MONSANTO

# Food and Feed Safety and Nutritional Assessment of Glyphosate-Tolerant Canola MON 88302 (OECD Unique Identifier MON-883Ø2-9)

**Conclusion Based on Data and Information Evaluated according to FDA's Policy on Foods from New Plant Varieties**

March 23, 2011

**FDA BNF 127**

**Monsanto 11-CA-220F**

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## CERTIFICATION

Monsanto Company is submitting this food and feed safety and nutritional assessment in compliance with the FDA's 1992 policy statement regarding foods derived from new plant varieties (57 FR 22984). At the agency's request, and where appropriate, this submission also complies with the recommendations contained in the proposed rule for Premarket Biotechnology Notice (PBN) Concerning Bioengineered Foods (66 FR 4706). Additionally this submission complies with the Codex Plant Guidelines (CAC/GL 45-2009) insofar as it is within the FDA's jurisdiction.

Specifically, as recommended in the proposed 21 CFR §192.25(a), the undersigned attests to the following:

1. It is the view of Monsanto Company (hereafter referred to as Monsanto) that: (a) canola MON 88302 is as safe and nutritious as other commercial canola; and (b) the intended uses of the food and feed derived from MON 88302 are in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.
2. Monsanto will make available to the FDA, upon request, relevant data or other information not included in this submission, either during the course of the FDA's evaluation of the submission, or for cause.
3. Upon request, Monsanto will make relevant data or other information not included in this submission available to the FDA either: (a) by allowing the FDA to review and copy these data or information at Monsanto's offices in St. Louis, MO, during customary business hours; or (b) by sending a copy of these data or information to the FDA.
4. Monsanto makes no claim of confidentiality regarding either the existence of this submission, or any of the data or other information contained herein. However, Monsanto reserves the right to make a claim of confidentiality regarding any relevant data or other information not included in this submission, but requested by the FDA, either in the course of its review of this submission, or for cause. Any such claim of confidentiality will be made at the time such data or information is provided, along with an explanation for the basis of the claim.
5. To the best of Monsanto's knowledge, this submission is representative and balanced, including information, unfavorable as well as favorable, that is pertinent to the evaluation of the safety, nutritional, or other regulatory issues that may be associated with MON 88302.

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Date: 3/23/11

Regulatory Affairs Manager
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## RELEASE OF INFORMATION

Monsanto is submitting the information in this assessment for review by the FDA as part of the regulatory process. By submitting this information, Monsanto does not authorize its release to any third party except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., § 552; the FDA complies with the provisions of FOIA and the FDA's implementation regulations (21 CFR Part 20); and this information is responsive to the specific request. Except in accordance with the Freedom of Information Act, Monsanto does not authorize the release, publication or other distribution of this information (including website posting) without Monsanto's prior notice and consent.

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## ABBREVIATIONS AND DEFINITIONS[1]

| | |
|---|---|
| ~ | Approximately |
| a.e. | Acid Equivalent |
| AA | Amino Acid |
| ADF | Acid Detergent Fiber |
| APHIS | Animal and Plant Health Inspection Service |
| Ave | Average |
| BBCH Scale | Bayer, BASF, Ciba-Geigy and Hoechst Cereal Grain Growth Scale |
| BLOCKS | A database of amino acid motifs found in protein families |
| BLOSUM | Blocks Substitution Matrix, used to score similarities between pairs of distantly related protein or nucleotide sequences |
| BSA | Bovine Serum Albumin |
| bw | Body Weight |
| C8-C24 | 8-24 Carbon-Chain Fatty Acids |
| CFIA | Canadian Food Inspection Agency |
| CFR | Code of Federal Regulations |
| CI | Confidence Interval |
| *cp4 epsps* | Codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS |
| CP4 EPSPS | 5-Enolpyruvylshikimate-3-phosphate synthase protein from the *Agrobacterium* sp. strain CP4 |
| CPI | Canola Protein Isolate |
| CSFII | Continuing Surveys of Food Intakes by Individuals |
| DDI | Daily Dietary Intake |
| DEEM-FCID | Dietary Exposure Evaluation Model-Food Commodity Intake Database |
| DTT | Dithiothreitol |
| dw | Dry Weight |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic Acid |
| ELISA | Enzyme-linked Immunosorbent Assay |
| EPA | Environmental Protection Agency |
| EPSP | 5-enolpyruvylshikimate-3-phosphate |
| EPSPS | 5-enolpyruvylshikimate-3-phosphate synthase enzyme |
| FA | Fatty Acid |
| FAO/WHO | Food and Agriculture Organization of the United Nations/World Health Organization |
| fw | Fresh Weight |
| *g* | g-force |
| GLP | Good Laboratory Practice |
| GRAS | Generally Recognized As Safe |

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[1] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

| | |
|---|---|
| HEPES | N-[2-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] |
| ILSI | International Life Sciences Institute |
| kDa | Kilodalton |
| LB | Laemmli buffer |
| LOD | Limit of Detection |
| LOQ | Limit of Quantitation |
| MMT | Million metric tons |
| MALDI-TOF-MS | Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry |
| MOE | Margin of Exposure |
| MW | Molecular Weight |
| MWCO | Molecular Weight Cutoff |
| NDF | Neutral Detergent Fiber |
| NFDM | Non-Fat Dry Milk |
| NIST | National Institute of Standards and Technology |
| NOAEL | No Observable Adverse Effect Level |
| OD | Optical Density |
| OECD | Organisation for Economic Co-operation and Development |
| ORF | Open Reading Frame |
| OSL | Over Season Leaf |
| PBS | Phosphate Buffered Saline |
| PBST | Phosphate Buffered Saline containing 0.05% (v/v) Tween-20 |
| PCR | Polymerase Chain Reaction |
| PEP | Phosphoenolpyruvate |
| $P_i$ | Inorganic phosphate |
| ppm | Parts Per Million |
| PRESS | Predicted Residual Sum Of Squares |
| PVDF | Polyvinylidene Difluoride |
| RBD | Refined, Bleached, and Deodorized |
| S3P | Shikimate-3-phosphate |
| Sarkosyl | *N*-lauroylsarcosine, sodium salt |
| SD | Standard Deviation |
| SDS-PAGE | Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis |
| SE | Standard Error |
| SGF | Simulated Gastric Fluid |
| sp. | Species |
| TDF | Total Dietary Fiber |
| T-DNA | Transfer DNA |
| Tm | Melting temperature |

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## NAME AND ADDRESS OF SUBMITTOR

The submitter of this safety and nutritional assessment summary for glyphosate-tolerant canola MON 88302 is:

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Communications with regard to this submission should be directed to Regulatory Affairs Manager, at the Monsanto address listed above, or by telephone at or by FAX at

## STATUS OF SUBMISSION TO USDA-APHIS

Monsanto will request a Determination of Nonregulated Status for MON 88302, including all progenies derived from crosses between MON 88302 and other canola, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) in the second half of 2011. Under regulations administered by USDA-APHIS (7 CFR 340), MON 88302 is currently considered a "regulated article." Monsanto will continue to conduct all field tests for MON 88302 in strict compliance with USDA field trial regulations until a Determination of Nonregulated Status is granted for MON 88302. Once MON 88302 is deregulated, authorization for import, interstate movement or environmental release of MON 88302 will no longer be required.

## STATUS OF SUBMISSION TO U.S. EPA

Monsanto submitted amended labelling to the U.S. Environmental Protection Agency (U.S. EPA) in February 2011 for EPA Registration Numbers 524-537 (Roundup WeatherMAX® Herbicide) and 524-549 (Roundup PowerMAX® Herbicide), that propose to modify the current use pattern of glyphosate in canola based on MON 88302. The post emergence (in-crop) use of glyphosate in Roundup Ready canola was first approved by the U.S. EPA in March 1999. Although the amended labelling increases the rate of application and widens the application period relative to canola development, this use of glyphosate does not present any new environmental exposure scenarios not previously evaluated for use on other Roundup Ready crops which have already been deemed acceptable by U.S. EPA.

## STATUS OF SUBMISSIONS TO OTHER GOVERNMENT AGENCIES

Regulatory submissions will be made to countries that import significant canola or food and feed products derived from canola and have functional regulatory review processes in place. This results in submissions to a number of additional governmental regulatory agencies including, but not limited to the Ministry of Agriculture, People's Republic of China; Japan's Ministry of Agriculture, Forestry, and Fisheries and the Ministry of Health, Labour, and Welfare; the Canadian Food Inspection Agency and Health Canada; the Intersectoral Commission for Biosafety of Genetically Modified Organisms, Mexico; the European Food Safety Authority, Food Standards Australia New Zealand, the Korea

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Food and Drug Administration, and the Rural Development Administration of Korea, as well as to regulatory authorities in other canola importing countries with functioning regulatory systems. As appropriate, notifications will be made to countries that import significant quantities of canola and canola products and do not have a formal regulatory review process for biotechnology-derived crops.

