, 1,000 bp flanking the 5′ end of the insert and 1,000 bp flanking the 3′ end of the insert were sequenced. This sequence information was used for further study of the T-DNA I insertion site and for open reading frame (ORF) analysis (see Sections 3.4.3 and 3.4.4).

3.4.3 Insert 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 (65037) using two primers, one specific to the 5′ flanking sequence of MON 94100 and one specific to the 3′ flanking 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 MON 94100. The comparison showed that 8 bp of canola genomic DNA were deleted during integration of the T-DNA I. Deletions such as this are relatively common with *Agrobacterium*-mediated transformations. MON 94100 plants show a normal phenotype; the changes at the insertion site have no discernible effect on the growth or productivity of the plants.

3.4.4 Open reading frame (ORF) analysis

An *in silico* analysis of the flanking regions was done to identify whether any novel ORFs had been created in MON 94100 as a result of the T-DNA I insertion. Any novel ORFs that correspond 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 databases[2].

Sequences spanning the 5′ and 3′ junctions of the MON 94100 T-DNA I insert were translated from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames. A total of 9 ORFs (six in the 5′ junction and three in the 3′ junction) were identified that encode putative polypeptides ranging from 8 – 91 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 4.5.3.

3.5 Stability of the genetic changes in MON 94100

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. 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 MON 94100 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 2 and Table 3). The four successive generations used to analyse stability were compared to the fully characterised R_3 generation. Control genomic DNA was isolated from the non-GM parental line 65037 and a conventional hybrid line (65037×55076) with similar background genetics to the R_3F_1 hybrid, as indicated in Table 3.

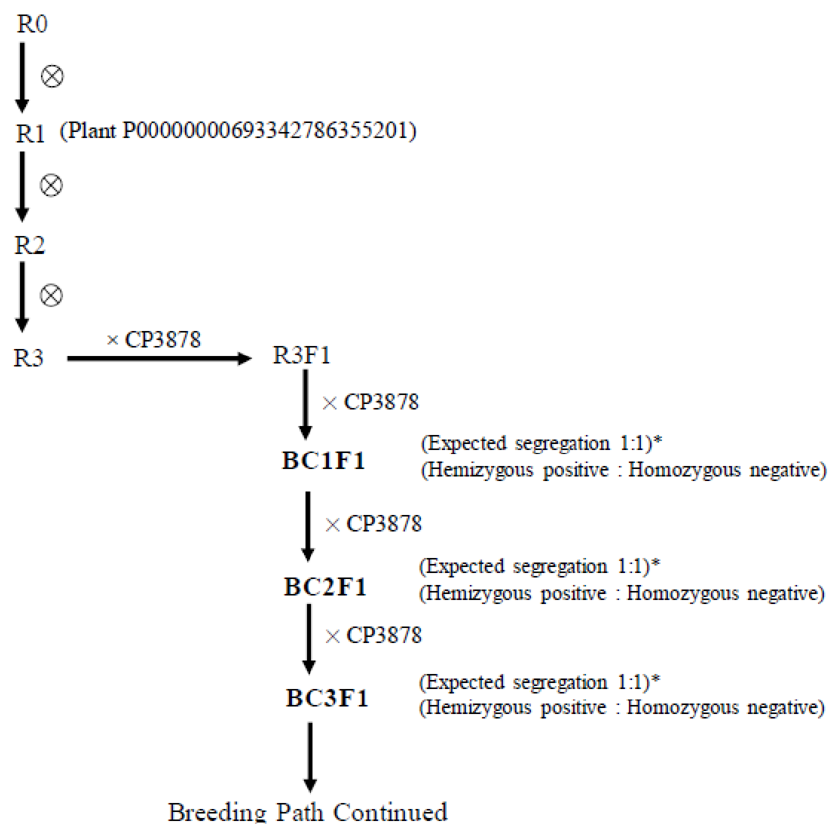
No junction sequences were detected in DNA obtained from the control lines (65037,

[2] AD_2019, TOX_2019, and PRT_2019

65037×55076). Molecular analysis of the MON 94100 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 MON 94100.

Since it was demonstrated that the insert is present at a single locus in the MON 94100 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 MON 94100. The breeding path followed for this analysis (Figure 5) was different from that represented in Figure 2. The inheritance pattern was assessed in the BC_1F_1, BCF_2F_1, and BC_3F_1 generations by PCR analysis. At the BC_1F_1, BCF_2F_1, and BC_3F_1 generations, the MON 94100 T-DNA I insert was predicted to segregate at a 1:1 ratio (hemizygous positive: homozygous negative) according to Mendelian principles of inheritance.



*Chi-square analysis was conducted on segregation data from BC_1F_1, BCF_2F_1, and BC_3F_1 generations.
⊗: Self-pollinated
×: Cross-pollinated

Figure 5: Breeding path for generating segregation data for MON 94100

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 MON 94100 and is inherited predictably according to Mendelian principles in subsequent generations.

Table 4. Segregation of the Expression Cassette During Development of MON 94100

Generation	Total Plants	Observed # Plant Positive	Observed # Plant Negative	1:1 Segregation Expected # Plant Positive	Expected # Plant Negative	χ^2	Probability
BC1F1	347	167	180	173.50	173.50	0.49	0.485
BC2F1	484	237	247	242.00	242.00	0.21	0.649
BC3F1	435	211	224	217.50	217.50	0.39	0.533

3.5.2 Phenotypic stability

Phenotypic stability of the dicamba herbicide tolerance trait was not provided to Health Canada by Bayer CropScience and thus was not directly analysed for Health Canada's assessment for MON 94100. However, it is known that the T-DNA I insert is genetically stable over multiple generations (as described in Section 3.5.1).

FSANZ Guideline 3.5.1 of the Application Handbook requires phenotypic data to demonstrate the stability of the genetic changes. Bayer CropScience will provide this data in their application to FSANZ (see Section 2 of SD2).

3.6 Antibiotic resistance marker genes

No antibiotic resistance marker genes are present in MON 94100. The T DNA I insert sequence analysis (Section 3.4.2) showed no plasmid backbone was integrated into the MON 94100 genome during transformation.

The *aadA* gene, which is part of the T-DNA II, was used as a marker to select for initial transformants but is not present in MON 94100.

3.7 Conclusion

Comprehensive molecular analyses of MON 94100 indicated a single T-DNA I, with an intact *dmo* expression cassette, was inserted into the canola 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 MON 94100. The introduced *dmo* gene was maintained over five generations and was inherited as expected of a single locus according to Mendelian principles.

4 Characterisation and safety assessment of novel 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 protein expected to be present in the plant as intended from translation of the introduced gene. A number of different analyses were done to characterise the newly expressed protein present in MON 94100.
- Possible translation products generated from ORFs as a result of the inserted T-DNA (see Section 3.4.4).

4.1 Description of the DMO protein

MON 94100 expresses a dicamba mono-oxygenase enzyme (DMO protein) to confer tolerance to dicamba herbicide. The wild-type *dmo* gene was derived from *S. maltophilia* (strain DI-6) isolated from soil at a dicamba manufacturing plant (Krueger et al., 1989). The DMO protein catalyses the demethylation of dicamba (3,6-dichloro-2-methoxy benzoic acid) to a non-herbicidal compound, DCSA and formaldehyde.