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## EXECUTIVE SUMMARY

### Food and Feed Safety Assessment of MON 88302

### MON 88302 Product Description

Monsanto Company has developed a second-generation glyphosate-tolerant canola product, MON 88302, designed to provide growers with improved weed control through greater flexibility for glyphosate herbicide application. MON 88302 produces the same 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein that is produced in commercial Roundup Ready® crop products, via the incorporation of a *cp4 epsps* coding sequence. The CP4 EPSPS protein confers tolerance to the herbicide glyphosate, the active ingredient in the family of Roundup agricultural herbicides.

MON 88302 utilizes an improved promoter sequence to enhance CP4 EPSPS expression in male reproductive tissues, compared to the FMV 35S promoter used to drive CP4 EPSPS production in the first-generation product, Roundup Ready canola (RT73). Tissues, such as pollen, that accumulate glyphosate and have a low level of CP4 EPSPS expression are considered to be at risk for glyphosate injury. By virtue of enhanced CP4 EPSPS expression in male reproductive tissues, MON 88302 provides tolerance to glyphosate during the sensitive reproductive stages of growth, and enables the application of glyphosate at later stages of development than is possible with the current product.

Weed competition can be a major limiting factor in canola production leading to significant yield reductions. Use of MON 88302 will enable growers to apply Roundup herbicide up to first flower, a later stage than Roundup Ready canola. This later stage application in canola will provide growers with greater flexibility enabling: (1) an increased opportunity to control weeds if glyphosate application is delayed due to weather or equipment failure; (2) an enhanced ability to tailor labelled glyphosate applications to weed development stage instead of the canola developmental stage; and (3) enhanced protection of canola plants at more advanced development stages at the time of glyphosate application. Use of MON 88302 will provide growers with the opportunity to ensure weeds that may impact yields are removed at the optimal time while minimizing the potential for crop injury.

### Molecular Characterization of MON 88302 Verifies the Integrity and Stability of the Inserted DNA

MON 88302 was developed through *Agrobacterium*-mediated transformation of hypocotyls from canola variety Ebony utilizing plasmid vector PV-BNHT2672. PV-BNHT2672 contains one T-DNA that is delineated by Left and Right Border regions. The T-DNA contains the *cp4 epsps* coding sequence under the control of the *FMV/Tsf1* chimeric promoter, the *Tsf1* leader and intron sequences, and the *E9* 3′ untranslated region. The chloroplast transit peptide CTP2 directs transport of the CP4 EPSPS protein to the chloroplast and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene. After transformation and subsequent rounds of self-pollination, homozygous

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$R_2$ plants containing only a single T-DNA insertion were identified resulting in production of glyphosate-tolerant canola MON 88302.

Molecular characterization by Southern blot analyses determined that MON 88302 contains one copy of the T-DNA at a single integration locus and all genetic elements are present. These data also demonstrated that MON 88302 does not contain detectable backbone sequences from the plasmid vector. The complete DNA sequence of the insert and adjacent genomic DNA sequences in MON 88302 confirmed the integrity of the inserted *cp4 epsps* expression cassette within the inserted sequences and identified the 5′ and 3′ insert-to-genomic DNA junctions. Southern blot analysis demonstrated that the insert in MON 88302 has been maintained over multiple generations of breeding, thereby confirming the stability of the insert. Further, results from segregation analyses show inheritance and stability of the insert were as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA in MON 88302 at a single chromosomal locus.

**Data Confirm the Safety of Expression Product in MON 88302**

The safety of CP4 EPSPS protein present in biotechnology-derived crops has been extensively assessed. Numerous Roundup Ready crops including Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready corn 2, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton and Roundup Ready alfalfa that produce the CP4 EPSPS protein have been reviewed by the FDA. The CP4 EPSPS protein expressed in MON 88302 is identical to the CP4 EPSPS in other Roundup Ready crops.

A multistep approach was conducted according to guidelines established by the Codex Alimentarius Commission and OECD which embody the principles and guidance of the FDA's 1992 policy on foods from new plant varieties, and was used to characterize the CP4 EPSPS protein present in MON 88302 as a result of the genetic modification. These steps include: 1) documentation of the history of safe use of the CP4 EPSPS protein and its homology with proteins that lack adverse effects on human or animal health; 2) characterization of the physicochemical and functional properties of CP4 EPSPS; 3) quantification of CP4 EPSPS expression in plant tissues; 4) examination of the similarity of CP4 EPSPS to known allergens, 5) evaluation of the digestibility of CP4 EPSPS in simulated gastrointestinal fluids; 6) evaluation of the stability of the CP4 EPSPS protein in response to typical food/feed preparation conditions such as heat treatment; 7) examination of the similarity of CP4 EPSPS to known toxins or other biologically active proteins known to have adverse effects on mammals; 8) investigation of potential mammalian toxicity through an animal assay and calculating margins of exposure; and 9) examination of the similarity of putative polypeptides encoded by the insert and flanking sequences to known allergens and toxins, or other biologically active proteins known to have adverse effects on mammals. Additionally, a stepwise approach to assess the potential allergenicity for the newly expressed protein (Codex Alimentarius, 2009) is included. The safety assessment supports the conclusion that dietary exposure to CP4 EPSPS protein derived from MON 88302 poses no meaningful risk to human or animal health.

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**Food and Feed Safety Assessments of MON 88302 Demonstrate Equivalence to the Conventional Crop**

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by the FDA. The CP4 EPSPS protein expressed in MON 88302 is identical to the CP4 EPSPS protein in other Roundup Ready crops and the mode of action of the CP4 EPSPS protein is well understood. Previous Roundup Ready crops reviewed by the FDA have had no biologically relevant compositional changes identified, and there is no reason to expect expression of the CP4 EPSPS protein in MON 88302 would affect nutritionally important nutrients, toxicants, and anti-nutrients present in seed from this new product.

Safety assessments of biotechnology-derived crops typically include comparisons of the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop to that of conventional counterparts. Compositional assessments were performed using the principles and analytes outlined in crop-specific OECD consensus documents, in this case for canola composition.

Compositional analysis comparing MON 88302 to the conventional control variety (Ebony) and commercial conventional reference varieties demonstrated that MON 88302 is compositionally equivalent to conventional canola. The background genetics of the conventional control were similar to that of MON 88302, but did not contain the *cp4 epsps* expression cassette. The commercial reference varieties were used to define the natural variability of key nutrients, toxicants, and anti-nutrients in canola varieties that have a history of safe consumption. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and total fat), fibers (acid detergent fiber [ADF], neutral detergent fiber [NDF], and total dietary fiber [TDF]), amino acids (18 components), fatty acids (FA; C8-C24,), vitamin E (α-tocopherol), and minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc) in seed. The toxicants assessed in seed included erucic acid and glucosinolates (alkyl glucosinolates [including 3-butenyl, 4-pentenyl, 2-hydroxy-3-butenyl, and 2-hydroxy-4-pentenyl glucosinolates], indolyl glucosinolates [including 3-indolylmethyl and 4-hydroxy-3-indoylmethyl], and total glucosinolates). The anti-nutrients assessed in seed included phytic acid and sinapine (as sinapic acid).

Combined-site analyses were conducted to determine statistically significant differences ($\alpha = 0.05$) between MON 88302 and the conventional control seed samples. Statistical results from the combined-site data were evaluated using considerations relevant to the safety and nutritional quality of MON 88302 when compared to the conventional control. Considerations used to assess the relevance of each combined-site statistically significant difference included: 1) the relative magnitude of the difference in the mean values of nutrient, toxicant, and anti-nutrient components between MON 88302 and the conventional control; 2) whether the MON 88302 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of the commercial reference varieties grown concurrently in the same trial; 3) evaluation of the reproducibility of the statistically significant ($\alpha = 0.05$) combined-site component differences at individual sites, and 4) an assessment of the differences within

the context of natural variability of commercial canola composition published in the scientific literature. If statistically significant differences detected in the individual site analyses were not observed in the combined-site analysis, they were not considered further for the compositional assessment of safety.

The levels of assessed components in MON 88302 were compositionally equivalent to the conventional control and within the range of variability of commercial reference varieties grown concurrently in the same field trial. The genetic modification in MON 88302 does not meaningfully impact seed composition and therefore the food and feed safety and nutritional quality of this product is comparable to conventional canola with a history of safe consumption.

Traditional canola processing is described in Section II of this document. The processing of MON 88302 is not expected to be any different from that of conventional canola. As summarized above, detailed compositional analyses of key components of MON 88302 have been performed and have demonstrated that MON 88302 is compositionally equivalent to conventional canola. Additionally, the mode of action of the CP4 EPSPS protein, as described in Section VI.A., is well understood, and there is no reason to expect interactions of this protein with important nutrients or endogenous toxicants that may be present in canola. Therefore, when MON 88302 is used on a commercial scale as a source of food or feed, these products are not expected to be different from the equivalent foods or feeds originating from conventional canola.

**Conclusion**

All data and information strongly support the conclusion that food and feed derived from MON 88302 and its progeny will be as safe and nutritious as food and feed derived from conventional canola. Therefore, the consumption of MON 88302 and its progeny, and the food and feed derived from it will be fully consistent with the FDA's Policy (FDA, 1992) and in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.

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# I. DESCRIPTION OF MON 88302

This section provides a description of MON 88302 being presented for food and feed safety and nutritional assessment. The description identifies the crop, the transformation event to be reviewed and the type and purpose of the modification, which will aid in understanding the nature of the food and feed products which may be developed from MON 88302. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraph 22 (Codex Alimentarius, 2009).

## I.A. MON 88302 Summary

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants" MON 88302 has been assigned the unique identifier MON-883Ø2-9.

Monsanto Company has developed a second-generation glyphosate-tolerant canola product, MON 88302, designed to provide growers with improved weed control through greater flexibility for glyphosate herbicide application. Weed competition can be a major limiting factor in canola production leading to significant yield reductions (CCC, 2006). Certain perennial weeds, such as Canada thistle, are known to be particularly important to control in canola production. For example, studies have demonstrated that only 10 Canada thistle plants per square meter have resulted in 10% yield loss while 40 plants per square meter have resulted in over 50% yield loss (CCC, 2006). Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaf weeds including Canada thistle (NDSU, 2005; Padgette et al., 1996). Glyphosate has been shown to have a favorable safety profile by the U.S. EPA (1993) which has concluded that use of glyphosate will not pose unreasonable risks to human or the environment.

The canola variety Ebony, a conventional canola variety developed by Monsanto Company, was used as the recipient for the DNA insertion to create MON 88302. MON 88302 was produced by incorporation of the *cp4 epsps* coding sequence from the common soil bacterium *Agrobacterium* sp. strain CP4. The *cp4 epsps* coding sequence directs the production of the 5-enolpyruvylshikimate-3-phosphate synthase (termed CP4 EPSPS) that is less sensitive to inhibition by glyphosate compared to the endogenous plant EPSPS. Hence, the CP4 EPSPS renders MON 88302 tolerant to glyphosate, the active ingredient in the Roundup family of agricultural herbicides. The transformation cassette in MON 88302 employs sequences from the promoter of the *TsfI* gene from *Arabidopsis thaliana* (Axelos et al., 1989) and enhancer sequences from the *35S* promoter from the figwort mosaic virus (Richins et al., 1987) to enhance CP4 EPSPS production in male reproductive tissues. Tissues, such as pollen, that accumulate glyphosate and have a low level of CP4 EPSPS expression are considered to be at risk for glyphosate injury (Feng et al., 2010). By virtue of enhanced CP4 EPSPS expression in male reproductive tissues, MON 88302 provides tolerance to glyphosate during the sensitive reproductive stages of growth (Feng et al., 2010). Use of MON 88302 enables the in-crop application of a Roundup agricultural herbicide at later canola developmental stages compared to the first-generation product, Roundup Ready canola. This later stage application in canola will provide growers with greater flexibility enabling: (1) an increased opportunity to control weeds if glyphosate application is delayed due to

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weather or equipment failure; (2) an enhanced ability to tailor labelled glyphosate applications to weed development stage instead of the canola developmental stage; and (3) enhanced protection of canola plants at more advanced development stages at the time of glyphosate application. Use of MON 88302 will provide growers with the opportunity to ensure weeds that may impact yields are removed at the optimal time while minimizing the potential for crop injury.

The data and information presented in this safety summary demonstrate that the food and feed derived from MON 88302 are as safe and nutritious as those derived from conventional canola varieties for which there is an established history of safe consumption. This safety assessment was conducted utilizing established methods for the evaluation of biotechnology-derived products as articulated in guidelines from the Codex Alimentarius Commission and the Organization for Economic Co-operation and Development (OECD) (Codex Alimentarius, 2009; OECD, 2002). These established methodologies embody the principles and guidance of the U.S. Food and Drug Administration's (FDA) 1992 policy on foods from new plant varieties (FDA, 1992). Therefore, the consumption of MON 88302 and the food and feed derived from it will be in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.

## I.B. Applications for Which MON 88302 is Not Suitable

Monsanto Company is aware of no food or feed uses of conventional canola that are not applicable to MON 88302.

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## II. DESCRIPTION OF THE HOST PLANT AND ITS USES AS FOOD OR FEED

This section includes data and information that provides a comprehensive description of the host plant. It also provides relevant phenotypic information on the host plant and on related species that may have contributed to the genetic background of the host plant. The history of use information provided describes how the plant is typically cultivated, transported and stored, any special processing required to assure the plant is safe to eat, and the plant's usual role in the diet. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraphs 23, 24, and 25 (Codex Alimentarius, 2009).

### II.A. Biology of *Brassica napus*

*Brassica napus* L. is a versatile crop that provides both food and feed to the global economy and whose biology is well understood and documented. There are numerous terms used to describe oil-producing *B. napus* varieties including oilseed rape, rapeseed, rape, low erucic acid rapeseed and canola. For purposes of this consultation document, *B napus* will be referred to as oilseed rape and the term canola will be used to denote *B. napus* varieties that produce low ($<2\%$) erucic acid oil and have levels of glucosinolates below the accepted standard of 30 µmoles/g in meal (OECD, 2001).

The Organisation for Economic Co-operation and Development Consensus Document on the Biology of *Brassica napus* (OECD, 1997) provides key information on:

- general description of *B. napus* biology, including taxonomy and morphology and use of *B. napus* as a crop plant

- agronomic practices in *B. napus* cultivation

- geographic centers of origin

- reproductive biology

- inter-species/genus introgression into relatives and interactions with other organisms

- summary of the ecology of *B. napus*

Additional information on the biology of *B. napus* can be found on the Canadian Food Inspection Agency website (CFIA, 2005) and the Australian Government Department of Health and Ageing (Office of the Gene Technology Regulator) website (OGTR, 2008). Information on the taxonomy of *B. napus* can be found in the U.S. Department of Agriculture Natural Resources Conservation Service PLANTS database (USDA-NRCS, 2010).

### II.A.1. History of Canola Development

Canola oil can be derived from any one of three species: *Brassica napus, Brassica rapa,* and *Brassica juncea* (OGTR, 2008; FDA, 1988; FDA, 2000). Most canola oil is from

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*Brassica napus* or oilseed rape, an oil-yielding plant. Oilseed rape is a member of the mustard (Brassicaceae) family, and has been cultivated by ancient civilizations in Asia and the Mediterranean primarily for its use as oil in lamps (Colton and Sykes, 1992). Later *B. napus* oil was used as an industrial lubricant, and today there is still demand for high erucic oil in a variety of industrial applications.

Until relatively recently, the presence of the naturally occurring toxicants, erucic acid in the oil fraction and glucosinolates in the meal has made rapeseed oil and meal derived from *B. napus* unattractive for human consumption and as an animal feed, respectively, particularly in western countries. High erucic acid rapeseed oil (as much as 50% of total fatty acids) has been shown to have cardiopathic potential resulting in a weakening of the heart muscle in experimental animals (Bozcali et al., 2009; Chien et al., 1983) while high levels of glucosinolates made oilseed rape meal unsuitable for use in animal nutrition because of anti-nutritional, goitrogenic (suppresses thyroid function), reproductive, and palatability problems (Fenwick et al., 1989). However, in the 1960s intensive breeding programs resulted in the development and introduction of low erucic acid or canola (Canadian oil, low acid) varieties of oilseed rape (OECD, 2001; OGTR, 2008). At approximately the same time low erucic acid varieties of *B. rapa* were introduced (OECD, 2001). Slightly later, in the 1980s, low erucic acid varieties of *B. juncea* were developed (CCC, 1999). However, nearly all canola varieties grown in the U.S. are *B. napus* varieties (Boyles et al., 2009).

Further breeding efforts lowered glucosinolates in canola varieties to acceptable levels and canola is now grown both for its high quality vegetable oil and its high quality animal feed.