The DMO protein expressed in MON 94100 differs from the wild-type *S. maltophilia* DMO protein by an additional alanine at position 2 and an amino acid substitution (cysteine in place of tryptophan) at position 112.

Alternative processing of the DMO protein occurred in MON 94100, giving rise to two forms of the enzyme i) a fully processed DMO protein and ii) a partially cleaved DMO protein known as DMO+27. DMO+27 expresses 27 additional N-terminal amino acids that are encoded by the pea Rubisco Small Subunit and Intervening Sequence of the DNA insert. Both forms of the enzyme are referred to as DMO protein.

Alternatively-processed DMO proteins are approved by Health Canada in MON 87708 soybean, MON 87419 corn and MON 88701 cotton. A comparison of the amino acid sequence from DMO proteins present in different plant lines and the wild-type DMO protein from *S. maltophilia* is shown in Figure 6. The alignment of the amino acid sequences of the different DMO proteins shows they are highly similar. DMO proteins expressed in MON 94100 canola are identical in amino acid sequence to the DMO proteins expressed in previously approved MON 87708 soybean. Each DMO protein has the same function and catalyzes the same enzymatic reaction.

The blue regions in Figure 6 indicate sequences of 100% amino acid identity. The DMO proteins in MON 94100, MON 87429, MON 87419, MON 88701 and MON 87708 share identity to the wild type DMO except for insertion of a single amino acid at position 2 from the N-terminus, or, a single amino acid change at position 112 (tryptophan to cysteine) or additional amino acids encoded at the N-terminus.

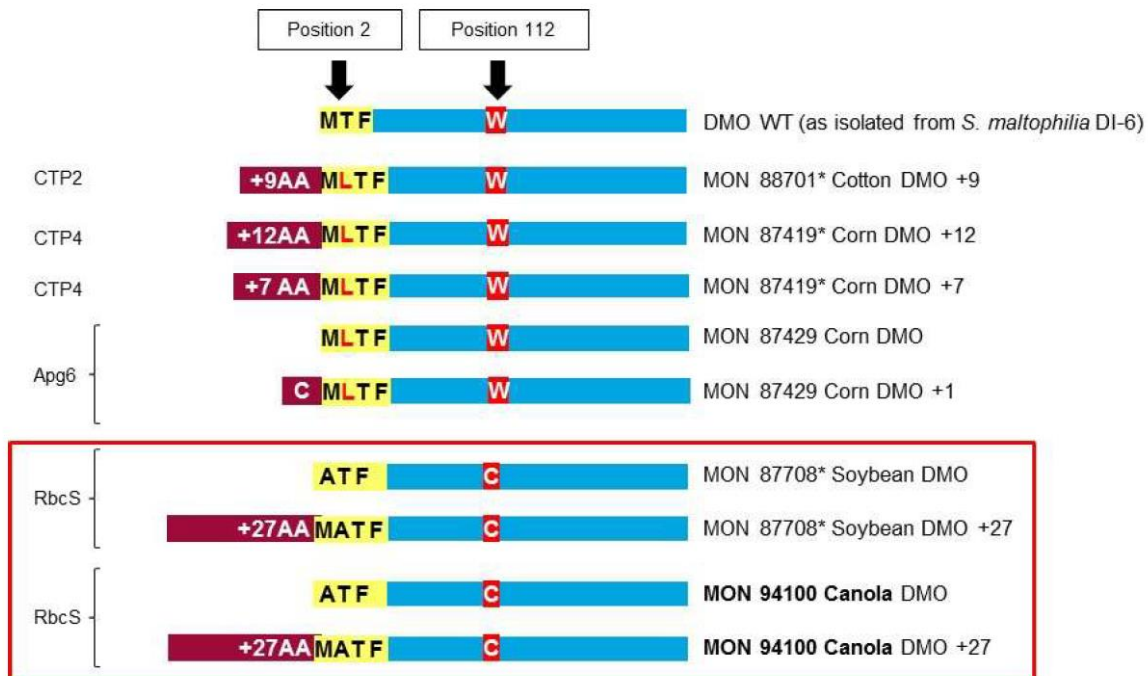


Figure 6: Protein sequence alignment of DMO proteins expressed in genetically modified commodities compared with wild-type DMO derived from S. maltophilia. Blue region indicates 100% amino acid identity. CTP2/4, Apg6 and RbcS – chloroplast targeting sequences (red boxes).

4.2 Characterisation of DMO protein expressed in MON 94100 and equivalence to the DMO protein expressed in MON 87708

The level of DMO protein in the seed of MON 94100 canola is very low, and the DMO protein in the canola seed has the same amino acid sequences as MON 87708-produced DMO protein, which has been characterised and used for safety testing. Demonstration of the physiochemical and functional equivalence of the MON 94100-produced and MON 87708-produced DMO proteins justifies the use of safety studies performed on the MON 87708-produced DMO protein to support the safety of the MON 94100-produced DMO protein. In order to confirm that the MON 94100-produced DMO protein is equivalent to the DMO protein expressed in MON 87708, a series of analytical techniques were 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 MON 94100-produced DMO protein was confirmed by a) N-terminal sequence analysis, b) liquid chromatography tandem mass spectrometry (LC-MS/MS), and c) Western blot analyses.

The DMO protein was extracted and purified from approximately 2 kg of ground defatted seed powder of MON 94100 canola through a series of extraction and filtration steps, followed by immunoaffinity chromatography using resin cross-linked with mouse monoclonal anti-DMO antibodies. The total protein concentration of the MON 94100-produced DMO was determined to be 0.47 mg/ml based on enzyme-linked immunosorbent assay (ELISA) analysis.

4.2.1 Molecular weight and immunoreactivity

The molecular weight (MW) of the DMO protein from MON 94100 and MON 87708 was determined by SDS-PAGE. The MON 94100-produced and MON 87708-produced DMO

proteins migrated to the same position on the gel and the apparent molecular weight of the DMO+27 and DMO was calculated to be 39.4 and 38.0 kDa, respectively. The average purity of the DMO protein was determined to be 92 %. Because the experimentally determined MW of the MON 94100-produced DMO protein was within the acceptance limits of equivalence (i.e., within the upper and lower limits of the 95 % prediction interval derived from data obtained from the apparent MW of the MON 87708-produced DMO protein), the MON 94100-produced and MON 87708-produced DMO proteins were determined to have equivalent apparent molecular weights.

Immunoreactivity was detected on Western blots using a polyclonal goat anti-DMO antibody followed by horseradish peroxidase-conjugated anti-goat IgG as the secondary antibody. The Western blot showed immunoreactive bands at the expected apparent MW for both the MON 94100-produced and MON 87708-produced DMO proteins (i.e., 38 kDa for DMO and 41 kDa for DMO+27). As expected, the signal intensity increased with protein load. Densitometric analysis was conducted on the bands that migrated at the expected apparent MW for the DMO proteins. The signal intensity (reported as optical density [OD]) of the bands of interest in the lanes loaded with MON 94100-produced and MON 87708-produced DMO proteins was measured. Although the expression ratio between DMO+27 and DMO bands in MON 94100-produced DMO and the MON 87708-produced DMO protein are different, the mean signal intensity of the MON 94100-produced DMO proteins was within 35 % of the mean signal intensity of the MON 87708-produced DMO proteins. Therefore, the MON 94100-produced DMO protein and MON 87708-produced DMO protein were determined to have equivalent immunoreactivity.

4.2.2 N-terminal sequence analysis

N-terminal sequencing was performed on the MON 94100-produced DMO protein. The expected N-terminal sequence for the canola DMO protein, deduced from the *dmo* gene present in MON 94100 was observed by LC-MS/MS, with the exception of the N-terminal methionine which was cleaved *in vivo* by methionine aminopeptidase and other aminopeptidases. The cleavage of the N-terminal methionine from proteins *in vivo* by methionine aminopeptidase is common in many organisms (Bradshaw et al., 1998; Wang et al., 2016). The N-terminal sequence for the canola DMO protein of MON 94100 was consistent with the N-terminal sequence for the soy DMO protein of MON 87708 observed by MALDI-TOF MS. The N-terminal sequence data confirms the identity of the DMO protein isolated from the seed of MON 94100.

The expected N-terminal sequence for the canola DMO+27 protein, deduced from the additional 27 amino acids at the N-terminus encoded by the target *dmo* gene was observed by LC-MS/MS analysis. The N-terminal sequence of the canola DMO+27 protein isolated from MON 94100 was consistent with the N-terminal sequence for the soy DMO+27 protein of MON 87708 observed by MALDI-TOF MS. The N-terminal sequence data confirms the identity of the DMO+27 protein isolated from the seed of MON 94100.

4.2.3 LC-MS/MS Mass Fingerprint Analysis

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

An aliquot of the purified MON 94100-produced DMO protein was digested by trypsin and the resulting peptide fragments analysed by LC-MS/MS. The MON 94100-produced DMO protein is a mixture of two forms of protein, DMO and DMO+27 due to incomplete processing of the target sequence.

There were 43 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 100 % (339 of 339 amino acids). A peptide map of the MON 94100-produced DMO is shown in Figure 7.

There were 50 unique peptides identified that corresponded to the expected masses deduced from the potential trypsin cleavage sites within the DMO+27 amino acid sequence. The experimentally determined coverage of the DMO+27 protein was 98 % (361 of 367 amino acids). A peptide map of the MON 94100-produced DMO+27 is shown in Figure 8.

An aliquot of the purified MON 87708-produced DMO protein was digested by trypsin and the resulting peptide fragments analysed by LC-MS/MS. The MON 87708-produced DMO protein is a mixture of two forms of protein, DMO and DMO+27 due to incomplete processing of the target sequence.

There were 16 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 62 % (211 of 339 amino acids). A peptide map of the MON 87708-produced DMO is shown in Figure 9.

There were 16 unique peptides identified that corresponded to the expected masses deduced from potential trypsin cleavage sites within the DMO+27 amino acid sequence. The experimentally determined coverage of the DMO+27 protein was 61 % (223 of 339 amino acids). A peptide map of the MON 87708-produced DMO+27 is shown in Figure 10.

```
  1 ATFVRNAWYV AALPEELSEK PLGRTILDTP LALYRQPDGV VAALLDICPH
 51 RFAPLSDGIL VNGHLQCPYH GLEFDGGGQC VHNPHGNGAR PASLNVRSFP
101 VVERDALIWI CPGDPALADP GAIPDFGCRV DPAYRTVGGY GHVDCNYKLL
151 VDNLMDLGHA QYVHRANAQT DAFDRLEREV IVGDGEIQAL MKIPGGTPSV
201 LMAKFLRGAN TPVDAWNDIR WNKVSAMLNF IAVAPEGTPK EQSIHSRGTH
251 ILTPETEASC HYFFGSSRNF GIDDPEMDGV LRSWQAQALV KEDKVVVEAI
301 ERRRAYVEAN GIRPAMLSCD EAAVRVSREI EKLEQLEAA
```

Figure 7: Tryptic Peptide Map of the MON 94100-produced DMO protein. The deduced amino acid sequence of the DMO protein is 339 amino acids as shown. Boxed regions correspond to peptides that were identified from the plant-produced protein sample using LC-MS/MS

```
1   MQVWPPIGKK KFETLSYLPP LTRDSRAMAT FVRNAWYVAA LPEELSEKPL
51  GRTILDTPLA LYRQPDGVVA ALLDICPHRF APLSDGILVN GHLQCPYHGL
101 EFDGGGQCVH NPHGNGARPA SLNVRSFPVV ERDALIWICP GDPALADPGA
151 IPDFGCRVDP AYRTVGGYGH VDCNYKLLVD NLMDLGHAQY VHRANAQTDA
201 FDRLEREVIV GDGEIQALMK IPGGTPSVLM AKFLRGANTP VDAWNDIRWN
251 KVSAMLNFIA VAPEGTPKEQ SIHSRGTHIL TPETEASCHY FFGSSRNFGI
301 DDPEMDGVLR SWQAQALVKE DKVVVEAIER RRAYVEANGI RPAMLSCDEA
351 AVRVSREIEK LEQLEAA
```

Figure 8: Tryptic Peptide Map of the MON 94100-produced DMO protein. The deduced amino acid sequence of the DMO+27 protein is 361 amino acids as shown. Boxed regions correspond to peptides that were identified from the plant-produced protein sample using LC-MS/MS

```
1   ATFVRNAWYV AALPEELSEK PLGRTILDTP LALYRQPDGV VAALLDICPH
51  RFAPLSDGIL VNGHLQCPYH GLEFDGGGQC VHNPHGNGAR PASLNVRSFP
101 VVERDALIWI CPGDPALADP GAIPDFGCRV DPAYRTVGGY GHVDCNYKLL
151 VDNLMDLGHA QYVHRANAQT DAFDRLEREV IVGDGEIQAL MKIPGGTPSV
201 LMAKFLRGAN TPVDAWNDIR WNKVSAMLNF IAVAPEGTPK EQSIHSRGTH
251 ILTPETEASC HYFFGSSRNF GIDDPEMDGV LRSWQAQALV KEDKVVVEAI
301 ERRRAYVEAN GIRPAMLSCD EAAVRVSREI EKLEQLEAA
```

Figure 9: Tryptic Peptide Map of the MON 87708-produced DMO protein. The deduced amino acid sequence of the DMO protein is 211 amino acids as shown. Boxed regions correspond to peptides that were identified from the plant-produced protein sample using LC-MS/MS

```
1   MQVWPPIGKK KFETLSYLPP LTRDSRAMAT FVRNAWYVAA LPEELSEKPL
51  GRTILDTPLA LYRQPDGVVA ALLDICPHRF APLSDGILVN GHLQCPYHGL
101 EFDGGGQCVH NPHGNGARPA SLNVRSFPVV ERDALIWICP GDPALADPGA
151 IPDFGCRVDP AYRTVGGYGH VDCNYKLLVD NLMDLGHAQY VHRANAQTDA
201 FDRLEREVIV GDGEIQALMK IPGGTPSVLM AKFLRGANTP VDAWNDIRWN
251 KVSAMLNFIA VAPEGTPKEQ SIHSRGTHIL TPETEASCHY FFGSSRNFGI
301 DDPEMDGVLR SWQAQALVKE DKVVVEAIER RRAYVEANGI RPAMLSCDEA
351 AVRVSREIEK LEQLEAA
```