*Brassica napus*, an amphidiploid (chromosome n=19, AA and CC genomes), is thought to be derived from a cross between two diploid *Brassica* species, *B. rapa* (chromosome n=10, AA genome) and *B. oleracea* (chromosome n=9, CC genome). *Brassica. napus* has the greatest sexual compatibility with *B. rapa* and *B. juncea* under natural field conditions, but has also been known to outcross with some wild relatives including *Raphanus raphanistrum* (wild radish) and *Hirschfeldia incana* (shortpod mustard) (OECD, 1997; OGTR, 2008).

There are spring and winter biotypes of canola varieties. Spring canola, a cool season crop, is grown in Canada, southern Australia, northern China, and in northern portions of the U.S. Great Plains. Spring canola is slow growing and does not compete well with weeds in its early growth stages. Closely related weeds like wild mustard, stinkweed and shepherd's purse are often problematic in commercial spring canola fields, and weeds must be controlled early in the spring canola life cycle to avoid yield loss due to competition (OECD, 1997). Winter canola is planted in the fall, requires vernalization (exposure to winter cold) to flower, and is grown in parts of Europe, Asia, northwestern U.S. and in the central portions of the U.S. Great Plains. Winter canola, once established, suppresses and out-competes most annual weeds (Boyles et al., 2009). In addition to various pre-emergent weed control options, varieties of canola having tolerance to glyphosate, glufosinate and imazamox herbicides for weed control in canola fields are widely available.

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## II.B. Characteristics of the Recipient Plant

The *B. napus* canola variety used as the recipient for the DNA insertion to create MON 88302 was Ebony, a non-transgenic conventional spring canola variety registered with the Canadian Food Inspection Agency in 1994 by Monsanto Company (CFIA, 2010). Ebony originated from a cross of varieties (Bienvenu × Alto) × Cesar. Selection criteria for the non-transgenic variety included yield, oil and protein content, and tolerance to the fungus *Leptosphaeria maculans*, commonly known as blackleg (CFIA, 1994). Ebony was used to produce the glyphosate-tolerant canola MON 88302 because it responds well to *Agrobacterium*-mediated transformation and tissue regeneration.

Ebony was used as the conventional canola comparator (referred to in this consultation document as the conventional control) in the safety assessment of MON 88302. MON 88302 and the conventional control have similar genetic backgrounds with the exception of the *cp4 epsps* expression cassette. In addition, commercial conventional canola varieties (referred to in this consultation document as commercial reference varieties) were used to establish ranges of natural variability or responses representative of commercial canola varieties. The commercial reference varieties used at each location were selected based on their availability and agronomic fit for the geographic region.

### II.B.1. Known Toxicity or Allergenicity of Recipient Plant

According to OECD (2001), oilseed rape contains two potential toxicants, erucic acid and glucosinolates, and the anti-nutrient components, phytic acid and sinapine. Because erucic acid has been historically associated with cardiopathic potential in animal species, the Codex Standard for Named Vegetable Oils (Codex Alimentarius, 2005) and the FDA (1988) specify that erucic acid in canola oil for human consumption cannot exceed more than 2% of total fatty acids. Erucic acid is a monounsaturated omega-9 fatty acid that is used in a number of applications including film emulsions, skin and healthcare emollients, surfactants and lubricants (USDA-ERS, 1996). High erucic acid oilseed rape varieties in which as much as 50% of the oil produced is erucic acid have been developed for these applications. Low erucic acid oilseed rape or canola varieties have also been developed that produce oil low in erucic acid content and are suitable for human consumption.

Glucosinolates are organic compounds that contain both sulfur and nitrogen reducing the palatability of canola meal. Some glucosinolates have the potential to form toxicants. While reducing erucic acid levels in the oil, breeders were also able to reduce levels of glucosinolates in the meal thus producing the "double low" canola varieties commonly grown today. The standard for glucosinolates in canola meal is 30 μmoles/g (OECD, 2001). Glucosinolates are found abundantly in plants of the *Brassica* genus and some are responsible for the pungent or biting flavors found in closely related *Brassica* species such as mustard and horseradish. In canola seed, glucosinolates can be categorized into two main chemical groups, alkyl and indolyl, with alkyl being the most common (CCC, 2009). While some glucosinolates contribute to human health such as those found in broccoli, most of the glucosinolate in canola can be hydrolyzed by the enzyme



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myrosinase, to form allyl isothiocyanate, a goitrogenic compound that depresses growth and thyroid function (Bell, 1984).

The anti-nutrient phytic acid is present in rapeseed and other commonly consumed foods and feeds, where it chelates mineral nutrients, including calcium, magnesium, potassium, iron, and zinc, rendering them biologically unavailable to mono-gastric animals consuming the seed (Liener, 2000). The second anti-nutrient, sinapine, is the choline ester of sinapic acid, and is the primary phenolic component in rapeseed. Sinapine imparts a bitter taste and reduces palatability of the seed (OECD, 2001) for some animal species. Maximum levels of anti-nutrients are not described (OECD, 2001) but are comparatively evaluated to levels observed in canola varieties that have a history of safe consumption.

There are no reports of allergic reactions to canola oil. There have been a limited number of reports (Alvarez et al., 2001; Suh et al., 1998) citing oilseed rape flour as an allergen. These studies reported that the four individuals with hypersensitivity to oilseed rape flour worked with animal feed preparation where oilseed rape flour is a component, suggesting the prevalence is low and confined to occupationally exposed populations. Studies have also been performed to determine the prevalence of allergic response to oilseed rape pollen. In a naturally exposed European population, i.e., villagers with surrounding fields planted with oilseed rape, only 0.2% of individuals were clinically proven to be allergic to oilseed rape pollen (Fell et al., 1992). The incidence of oilseed rape hypersensitivity in the occupationally exposed population, i.e., scientists or farm workers that handle the plant and the pollen on a daily basis, was 31%, but most of these individuals were hypersensitive to multiple allergens; only 3% were sensitive to oilseed rape pollen alone (Fell et al., 1992). In a more recent study of a naturally exposed general farming population visiting clinics for seasonal allergies, oilseed rape hypersensitivity was found to be relatively uncommon comprising only 2% of the population tested. Allergies to house dust mites (25%), grass pollen (25%) and cereal pollen (20%) were much higher, and it was concluded that oilseed rape pollen does not cause significant allergy even in areas of high production (Trinidade et al., 2010).

## II.C. Canola as a Food Source

Canola is grown principally for its oil which is extracted from the seed, and has both food and industrial applications. Processing canola seed yields approximately 40% oil and 60% meal (Colton and Sykes, 1992). Due to the small seed size and high oil content that contributes to the difficulty in economically removing the protective hull surrounding the rapeseed, the hull is usually left on the seed in large commercial oil extraction operations. Canola seeds are flaked by a rolling process in preparation for oil extraction after which the flakes are placed in a cooker. Heating reduces the viscosity of the oil and inactivates enzymes, such as myrosinase, that can break down glucosinolates to produce isothiocyanates and nitriles that are harmful when fed to animals (Booth, 2004). After heating, canola seeds undergo mechanical extraction to produce a cake with an oil content of less than 20%, followed by solvent extraction using hexane to remove the bulk of the remaining oil. This crude oil undergoes further processing that may include: a) water or acid "degumming" to remove phospholipids; b) physical or acid refining to

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remove free fatty acids; c) bleaching to remove pigments and oxidation products present in the oil; d) winterization to remove some saturated fatty acids that crystallize out at lower temperatures that make the oil appear cloudy; e) hydrogenation to increase oxidative stability and melting points of triglycerides; f) interesterification to prevent phase separation in fats and g) deodorization to remove undesirable odors and off flavors (Booth, 2004; Carr, 1995).

Canola oil is high quality oil that is used in a variety of foods including frying and baking oils, salad oils, margarines and shortenings, and is the most valuable component of canola seed. It is the world's third largest source of vegetable oil with 15% of world vegetable oil consumption after soybean oil at 28% and palm oil at 32% (ASA, 2010; USDA-ERS, 2010a). Canola oil contains a low level (<10% of total fatty acids), of saturated fatty acids; a high level (approximately 60%) of the monounsaturated fatty acid, oleic acid, a moderate level (approximately 20%) of linoleic acid, and an appreciable amount (approximately 10%) of alpha-linolenic acid (CCC, 2010). Dietary guidance calls for limiting saturated fats in the diet in favor of monounsaturated and polyunsaturated fats. Canola oil helps achieve this guidance by replacing saturated fats with unsaturated fats. Furthermore, canola oil provides alpha-linolenic acid, which is essential to human health and must be supplied in the diet. Canola oil has well established heart health benefits and the FDA has issued a qualified health claim based on its ability to reduce the risk of coronary heart disease (FDA, 2006).