Figure 10: Tryptic Peptide Map of the MON 87708-produced DMO protein. The deduced amino acid sequence of the DMO+27 protein is 223 amino acids as shown. Boxed regions correspond to peptides that were identified from the plant-produced protein sample using LC-

MS/MS

4.2.4 Functional Activity

The DMO enzyme converts dicamba to DCSA and formaldehyde. The functional activity of the MON 94100-produced and MON 87708-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 MON 94100-produced and MON 87708-produced DMO proteins were determined to be 566 and 217 nmol per minute per mg, respectively.

Based on the results of these enzyme activity assays, the MON 94100-produced DMO protein and MON 87708-produced DMO protein are functionally equivalent.

4.2.5 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 MON 94100, the plant-produced protein was analysed with a proprietary glycoprotein detection kit. Transferrin, a glycosylated protein, was used as a positive control in the assay. To assess equivalence of the MON 94100-produced and MON 87708-produced DMO proteins, the MON 87708-produced DMO protein was also analysed.

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 MON 94100-produced and MON 87708-produced DMO protein samples.

Based on the results of these analyses, the MON 9400-produced and MON 87708-produced DMO proteins have equivalent glycosylation status.

4.2.6 Conclusion

Analyses of molecular weight, immunoreactivity, and functional enzyme activity confirmed that the DMO protein produced in the grain of MON 94100 plants was as expected from the expression of the inserted *dmo* gene. The results of the N-terminal amino acid sequencing and peptide mass fingerprinting of DMO protein was further confirmation of its identity. Comparison of the MON 94100-produced and MON 87708-produced DMO proteins demonstrated that both proteins are equivalent in terms of molecular size, immunoreactivity, and specific enzyme activity. Separate analysis of the MON 94100-produced and MON 87708-produced DMO proteins showed that they are not glycosylated. Based on these results, the conclusion is that the MON 87708-produced DMO protein is a suitable substitute for the MON 94100-produced DMO protein in safety assessment studies.

4.3 Expression of the DMO protein in canola tissues

Levels of the DMO protein(s) expressed in MON 94100 canola tissues were determined in field expression studies. MON 94100 plants were grown from verified seeds[3] at three field sites in the U.S[4] and two sites in Canada[5] during the 2018 growing season. There were four replicated plots at each site. Leaf [over season leaf 1 (OSL1) and over season leaf 4 (OSL4)], forage, root (Root1 and Root2), raceme and grain samples were treated throughout

[3] The identity of the starting seed was verified by event-specific PCR prior to planting.
[4] Idaho, South Dakota, Washington
[5] Saskatchewan and Manitoba

the growing season with dicamba herbicide, and were collected from the replicated plots (Table 5). Forage and grain samples from canola not treated with dicamba were also collected. To facilitate protein extraction, tissues were ground, lyophilized and stored at -80°C until analysed. Protein was extracted using extraction buffer (phosphate buffered saline buffer with Tween-20), metal beads, shaken and then clarified by centrifugation.

The level of DMO protein was determined for each sample type using a validated enzyme linked immunosorbent assay (ELISA). Mouse anti-DMO capture antibody was immobilized onto microtiter plates to allow binding with the DMO containing sample (antigen) or reference standard (*E. coli* produced transgenic DMO protein). This was followed by the addition of a biotinylated mouse anti-DMO antibody and an avidin-horseradish peroxidase conjugate. To generate colour, 3,3',5,5'- tetramethylbenzidine (TMB) was added as a substrate. The enzymatic reaction was stopped by the addition of phosphoric acid.

DMO ELISA plates were analysed on a microplate spectrophotometer. Protein concentrations were determined by optical absorbance at a wavelength of 450 nm. Absorbance readings and protein standard concentrations were fitted with a five-parameter logistic curve fit. Following the interpolation from the DMO protein standard curve, the protein levels (ng/ml) in the tissues were converted to a µg/g dry weight value. This conversion utilized a sample dilution factor, tissue-to-buffer ratio and an extraction efficiency conversion factor.

Table 5 shows the across-site means, standard error, and ranges reported for DMO protein levels for tissues treated with dicamba. Similar values were obtained for samples not treated with dicamba. On a dry weight (DW) basis, the mean DMO protein level in MON 94100 across all sites was highest in the root (Root1) at 5.0 µg/g DW, and lowest in grain at 0.64 µg/g DW. The grain is the tissue used for food.

Table 5: Summary of DMO Protein Levels in Treated Canola Tissues Collected from MON 94100 Produced in United States and Canadian Field Trials in 2018

Tissue Type[1]	Development Stage[2]	Mean (SE) Range (µg/g fw)[3]	Mean (SE) Range (µg/g dw)[4]	LOQ/LOD (µg/g dw)[5]
OSL1	13-16 BBCH	0.29 (0.022) 0.11 - 0.42	2.5 (0.19) 0.95 - 3.6	0.094/0.008
forage	19-59 BBCH	0.20 (0.029) 0.035 - 0.55	2.5 (0.35) 0.42 - 6.7	0.094/0.006
Root1	19-59 BBCH	0.77 (0.057) 0.28 - 1.3	5.0 (0.37) 1.8 - 8.3	0.094/0.006
raceme	58-59 BBCH	0.58 (0.040) 0.23 - 0.86	4.1 (0.29) 1.6 - 6.2	0.094/0.040
OSL4	60-62 BBCH	0.41 (0.032) 0.22 - 0.70	3.0 (0.23) 1.6 - 5.1	0.094/0.008
Root2	67-80 BBCH	1.1 (0.061) 0.68 - 1.7	4.5 (0.26) 2.8 - 7.0	0.094/0.006
grain	99 BBCH	0.59 (0.063) 0.35 - 1.6	0.64 (0.068) 0.38 - 1.8	0.094/0.004

[1]OSL = over season leaf
[2]The crop development stage at which each tissue was collected.
[3]Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SE, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=20).
[4] Protein levels are expressed as the arithmetic mean and standard error (SE) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw).
[5]LOQ=limit of quantitation defined as tissue LOD=limit of detection.

4.4 Potential Toxicity of the DMO protein

The assessment of potential toxicity of a novel protein is to establish, using a weight of evidence approach, that the novel protein will behave like any other dietary protein. The assessment focuses on 1) whether the novel protein has a history of safe human consumption and if the protein has structural similarity, based on the amino acid sequence to known toxins; 2) structural properties of the novel protein, including whether it is resistant to heat or processing and/or digestion; 3) an assessment of potential acute toxic effects in animals may be considered; and 4) the anticipated dietary exposure levels.

The safety of the DMO protein has been reviewed previously by Health Canada in approved products including MON 87708 soybean, MON 88701 cotton, MON 87419 corn and MON 87429 corn[6]. Results in published literature also support the safety of DMO (Wang et al., 2016).

The results presented in Section 4.2 verify the equivalence of the expressed DMO proteins in MON 94100 and in MON 87708. As the forms of the DMO proteins are 100% identical throughout their amino acid sequence, data previously submitted for the DMO protein expressed in MON 87708 soybean[7] were referenced to support the safety of the DMO

[6] FSANZ has also reviewed the safety of the DMO protein in MON 87708 soybean (Application A1063), MON 88701 cotton (Application A1080) and MON 87429 corn (Application A1192).
[7] Dicamba Tolerant Soybean MON 88708: https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/approved-products/novel-food-information-dicamba-tolerant-soybean-87708.html

protein in MON 94100.

4.4.1 Similarities with known protein toxins

Bioinformatics analyses are useful for assessing whether introduced proteins share any amino acid sequence similarity with known allergenic or toxic proteins.

The applicant provided updated results from *in silico* analyses comparing the DMO+27 protein amino acid sequence to the sequences of known protein toxins identified in the National Center for Biotechnology Information (NCBI) protein database (search date: January 2019). At the date of the search, there were 34,642 sequences in the toxin database. A FASTA algorithm (Pearson and Lipman, 1988) was used with a BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992) and the E-value[8] threshold conservatively set to 1 x 10^{-5} (1e-5). No alignments with an E score of ≤ 1 x 10^{-5} were obtained and suggests the DMO protein did not share structurally relevant similarities with known toxins. This is consistent with conclusions reached in the previous assessment of the DMO+27 protein by the Health Canada.

4.4.2 Thermolability of the DMO protein

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

The DMO protein preparations (derived from MON 87708 soybean seed containing DMO, DMO+27 and all forms of the trimer) were incubated at 25, 37, 55, 75 or 95°C for 15 or 30 minutes. DMO protein maintained at 4°C served as a control. Following treatment, samples were analysed by SDS-PAGE and tested for enzyme activity by incubating with dicamba substrate at 30°C for 15 minutes. The quantity of dichlorosalicyclic acid (DCSA) metabolite was measured using high performance liquid chromatography (HPLC) separation followed by florescence detection using a DCSA standard curve. Rates of reaction were measured as nmol of DCSA produced per minute per mg MON 87708 DMO protein. Non-heat treated DMO protein served as a control in the experiment.

SDS-PAGE analysis indicated that loss of visually detectable protein as determined by gels stained with Brilliant Blue, required heat treatment of 95°C for ≥ 30 minutes. Results of the activity assay indicated that DMO proteins lost functional activity when heated above temperatures of 55°C for ≥ 15 minutes.

The results demonstrate that the DMO proteins are functionally and structurally unstable at elevated temperatures. The DMO protein is expected to be inactivated and degraded as canola oil is exposed to high temperatures during its processing.

4.4.3 Susceptibility of the DMO protein to digestion with pepsin and pancreatin

The *in vitro* digestibility of DMO protein preparations (derived from MON 87708 soybean seed) were evaluated at multiple time points, after incubation at 37°C in simulated gastric fluid (SGF; 104 µg DMO:1044 units of pepsin, pH 1-2) or in simulated intestinal fluid (SIF; 72 µg DMO:3.98 mg pancreatin, pH 7.5). SGF protein digests were run on SDS-PAGE and visualized with Brilliant Blue staining. Western blotting of the gels was performed for both SGF and SIF samples using a polyclonal anti-DMO primary antibody and a rabbit anti-goat

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[8] The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. Typically, alignments between two sequences will need to have an *E*-score of $1e^{-5}$ (1×10^{-5}) or smaller to be considered to have significant homology.

IgG secondary antibody conjugated to horseradish peroxidase.

Western blot analysis showed that greater than 95 % of DMO proteins were digested after incubation in SGF for 30 seconds or SIF for 5 minutes.[9]

The results demonstrate that DMO protein was highly susceptible to protein digestion in conditions similar to those found in the human gastrointestinal system.

4.4.4 Acute toxicity studies

In an acute oral toxicity study, conducted in compliance with Good Laboratory Practice (GLP), male and female CD-1 mice (5 mice per sex per group, 8 weeks of age) were administered either 140 mg DMO protein preparation/kg body weight (derived from MON 87708) or 205 mg bovine serum albumin protein/kg body weight (control) by oral gavage. No treatment related deaths, clinical signs of toxicity, changes in body weight or food consumption were noted during the 14-day observation period. Necropsies conducted on day 14 did not reveal any treatment-related pathology on gross examination. Thus, an oral LD_{50} of > 140 mg/kg bw was established.

4.4.5 Dietary Exposure to DMO protein expressed in MON 94100

The applicant states that the primary human food produced from canola is refined, bleached and deodorized (RBD) oil.

No data was provided on the level of DMO protein in RBD oil. According to the applicant, there is minimal, if any dietary exposure to DMO protein from consumption of foods derived from MON 94100. The petitioner states that because RBD oil contains extremely low amounts of protein (<0.2 ppm, Martin Hernandez 2008), oil produced from MON 94100 will contain negligible levels of DMO protein.

The applicant's opinion is supported by data from field expression studies. Total protein content in the MON 94100 seed was estimated to be about 28.13 % DW of the seed (equal to 281 300 µg/g DW). The mean DMO protein in the seed was estimated to be 0.64 µg/g DW. The DMO protein represents 0.00022 % of total seed protein.[10] Considering the level of DMO protein determined to be in the seed, any protein still present in oil after processing produced from MON 94100 would contain extremely low levels of DMO protein (0.4 ppt),[11] and is considered negligible.

4.5 Potential allergenicity of DMO protein

The allergenic potential of an introduced protein can be assessed by comparing the biochemical characteristics of the introduced protein to biochemical characteristics of known allergens (Codex, 2009). Using a weight of evidence approach, a protein is not likely to be associated with food allergenicity if: 1) the protein is from a non-allergenic source; 2) the protein does not share structural similarities to known allergens based on the amino acid sequence; and 3) the protein does not show resistance to pepsin digestion.

The DMO protein in MON 94100 is encoded by the *dmo* gene from *S. maltophilia*. The applicant states that *S. maltophila* is not reported to be a source of known allergens. *S. maltophilia* is a donor organism for several dicamba-tolerant events with no allergenicity issues previously identified by Health Canada. A search of the NCBI PubMed database[12]

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[9] Limit of detection of assay was 0.3ng.

[10] (0.64 µg /g DMO protein ÷ 281, 300 µg /g total protein) x 100 = 0.00022%

[11] Assuming 0.2 mg total protein in 1 kg refined oil x 0.00022% (DMO protein estimated to be of total protein) = 0.0000004 mg DMO protein/kg oil = 0.4ppt DMO protein in oil

(July 2020) for “S. maltophila” AND “allergen” did not reveal any reports of the organism as an allergenic source organism.