**II.D. Canola as a Feed Source**

The solid residue or meal left after oil extraction of canola is used as high protein animal feed and is an important feed product derived from canola. Canola meal is used in poultry, pig, beef and dairy cattle feeds, and can also be used in aquaculture diets for salmon, catfish and trout (CCC, 2009). Canola meal contains approximately 40% protein with a good balance of essential amino acids, and approximately 13% crude fiber (Bell, 1995). The standard for glucosinolates in canola meal is 30 µmoles/g (OECD, 2001), but most low glucosinolate canola varieties today have significantly lower levels of glucosinolates.

Compared to other oilseed-based feed sources canola meal has slightly less digestible energy value. Therefore, animals with requirements for intermediate energy levels such as dairy cattle and laying chickens perform well on canola meal, while high energy animals such as broiler chickens perform better on diets with soy meal (CCC, 2009). However, after soybean meal, canola meal is the second most widely traded protein ingredient for animal feed in the world.

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# III. DESCRIPTION OF THE DONOR ORGANISMS

This section describes the donor organism for the introduced protein. It contains information describing if the donor organism exhibits characteristics of pathogenicity or toxin production, is a known allergenic source, or has other traits that affect food and feed safety. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraph 26 (Codex Alimentarius, 2009).

## III.A. Identity and Sources of the Genetic Material Introduced into MON 88302

The donor organism, *Agrobacterium* sp. strain CP4, was isolated based on its tolerance to glyphosate brought about by the production of a naturally glyphosate-tolerant EPSPS protein (Padgette et al., 1996). The bacterial isolate, CP4, was identified by the American Type Culture Collection (ATCC) as an *Agrobacterium* species. This identification was made based on morphological and biochemical characteristics of the isolate and its similarity to a reference strain of *Agrobacterium*. The taxonomy of *Agrobacterium* sp. is:

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Alphaproteobacteria

Order: Rhizobiales

Family: Rhizobiaceae

Genus: Agrobacterium

*Agrobacterium* sp. strain CP4 is related to microbes commonly present in the soil and in the rhizosphere of plants. *Agrobacterium* species are not commonly known for human or animal pathogenicity, and are not commonly allergenic. Furthermore, according to a report of a joint FAO/WHO Expert Consultation (FAO/WHO, 2001), there is no known population of individuals sensitized to bacterial proteins.

MON 88302 was developed through *Agrobacterium*-mediated transformation of conventional canola using plasmid vector PV-BNHT2672. PV-BNHT2672 is approximately 9.7 kb and comprised of a *cp4 epsps* expression cassette, which contains the following genetic elements: P-*FMV/Tsf1* chimeric promoter derived from enhancer sequences of the 35S promoter of the figwort mosaic virus (Richins et al., 1987) and the promoter from the *Tsf1* gene of *Arabidopsis thaliana* (Axelos et al., 1989); the leader and intron sequences from the *Tsf1* gene of *Arabidopsis thaliana* (Axelos et al., 1989); the EPSPS chloroplast transit peptide coding sequence from the *shkG* gene of *Arabidopsis thaliana* (Herrmann, 1995; Klee et al., 1987); the codon optimized coding sequence of *cp4 epsps* from *Agrobacterium* sp. strain CP4 (Barry et al., 2001; Padgette et al., 1996); and the polyadenylation sequence derived from the 3' untranslated region of the pea

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(*Pisum sativum*) ribulose 1,5 bisphosphate carboxylase small subunit (*rbcS2*) *E9* gene (Coruzzi et al., 1984).

There is no evidence of human or animal pathogenicity for any of the donor organisms of the coding and noncoding DNA sequences present in MON 88302. DNA has always been present in food and, upon consumption, is quickly degraded to nucleic acids by restriction nucleases present in the gastrointestinal tract of humans and animals. According to the FDA (1992), nucleic acids are present in the cells of every living organism, do not raise concerns as a component of food, and are generally recognized as safe. Results from an International Life Sciences Institute (ILSI) workshop on safety considerations of DNA in food were reported (Jonas et al., 2001) and confirmed that: 1) all DNA including recombinant DNA is composed of the same four nucleotides; 2) there are no changes to the chemical characteristics or the susceptibility to degradation by chemical or enzymatic hydrolysis of recombinant DNA as compared to nonrecombinant DNA; and 3) there is no evidence that DNA from dietary sources has ever been incorporated into the mammalian genome.

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# IV. DESCRIPTION OF THE GENETIC MODIFICATION

This section provides a description of the transformation process and plasmid vector used in the development of MON 88302. Molecular analyses are an integral part of the characterization of crop products with new traits introduced by methods of biotechnology. Vectors and methods are selected for transformation to achieve high probability of obtaining the trait of interest and integration of the introduced DNA into a single locus in the plant genome. This helps ensure that only the intended DNA encoding the desired trait(s) is integrated into the plant genome and facilitates the molecular characterization of the product. Information provided here allows for the identification of the genetic material present in the plasmid vector delivered to the host plant and for an analysis of the data supporting the characterization of the DNA inserted in the plant found in Section V. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraphs 27, 28, and 29 (Codex Alimentarius, 2009).

MON 88302 was developed through *Agrobacterium*-mediated transformation of hypocotyls from Ebony canola variety utilizing plasmid vector PV-BNHT2672. This section describes the plasmid vector, the donor gene, and the regulatory elements used in the development of MON 88302 as well as the deduced amino acid sequence of the CP4 EPSPS protein produced in MON 88302. In this section, transfer DNA (T-DNA) refers to DNA that is transferred to the plant during transformation. An expression cassette is comprised of sequences to be transcribed and the regulatory elements necessary for the expression of those sequences.

## IV.A. Plasmid Vector PV-BNHT2672

PV-BNHT2672 was used in the transformation of canola to produce MON 88302 and is shown in Figure IV-1. The elements included in this plasmid vector are described in Table IV-1. PV-BNHT2672 is approximately 9.7 kb and contains one T-DNA that is delineated by Left Border and Right Border regions. The T-DNA contains the *cp4 epsps* coding sequence under the control of the *FMV/Tsf1* chimeric promoter, the *Tsf1* leader and intron sequences, and the *E9* 3′ untranslated region. The chloroplast transit peptide CTP2 directs transport of the CP4 EPSPS protein to the chloroplast and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene (Herrmann, 1995; Klee et al., 1987).

The backbone region of PV-BNHT2672, located outside of the T-DNA, contains two origins of replication for maintenance of plasmid vector in bacteria (*ori V* and *ori-pBR322*), a bacterial selectable marker gene (*aadA*), and a coding sequence for repressor of primer protein for maintenance of plasmid vector copy number in *Escherichia coli (E. coli)* (*rop*). A description of the genetic elements and their prefixes (e.g., B-, P-, L-, I-, TS-, CS-, T-, and OR-) in PV-BNHT2672 is provided in Table IV-1.

## IV.B. Description of the Transformation System

MON 88302 was developed through *Agrobacterium*-mediated transformation of canola hypocotyls, based on the method described by Radke et al., (1992), utilizing

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PV-BNHT2672 (Figure IV-1). In summary, hypocotyl segments were excised from dark grown seedlings of germinated Ebony seed. After co-culturing with the *Agrobacterium* carrying the vector, the hypocotyl segments were placed on medium for callus growth containing carbenicillin, ticarcillin disodium and clavulanate potassium to inhibit the growth of excess *Agrobacterium*. The hypocotyls were then placed in selection media containing glyphosate to inhibit the growth of untransformed cells and plant growth regulators conducive to shoot regeneration. Rooted $R_0$ plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The $R_0$ plants generated through the *Agrobacterium*-mediated transformation were transferred to soil for growth and then selfed to produce $R_1$ seed. $R_0$ and $R_1$ plants were evaluated for tolerance to glyphosate and screened for the presence of the T-DNA (*cp4 epsps* expression cassette) and absence of plasmid vector backbone (*Ori V*). Subsequently, the *cp4 epsps* homozygous $R_1$ plant was self-pollinated to give rise to $R_2$ plants. 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. MON 88302 was selected as the lead event based on superior phenotypic characteristics and its comprehensive molecular profile. Regulatory studies on MON 88302 were initiated to further characterize the genetic insertion and the expressed protein, and to establish the food, feed, and environmental safety relative to conventional canola. The major steps involved in the development of MON 88302 are depicted in Figure IV-2.