4.5.1 Similarity with known allergens

The DMO+27 amino acid sequence was compared against 2,081 sequences of known allergens in the allergen, gliadin and glutenin sequences (AD_2019) retrieved from the Comprehensive Protein Allergen Resource (COMPARE, 2019) database from the Health and Environmental Sciences Institute (HESI) using the FASTA search algorithm (E score ≤1×10^{-5}) to assess overall structural similarity. As well, a sliding window search for sequences of 80 amino acids with a linear identity of greater than 35% was conducted to compare DMO amino acid sequences to those of known allergens. In addition, a search for eight contiguous amino acids was performed to assess for the presence of potential immunologically significant epitopes. No matches were identified for the DMO+27 protein. As such, the DMO proteins are not considered to have significant amino acid sequence identity with known allergens. This is consistent with conclusions reached in the previous assessment of the DMO+27 protein by the Health Canada.

4.5.2 In vitro digestibility

Several food allergens are observed to be resistant to gastrointestinal digestion. As described above, the results of an *in vitro* digestibility study showed that DMO proteins isolated from MON 87708 soybean seeds and considered equivalent to DMO protein in MON 94100 are digested in simulated gastric fluid (pepsin, pH 1-2) within 30 seconds and in simulated intestinal fluid (pancreatin, pH 7.5) within 5 minutes. As the majority of DMO proteins are expected to be digested in the stomach, it is unlikely that functionally active DMO proteins derived from dietary intake will pose a food allergy concern.

4.5.3 Bioinformatics analysis of ORFs created by the transformation

As described in Section 3.4.4, the putative peptides/polypeptides encoded by the 9 identified ORFs were analysed using a bioinformatics strategy to determine similarity to known protein toxins, allergens, or other proteins with known biological activity.

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

The FASTA algorithm was used to compare the overall structural similarity between the query sequences to the allergen (AD_2019), toxin (TOX_2019), and protein (PRT_2019) databases.

An *E*-score of 1e-5 was set as an initial high cut-off value for alignment significance to identify potential allergens, toxins, and proteins of concern. Any aligned sequence that yielded an *E*-score of ≤1e-5 was analysed further to determine if it represented relevant sequence homology.

Regarding potential allergenicity, none of the putative peptides/polypeptides encoded by the 9 identified ORFs yielded an alignment with an *E*-score of ≤1e-5 when using the AD_2019 database to run a FASTA search. No alignments met or exceeded the significance threshold of ≥35 % over 80 amino acids, and no eight amino acid matches were identified.

Regarding potential toxicity, none of the putative peptides/polypeptides encoded by the 9 identified ORFs yielded an alignment with an *E*-score of ≤1e-5 when using the TOX_2019 database to run a FASTA search.

Lastly, regarding potential adverse biological activity, none of the putative peptides/polypeptides

encoded by the 9 identified ORFs yielded an alignment with an *E*-score of ≤1e-5 when using the PRT_2019 database to run a FASTA search.

These results indicate that in the unlikely occurrence that any of these putative peptides/polypeptides are expressed in MON 94100, none would share significant similarity or identity with known allergens, toxins, or other biologically active proteins that could affect human health.

4.6 Conclusion

The genetic modification in MON 94100 results in the expression of one novel enzyme, DMO, in a range of plant tissues. The mean level of the proteins in herbicide-sprayed plants grown in the field were highest in the root at 5.0 µg/g (not consumed by humans), and lowest in the grain at 0.64 µg/g on a dry weight basis.

The equivalence of the MON 94100 DMO protein and the DMO protein previously assessed in MON 87708 was demonstrated through a series of analytical techniques.

The DMO protein was reviewed previously by Health Canada and the evidence suggested that the protein is not toxic or allergenic. This is supported by an updated bioinformatics analyses of the DMO protein confirming the absence of significant similarity to known toxins, allergens, gliadins and glutenins. Experimental data suggests that the protein is readily digestible in simulated gastric fluid and simulated intestinal fluid; the protein's functionality is heat sensitive at temperatures ≥ 55°C, suggesting that the protein is likely inactivated and degraded during processing; and an acute 14-day oral toxicity study in mice failed to demonstrate any signs of clinical toxicity, at the highest dose tested, 140 mg/kg body weight.

Dietary exposure to canola is limited to canola oil which contains very low amounts of protein (<0.2 ppm, Martin Hernandez 2008). The DMO protein comprises only 0.00022 % of total protein in the MON 94100 seed. On this basis, the dietary exposure to the DMO protein from MON 94100 is considered negligible. For this reason, a margin of exposure could not be determined.

Taken together, the evidence supports the conclusion that the DMO protein present in MON 94100 is innocuous and raises no food safety concerns.

5 Novel herbicide metabolites in GM herbicide-tolerant plants

The review of novel herbicide metabolites falls under the purview of Health Canada's Pest Management Regulatory Agency (PMRA). As such, this aspect which is standard for the FSANZ assessment, was not addressed by the Food Directorate.

FSANZ Guideline 3.5.1 of the Application Handbook requires that data must be provided on the identity and levels of herbicide and any novel metabolites that may be present in the food produced using gene technology. Bayer CropScience will provide this data in their application to FSANZ (see Section 3 of SD2).

6 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).

6.1 Key Components

The Organisation for Economic Co-operation and Development (OECD) document on Low Erucic Acid Rapeseed (Canola) issued in 2011 provides the compositional considerations for new varieties of canola (OECD, 2011). The key food nutrients, anti-nutrients, and toxicants suggests by OECD to be analysed when reviewing new canola varieties include crude protein, crude fat, ash, amino acids, fatty acids, vitamin K1, vitamin E, glucosinolates, tannins, sinapine, and phytic acid.

6.2 Study design

A compositional study was undertaken to compare the composition of MON 94100 canola treated with dicamba to the conventional control. The applicant has stated that the *B. napus* canola variety was used as the non-genetically modified conventional control (herein referred to as the control). MON 94100 and this control have similar genetic backgrounds with the exception of the *dmo* gene expression cassette.

MON 94100 and the control were planted at each of five field sites in the United States and Canada during the 2018 season. The field sites included: Bonneville County, Idaho; Jerome County, Idaho; Portage la Prairie Rural Municipality, Manitoba; Brookings County, South Dakota; and Grant County, Washington. The applicant has stated that all the field sites were grown under typical agronomic field conditions for the regions.

At each of the five field sites that produced grain for the compositional analysis, plots of MON 94100 and control were arranged in a randomised complete block design with four replications each. Randomisation of field plots was done independently at each site. Four seeder passes were planted in each plot, and plots were spaced approximately 3 meters apart.

MON 94100 was treated with dicamba, and grain of both MON 94100 and control were harvested at physiological maturity. As per the statistical report, seeds were collected from four replicated plots per substance grown. A representative one-kilogram grain sample was collected from each plot, resulting in 20 samples per entry (five locations with four replications each). Prior to shipping, grain was dried down to less than 10 % moisture and cleaned of excess debris, if necessary. These were then shipped at ambient temperature from the field site to Monsanto Company and a subsample for compositional analysis was obtained from each grain sample collected. The applicant has indicated that these subsamples were ground and stored in a freezer set to maintain a temperature of -20 degrees Celsius until their shipment on dry ice to the laboratory for analysis.