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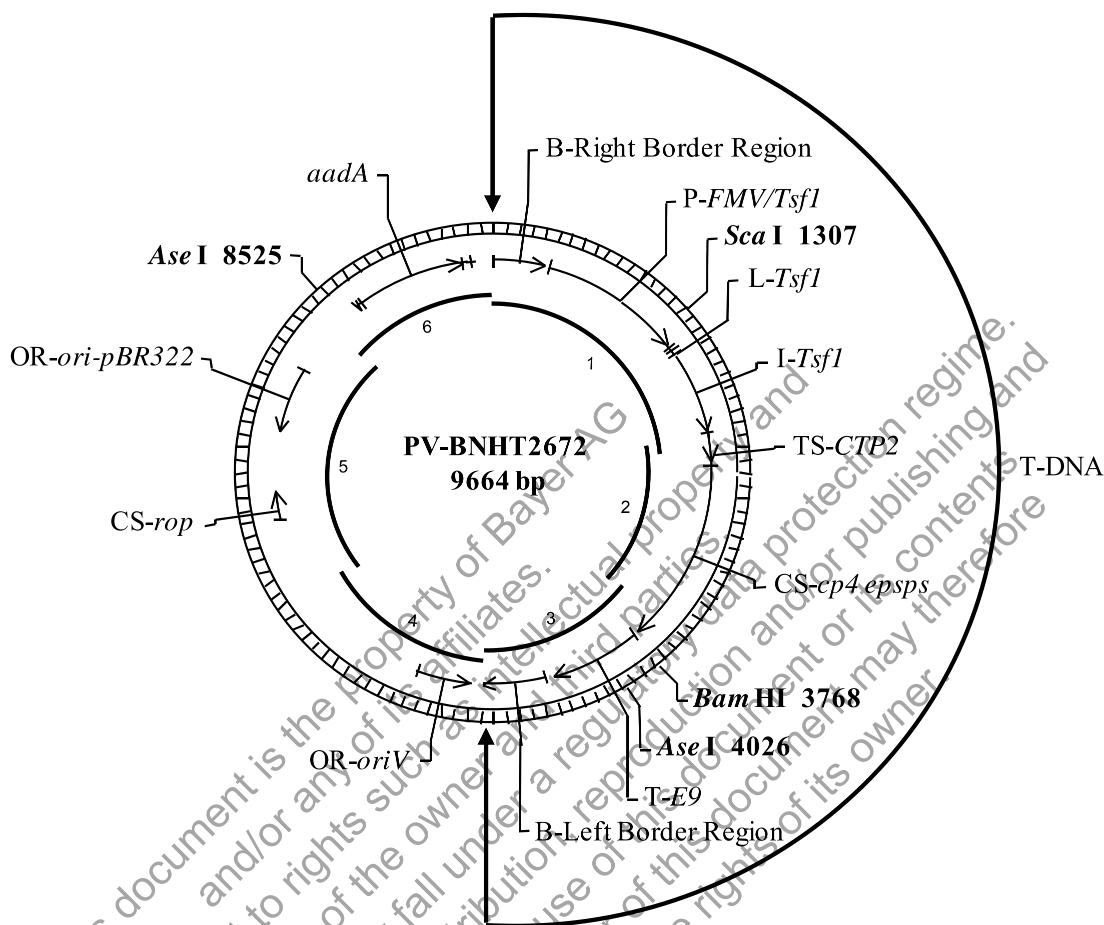


| Probe | DNA Probe | Start Position (bp) | End Position (bp) | Total Length (kb) |
|---|---|---|---|---|
| 1 | T-DNA Probe 1 | 1 | 2287 | ~2.3 |
| 2 | T-DNA Probe 2 | 2231 | 3618 | ~1.4 |
| 3 | T-DNA Probe 3 | 3562 | 4910 | ~1.3 |
| 4 | Backbone Probe 4 | 4911 | 6564 | ~1.7 |
| 5 | Backbone Probe 5 | 6512 | 8383 | ~1.9 |
| 6 | Backbone Probe 6 | 8329 | 9664 | ~1.3 |

**Figure IV-1. Circular Map of PV-BNHT2672 Showing Probes 1-6**

A circular map of PV-BNHT2672 used to develop MON 88302 is shown. Genetic elements and restriction sites (in bold) used in Southern analyses (with positions relative to the first base pair of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map and listed in the table. PV-BNHT2672 contains a single T-DNA.

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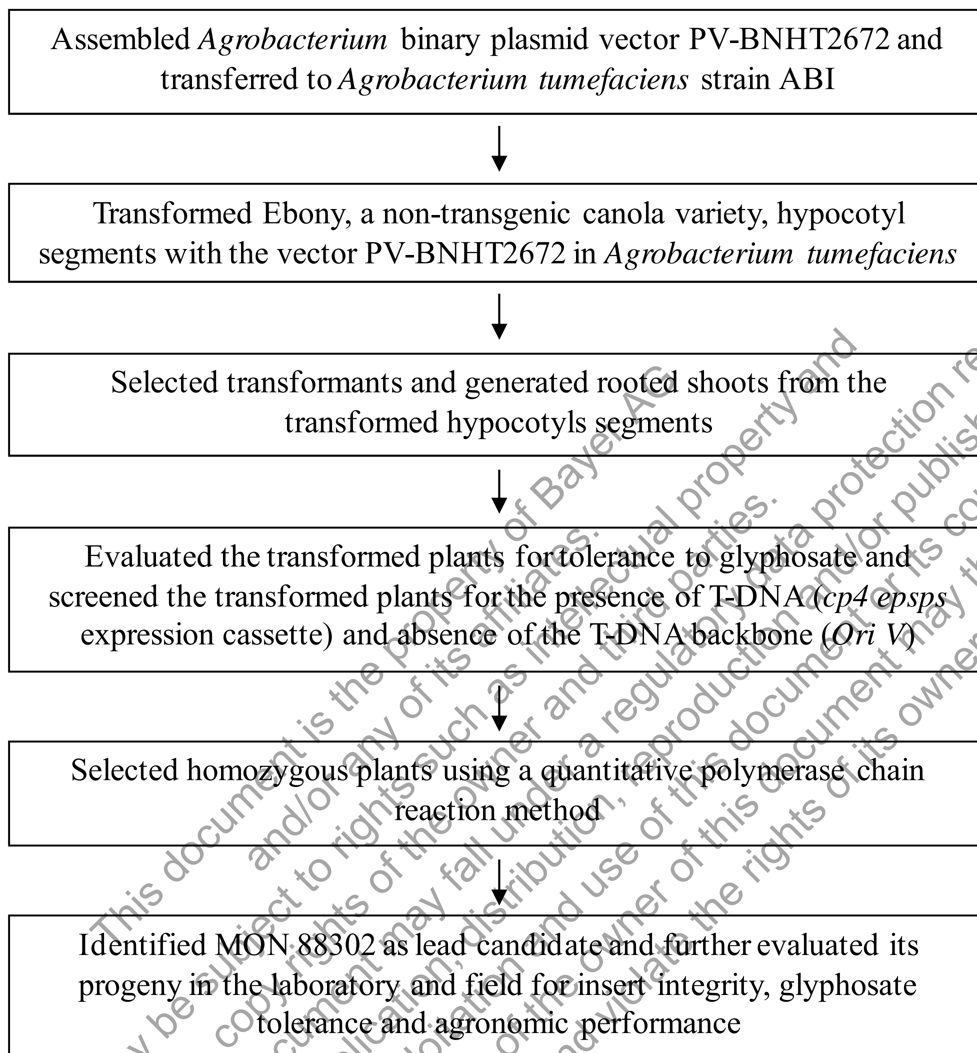


**Figure IV-2. Schematic of the Development of MON 88302**

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## IV.C. The *cp4 epsps* Coding Sequence and CP4 EPSPS Protein

The *cp4 epsps* expression cassette, or T-DNA in this petition, encodes a 47.6 kDa CP4 EPSPS protein consisting of a single polypeptide of 455 amino acids (Figure IV-3) (Padgette et al., 1996). The *cp4 epsps* coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry et al., 2001; Padgette et al., 1996). The CP4 EPSPS protein is similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Barry et al., 2001; Padgette et al., 1996).