The methods of compositional analysis include validated methods from the Association Of Official Analytical Collaboration AOAC International, the American Oil Chemists' Society (AOCS), or other published scientific methods

6.3 Analyses of key components in grain

In total, 56 analytes were measured in canola including those recommended in the OECD Consensus Document on Compositional Considerations for New Varieties of Low Erucic Acid Rapeseed (Canola): Key Food and Feed Nutrients, Anti-Nutrients and Toxicants (OECD, 2011). The analytes measured included carbohydrates (by calculation), moisture (not provided), protein, total fat, ash, acid detergent fibre (ADF), neutral detergent fibre (NDF), 21 fatty acids, 18 amino acids, calcium, phosphorus, vitamin E (alpha-tocopherol), vitamin K1, total glucosinolates, total alkyl glucosinolates, total idolyl glucosinolates, phytic acid, tannins, and sinapine.

Statistical analysis was based on compositional data combined across all field sites and included reporting and comparison of minimum, maximum, and range values as well as the least square mean and standard errors for MON 94100 and control substances for each component.

To estimate the least square mean and standard errors, the linear mixed model was built separately for each component. The Mixed model for the analysis of a randomised complete block design at multiple locations is a standard model for this experimental design (Littell et al., 2006). The model was adjusted by the random effects for the Site, Replicate nested with the Site, and the interaction between the Site and the Substance. Model assumptions were verified by using a residual analysis. Studentized residuals were used to detect potential outliers in the dataset. The normality and common variance assumptions were checked by visual examination of residual plots and histograms. No extreme violations were observed for any characteristic.

Comparisons in least-square means (SE) between MON 94100 and the conventional control were defined with the model and tested using t-tests. P-values were used to detect the statistically significant differences at the 5 % level ($\alpha = 0.05$). All statistically analysis were conducted using Statistical Analysis Software (SAS®)[13] software.

6.3.1 Protein and amino acids

The results for the protein and amino acids in canola grain are shown in Table 6. The protein and all of the amino acids were not significantly different in MON 94100 grain compared to the control on a dry weight (DW) basis. There are no nutritional safety concerns with the protein or amino acid profile of MON 94100 grain.

[13] SAS website - http://www.sas.com/technologies/analytics/statistics/stat/index.html

Table 6: Mean (±SE) levels of protein and amino acids in grain from MON 94100 (treated) and the control, expressed as percentage dry weight (% DW)

Analyte (% DW)	MON 94100 (treated) Mean (SE) Range	Control Mean (SE) Range	p-Value
Protein	28.13 (1.15) 22.67-32.56	27.71 (1.15) 23.30-32.09	0.066
Alanine	1.24 (0.048) 1.07-1.44	1.22 (0.048) 1.03-1.39	0.388
Arginine	1.90 (0.10) 1.54-2.43	1.88 (0.10) 1.44-2.59	0.626
Aspartic acid	2.06 (0.11) 1.66-2.41	2.05 (0.11) 1.55-2.35	0.657
Cystine	0.69 (0.022) 0.57-0.79	0.68 (0.022) 0.60-0.81	0.194
Glutamic acid	4.97 (0.23) 4.06-5.95	4.85 (0.23) 4.00-5.75	0.064
Glycine	1.39 (0.057) 1.20-1.61	1.37 (0.057) 1.13-1.57	0.447
Histidine	0.68 (0.023) 0.58-0.78	0.68 (0.023) 0.59-0.79	0.904
Isoleucine	1.16 (0.054) 0.93-1.36	1.15 (0.054) 0.94-1.33	0.508
Leucine	2.01 (0.094) 1.61-2.36	1.99 (0.094) 1.62-2.31	0.520
Lysine	1.45 (0.051) 1.29-1.72	1.46 (0.051) 1.26-1.75	0.940
Methionine	0.52 (0.021) 0.41-0.62	0.51 (0.021) 0.46-0.59	0.548
Phenylalanine	1.16 (0.057) 0.92-1.37	1.15 (0.057) 0.90-1.33	0.473
Proline	1.73 (0.068) 1.40-2.06	1.69 (0.068) 1.49-2.02	0.090
Serine	1.19 (0.049) 1.00-1.38	1.17 (0.049) 0.97-1.34	0.217
Threonine	1.17 (0.042) 1.03-1.34	1.16 (0.042) 0.98-1.30	0.558
Tryptophan	0.39 (0.918) 0.30-0.46	0.38 (0.018) 0.30-0.45	0.148
Tyrosine	0.85 (0.034) 0.73-0.97	0.84 (0.034) 0.70-0.95	0.675
Valine	1.41 (0.058) 1.18-1.65	1.40 (0.058) 1.15-1.58	0.542

6.3.2 Total fat and fatty acids

The results for the total fat and fatty acids in canola grain are shown in Table 7. The total fat and 11 fatty acids were not significantly different in MON 94100 compared to the control on a dry weight (DW) basis or as a percentage of total fatty acids (% Total FA) basis, respectively. The following fatty acids had more than 50 % of the observations below the limit of quantification (LOQ) and were excluded from the statistical analysis: caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, heptadecanoic acid, heptadecenoic acid, eicosanienoic acid, erucic acid, and docosadienoic acid. These are consistent with the fatty acids listed in OECD Consensus Document (2011) as being “not detected” or found at very low levels. There are no nutritional concerns with the total fat or fatty acid profile in MON 94100 grain.

Table 7. Mean (±SE) levels of total fat (% DW) and fatty acids in grain from MON 94100 (treated) and the conventional control, expressed as a percentage of total fatty acids (% Total FA).

Analyte	MON 94100 Mean (SE) Range	Control Mean (SE) Range	p-Value
Total fat (% DW)	43.22 (1.41) 37.28-48.43	43.40 (1.41) 38.73-47.87	0.791
Palmitic acid (% Total FA)	4.29 (0.070) 3.98-4.68	4.28 (0.070) 3.98-4.55	0.941
Palmitoleic acid (% Total FA)	0.24 (0.0066) 0.22-0.27	0.25 (0.0066) 0.22-0.29	0.167
Stearic Acid (% Total FA)	1.75 (0.060) 1.55-1.96	1.74 (0.060) 1.50-2.00	0.793
Oleic acid (% Total FA)	65.29 (0.39) 63.18-67.57	65.11 (0.39) 63.31-66.74	0.608
Linoleic acid (% Total FA)	18.87 (0.36) 17.06-20.43	18.95 (0.36) 17.65-20.52	0.754
Linolenic acid (% Total FA)	7.23 (0.24) 6.35-8.22	7.32 (0.24) 6.43-8.19	0.500
Arachidic acid (% Total FA)	0.55 (0.017) 0.50-0.63	0.56 (0.017) 0.48-0.61	0.700
Eicosenoic acid (% Total FA)	1.16 (0.016) 1.07-1.23	1.18 (0.016) 1.12-1.24	0.096
Behenic acid (% Total FA)	0.27 (0.0089) 0.23-0.30	0.27 (0.0089) 0.24-0.31	0.295
Lignoceric acid (% Total FA)	0.17 (0.011) 0.13-0.22	0.18 (0.011) 0.14-0.23	0.542
Nervonic acid (% Total FA)	0.18 (0.025) 0.090-0.31	0.18 (0.025) 0.098-0.28	0.893

6.3.3 Carbohydrates and fibre

The results for the carbohydrates and fibre in canola grain are shown in Table 8. The carbohydrates, ADF, and NDF were not significantly different in MON 94100 compared to the control on a dry weight (DW) basis. There are no nutritional safety concerns with these analytes.