## IV.D. Regulatory Sequences

The *cp4 epsps* coding sequence in MON 88302 is under the regulation of the *FMV/Tsf1* chimeric promoter, the *Tsf1* leader and intron sequences, and the *E9* 3′ untranslated region. The *FMV/Tsf1* chimeric promoter, which directs transcription in plant cells, contains enhancer sequences from the promoter of the figwort mosaic virus 35S RNA (Richins et al., 1987) combined with the promoter from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1α (Axelos et al., 1989). The *Tsf1* leader sequence is the 5' untranslated region from the *Tsf1* gene of *Arabidopsis thaliana* (Axelos et al., 1989). The *E9* 3' untranslated region is the 3' untranslated region of the pea (*Pisum sativum*) ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS2*) *E9* gene (Coruzzi et al., 1984) and is present to direct polyadenylation of the *cp4 epsps* transcript. The chloroplast transit peptide CTP2 directs transport of the CP4 EPSPS protein to the chloroplast and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene (Herrmann, 1995; Klee et al., 1987).

## IV.E. T-DNA Border Regions

PV-BNHT2672 contains Right Border and Left Border regions (Figure IV-1 and Table IV-1) that were derived from *Agrobacterium tumefaciens* plasmids. The border regions each contain a 24-25 bp nick site that is the site of DNA exchange during transformation (Barker et al., 1983; Depicker et al., 1982; Zambryski et al., 1982). The border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer T-DNA into the canola genome.

## IV.F. Genetic Elements Outside the T-DNA Border Regions

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV-BNHT2672 in bacteria. The origin of replication, *ori V,* is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host plasmid RK2 (Stalker et al., 1981). The origin of replication, *ori-pBR322,* is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pBR322 (Sutcliffe, 1979). Coding sequence *rop* encodes the repressor of primer (ROP) protein which is necessary for the maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989). The selectable marker *aadA* is a bacterial promoter and coding sequence for an enzyme from transposon Tn7 that confers spectinomycin and

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streptomycin resistance (Fling et al., 1985) in *E. coli* and *Agrobacterium* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the canola genome. The absence of detectable backbone sequence in MON 88302 has been confirmed by Southern blot analyses (see Section V.B).

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**Table IV-1. Summary of Genetic Elements in PV-BNHT2672**

| Genetic Element | Location in Plasmid | Function (Reference) |
|---|---|---|
| **T-DNA** | | |
| **B[1]-Right Border Region** | 1-357 | DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982) |
| Intervening Sequence | 358-427 | Sequence used in DNA cloning |
| **P[2]-*FMV/Tsf1*** | 428-1467 | Chimeric promoter consisting of the promoter of the *Tsf1* gene from the *Arabidopsis thaliana* encoding elongation factor EF-1α (Axelos et al., 1989) and enhancer sequences from the 35S promoter from the figwort mosaic virus (Richins et al., 1987) |
| **L[3]-*Tsf1*** | 1468-1513 | 5' untranslated leader (exon 1) from the *Arabidopsis thaliana Tsf1* gene encoding elongation factor EF-1α (Axelos et al., 1989) |
| **I[4]-*Tsf1*** | 1514-2135 | Intron from the *Arabidopsis thaliana Tsf1* gene encoding elongation factor EF-1α (Axelos et al., 1989) |
| Intervening Sequence | 2136-2144 | Sequence used in DNA cloning |
| **TS[5]-*CTP2*** | 2145-2372 | Targeting sequence from the *shkG* gene encoding the chloroplast transit peptide region of *Arabidopsis thaliana* EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast |
| **CS[6]-*cp4 epsps*** | 2373-3740 | Codon optimized coding sequence of the *aroA* gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgette et al., 1996) |
| Intervening Sequence | 3741-3782 | Sequence used in DNA cloning |
| **T[7]-*E9*** | 3783-4425 | 3' untranslated sequence from the *rbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit (Coruzzi et al., 1984) |
| Intervening Sequence | 4426-4468 | Sequence used in DNA cloning |
| **B-Left Border Region** | 4469-4910 | DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983; Zambryski et al., 1982) |

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**Table IV-1. Summary of Genetic Elements in PV-BNHT2672 (continued)**

| Genetic Element | Location in Plasmid | Function (Reference) |
|---|---|---|
| **Vector Backbone** | | |
| Intervening Sequence | 4911-4996 | Sequence used in DNA cloning |
| **OR[8]- *oriV*** | 4997-5393 | Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981) |
| Intervening Sequence | 5394-6901 | Sequence used in DNA cloning |
| **CS-*rop*** | 6902-7093 | Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989) |
| Intervening Sequence | 7094-7520 | Sequence used in DNA cloning |
| **OR-*ori-pBR322*** | 7521-8109 | Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979) |
| Intervening Sequence | 8110-8639 | Sequence used in DNA cloning |
| ***aadA*** | 8640-9528 | Bacterial promoter, coding sequence, and 3′ untranslated region for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyl-transferase from the transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistance |
| Intervening Sequence | 9529-9664 | Sequence used in DNA cloning |

[1] B, Border
[2] P, Promoter
[3] L, Leader
[4] I, Intron
[5] TS, Targeting Sequence
[6] CS, Coding Sequence
[7] T, Transcription Termination Sequence
[8] OR, Origin of Replication