Table 8. Mean (±SE) levels of carbohydrates in grain from MON 94100 (treated) and the conventional control, expressed as percentage dry weight (% DW)

Analyte (% DW)	MON 94100 Mean (SE) Range	Control Mean (SE) Range	p-Value
Carbohydrates by calculation[1]	24.79 (0.46) 23.13-26.42	24.82 (0.46) 23.26-26.59	0.845
Acid detergent fibre	13.92 (0.79) 11.37-18.28	13.93 (0.79) 10.94-18.31	0.977
Neutral detergent fibre	18.86 (0.93) 15.31-23.84	18.96 (0.93) 15.24-23.50	0.796

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

6.3.4 Ash and minerals

The results for the ash and minerals in canola grain are shown in Table 9. The ash, calcium, and phosphorus were not significantly different in MON 94100 compared to the control on a dry weight (DW) basis. There are no nutritional safety concerns with these analytes.

Table 9. Mean (±SE) levels of ash and minerals in grain from MON 94100 (treated) and the conventional control, expressed as percentage dry weight (% DW)

Analyte (% DW)	MON 94100 Mean (SE) Range	Control Mean (SE) Range	p-Value
Ash	3.86 (0.26) 2.99-4.56	3.95 (0.26) 3.18-5.00	0.335
Calcium	0.36 (0.036) 0.28-0.47	0.39 (0.036) 0.30-0.63	0.204
Phosphorous	0.80 (0.066) 0.56-1.00	0.80 (0.066) 0.59-1.01	0.981

6.3.5 Vitamins

The results for the vitamins in canola grain are shown in Table 10. The vitamin E and vitamin K_1 were not significantly different in MON 94100 compared to the control on a dry weight (DW) basis. There are no nutritional safety concerns with these analytes.

Table 10. Mean (±SE) amount of vitamins in grain from MON 94100 and the control, expressed as milligram per kilogram dry weight (mg/kg DW)

Analyte	MON 94100 Mean (S.E.)[2] Range	Control Mean (S.E.) Range	p-Value
Vitamin E (mg/g DW)[1]	0.10 (0.0076) 0.067-0.14	0.096 (0.0076) 0.066-0.13	0.105
Vitamin K1 (µg/g DW)	0.70 (0.081) 0.47-1.00	0.77 (0.081) 0.54-1.12	0.097

6.3.6 Anti-nutrients

The results for the anti-nutrients in canola grain are shown in Table 11. The total glucosinolates, total alkyl glucosinolates, total indolyl glucosinolates, phytic acid, and tannins were not significantly different in MON 94100 compared to the control on a dry weight (DW) basis. There are no nutritional safety concerns with these analytes.

Sinapine was statistically significantly higher in MON 94100 than in the control by 2.7 % DW. Sinapine is an anti-nutrient that can impart a fishy odour and flavour in the tissues and eggs of some animals fed canola meal (OECD, 2011). The magnitude of difference was small and the level is within the range reported in the International Life Institute Crop Composition Database (ILSI). Given the small magnitude of difference between MON 94100 and the control and similar values to those in the ILSI database, there are no nutritional safety concerns with sinapine levels in MON 94100 grain.

Table 11. Mean (±SE) levels of key anti-nutrients in grain from MON 94100 (treated) and the conventional control, expressed as µmol per gram dry weight (µmol/g DW) or as percentage dry weight (% DW)

Analyte	MON 94100 Mean (SE) Range	Control Mean (SE) Range	p-Value
Total glucosinolates (µmol/g DW)	11.49 (0.67) 8.49-14.68	10.98 (0.67) 8.02-12.96	0.104
Total alkyl glucosinolates (µmol/g DW)	5.14 (0.53) 2.62-8.61	4.85 (0.53) 2.65-6.99	0.140
Total indolyl glucosinolates (µmol/g DW)	5.93 (0.23) 4.72-6.85	5.75 (0.23) 4.73-7.10	0.254
Phytic acid (% DW)	1.80 (0.16) 1.09-2.59	1.78 (0.16) 0.86-2.34	0.813

Analyte	MON 94100 Mean (SE) Range	Control Mean (SE) Range	p-Value
Sinapine (% DW)	0.75 (0.017) 0.67-0.82	0.73 (0.017) 0.63-0.81	**0.043**
Tannins (% DW)	0.054 (0.0029) 0.019-0.065	0.055 (0.0029) 0.043-0.071	0.716

6.3.7 Secondary metabolites

No secondary metabolites were analysed as part of the compositional analysis.

6.4 Conclusion

To evaluate if there were any unanticipated consequences of the genetic modifications to MON 94100, the nutritional and anti-nutritional components of MON 94100 grain were analysed and compared to a non-genetically modified (non-GM) conventional control. This was done in part of a field trial conducted in 2018 at four different sites in the United States (Bonneville County, Idaho; Jerome County, Idaho; Brookings County, South Dakota; and Grant County, Washington) and one site in Canada (Portage la Prairie Rural Municipality, Manitoba). The field trial was a randomised complete block design with four blocks at each site and each block containing MON 94100 and the control.

The compositional analytes measured in MON 94100 and the non-GM control canola grain were: carbohydrates (by calculation), protein, total fat, acid detergent fibre, neutral detergent fibre, ash, minerals (calcium and phosphorus), vitamins (vitamin E and vitamin K_1), fatty acids (21), amino acids (18), total glucosinolates, total alkyl glucosinolates, total indolyl glucosinolates, sinapine, phytic acid, and tannins.

Of the analytes measured, only sinapine showed a statistically significant treatment effect in MON 94100 grain compared to the control (higher in MON 94100 by 2.7 % DW). The difference between MON 94100 and the control is acceptable as the analyte level remained with the ILSI Crop Composition Database range for this analyte. The remaining analytes were not statistically significantly different in MON 94100 compared to the control.
Grain from MON 94100 can therefore be regarded as equivalent in composition to grain from conventional canola.

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 wellbeing. In most cases, this can be achieved through a detailed understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food, such as that presented in Section 6 of this report.

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 or other animal species will add little to the safety assessment (Bartholomaeus et al., 2013; OECD, 2003). If the compositional analysis indicates biologically significant changes, either intended or unintended, to the levels of certain nutrients in the GM food, additional nutritional studies should be undertaken to assess the potential impact of the changes on the whole diet.

MON 94100 is the result of genetic modifications to confer tolerance to dicamba herbicide, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated MON 94100 is compositionally equivalent to conventional varieties.

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