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```
  1   MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51   LKKSGMTLIG SELRPLKVMS SVSTACMLHG ASSRPATARK SSGLSGTVRI
101   PGDKSISHRS FMFGGLASGE TRITGLLEGE DVINTGKAMQ AMGARIRKEG
151   DTWIIDGVGN GGLLAPEAPL DFGNAATGCR LTMGLVGVYD FDSTFIGDAS
201   LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP ITYRVPMASA
251   QVKSAVLLAG LNTPGITTVI EPIMTRDHTE KMLQGFGANL TVETDADGVR
301   TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR
351   TGLILTLQEM GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM
401   IDEYPILAVA AAFAEGATVM NGLEELRVKE SDRLSAVANG LKLNGVDCDE
451   GETSLVVRGR PDGKGLGNAS GAAVATHLDH RIAMSFLVMG LVSENPVTVD
501   DATMIATSFP EFMDLMAGLG AKIELSDTKA A
```

**Figure IV-3. Deduced Amino Acid Sequence of the MON 88302 CP4 EPSPS Precursor Protein**

The amino acid sequence of the CP4 EPSPS precursor protein was deduced from the full-length coding nucleotide sequence present in PV-BNHT2672. The 76 amino acid CTP2, the transit peptide of the *Arabidopsis thaliana* EPSPS protein, is underlined. CTP2 targets CP4 EPSPS protein to the chloroplasts. At the chloroplast the CTP2 is cleaved producing the mature 455 amino acid CP4 EPSPS protein that begins with the methionine at position 77.

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# V. CHARACTERIZATION OF THE GENETIC MODIFICATION

This section contains a comprehensive molecular characterization of the genetic modification present in MON 88302. It provides information on the DNA insertion(s) into the plant genome of MON 88302, and additional information relative to the arrangement and stability of the introduced genetic material. The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 30, 31, 32, and 33 (Codex Alimentarius, 2009).

Characterization of the DNA insert in MON 88302 was conducted by Southern blot, PCR and DNA sequence analyses. The results of this characterization demonstrate that MON 88302 contains a single copy of the *cp4 epsps* expression cassette, i.e., the T-DNA that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions were based on several lines of evidence: 1) Southern blot analyses assayed the entire canola genome for the presence of T-DNA and the absence of the plasmid backbone sequences derived from PV-BNHT2672, and demonstrated that only a single copy of the T-DNA was inserted at a single site; 2) DNA sequence analyses determined the exact sequence of the inserted DNA and the DNA sequences flanking the 5' and 3' ends of the insert, and allowed a comparison to the T-DNA sequence in the plasmid vector to confirm that only the expected sequences were integrated; and 3) DNA sequences flanking the 5' and 3' ends of the insert were compared to the sequence of the insertion site in conventional canola to identify any rearrangements that occurred at the insertion site during transformation. Taken together, the characterization of the genetic modification demonstrates that a single copy of the T-DNA was inserted at a single locus of the canola genome and that no plasmid vector backbone sequences are present in MON 88302.

Southern blot analyses were used to determine the copy number and insertion sites of the integrated DNA as well as the presence or absence of plasmid vector backbone sequences. The Southern blot strategy was designed to ensure that all potential transgenic segments would be identified. The entire canola genome was assayed with probes that spanned the complete plasmid vector to detect the presence of the insert as well as confirm the absence of any plasmid vector backbone sequences. This was accomplished by using probes that were not more than 2.5 kb in length to ensure a high level of sensitivity. This high level of sensitivity was demonstrated for each blot by detection of a positive control added at 0.1 copies per genome equivalent. Two sets of restriction enzymes were specifically chosen to fully characterize the T-DNA and detect any potential fragments of the T-DNA and backbone sequences. The restriction enzyme sets were chosen such that each enzyme set cleaves once within the inserted T-DNA and at least once within the known DNA flanking the 5' or 3' end of the insert. As a consequence, at least one segment containing a portion of the insert with the adjacent 5' flanking DNA generated by one set of the enzyme(s) is of a predictable size and overlaps with another predictable size segment containing a portion of the insert with the adjacent 3' flanking DNA generated by another set of the enzyme(s). This two-set-enzyme design ensures that the entire insert is identified in a predictable hybridization pattern. This strategy also maximizes the possibility of detecting an insertion elsewhere in the genome

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that could be overlooked if that the fragment co-migrated on the gel with an expected fragment.

To determine the number of copies and insertion sites of the T-DNA, and the presence or absence of the plasmid vector backbone sequences, duplicated samples that consisted of equal amounts of digested DNA were run on the agarose gel. One set of samples was run for a longer period of time (long run) than the second set (short run). The long run allows for greater resolution of large molecular weight DNA, whereas the short run allows for retaining the small molecular weight DNA on the gel. The molecular weight markers on the left of the figures were used to estimate the sizes of the bands present in the long run lanes of the Southern blots, and the molecular weight markers on the right of the figures were used to estimate the sizes of bands present in the short run lanes of the Southern blots (Figure V-2 through Figure V-6). Southern blot analyses determined that a single copy of the T-DNA was inserted at a single locus of the canola genome, and no additional genetic elements, including backbone sequences, from PV-BNHT2672 were detected in MON 88302.

PCR and DNA sequence analyses complement the Southern analyses. PCR and DNA sequence analyses performed on MON 88302 determined the complete DNA sequence of the insert and flanking genomic DNA sequences in MON 88302, confirmed the predicted organization of the genetic elements within the insert, and determined the sequences flanking the insert. In addition, DNA sequence analyses confirmed that each genetic element in the insert is intact and the sequence of the insert is identical to the corresponding sequence in PV-BNHT2672. Furthermore, genomic organization at the MON 88302 insertion site was determined by comparing the 5' and 3' flanking sequences of the insert to the sequence of the insertion site in conventional canola.

The stability of the T-DNA present in MON 88302 across multiple generations was demonstrated by Southern blot fingerprint analysis. Genomic DNA from multiple generations of MON 88302 (Figure V-9) was digested with one of the enzyme sets used for the insert and copy number analyses and was hybridized with two probes that detect restriction segments that encompass the entire insert. This fingerprint strategy consists of two insert segments each containing its adjacent genomic DNA that assesses not only the stability of the insert, but also the stability of the DNA directly adjacent to the insert.

Segregation analysis was conducted to determine the inheritance and stability of the T-DNA insert in MON 88302. Results from this analysis demonstrated the inheritance and stability of the insert was as expected across multiple generations (Figure V-11, Table V-3), which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA at a single chromosomal locus.

The Southern blot analyses confirmed that the T-DNA reported in Figure V-1 represents the only detectable insert in MON 88302. A circular map of PV-BNHT2672 annotated with the probes used in the Southern blot analysis is presented in Figure IV-1 and the genetic elements within the MON 88302 insert are summarized in Table V-2. A linear map depicting restriction sites within the insert as well as within the DNA immediately flanking the insert in MON 88302 is shown in Figure V-1. Based on the plasmid map

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and the linear map of the insert, a table summarizing the expected DNA segments for Southern analyses is presented in Table V-1. The results from the Southern blot analyses are presented in Figure V-2 through Figure V-6. PCR amplification of the MON 88302 insert and the insertion site in conventional control (Ebony) for DNA sequence analysis are shown in Figure V-7 and Figure V-8, respectively. The generations used in the generational stability analysis are depicted in the breeding history shown in Figure V-9 and the results from the generational stability analysis are presented in Figure V-10. The breeding path for generating the segregation data is shown in Figure V-11 and the results for the segregation analysis are presented in Table V-3. Materials and methods used for the characterization of the insert in MON 88302 are found in Appendix A.

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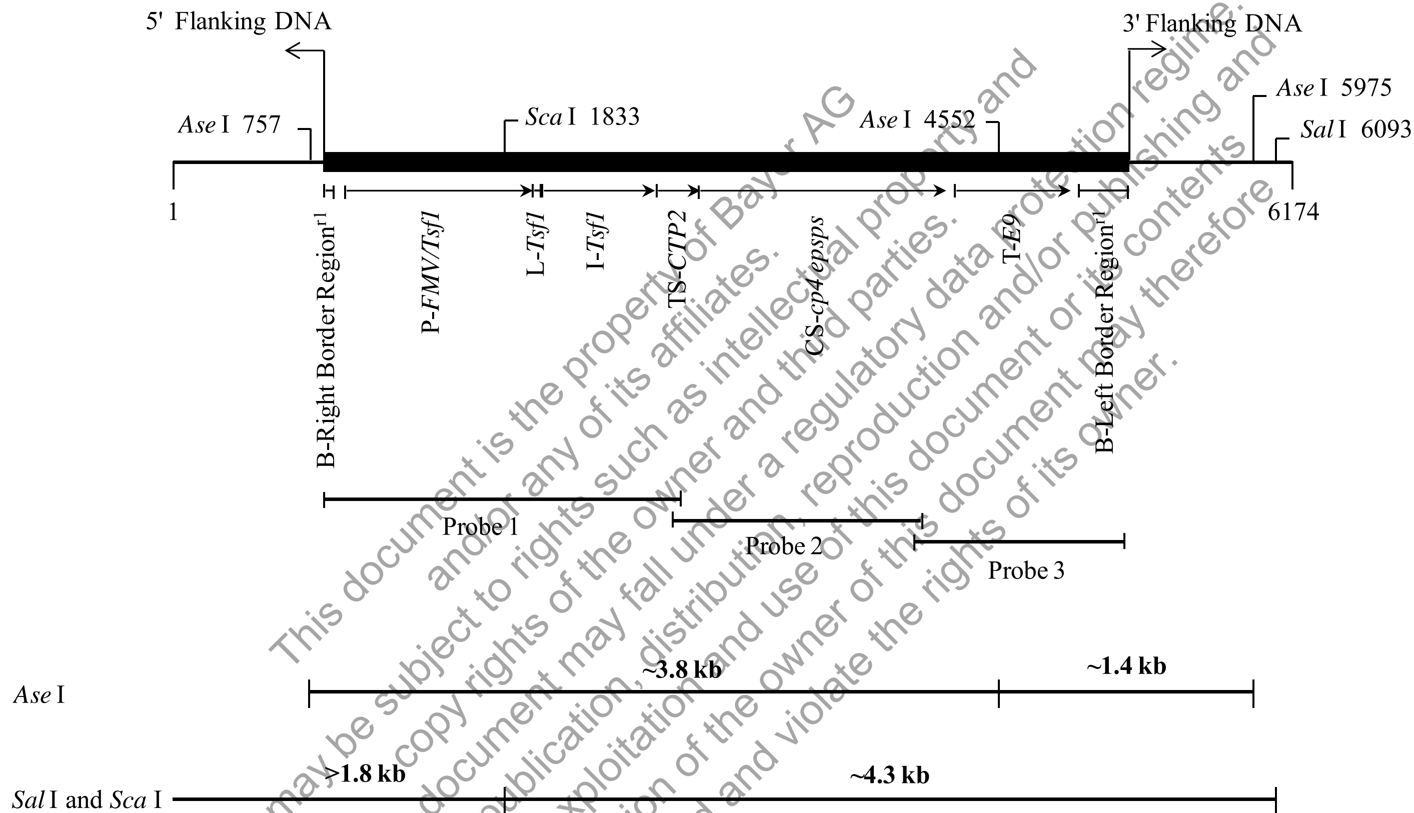


**Figure V-1. Schematic Representation of the Insert and Flanking DNA in MON 88302**

A linear map of the insert and DNA flanking the insert in MON 88302 is shown. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking DNA. Identified on the linear map are genetic elements within the insert, as well as the sites of the restriction enzymes used in the Southern analyses with positions relative to the first base pair of the DNA sequence represented in this map. The relative sizes and locations of the T-DNA probes and the expected sizes of restriction fragments are indicated in the lower portion of the scheme. This schematic diagram is not drawn to scale. Locations of genetic elements and T-DNA probes are approximate. Probes are also shown in Figure IV-1.

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**Table V-1. Summary Chart of the Expected DNA Segments Based on Hybridizing Probes and Restriction Enzymes Used in MON 88302 Analysis**

| Southern Blot Analysis | | T-DNA | | Backbone | | | Insert Stability |
|---|---|---|---|---|---|---|---|
| **Figure Number** | | V-2 | V-3 | V-4 | V-5 | V-6 | V-10 |
| **Probe Used** | | 1, 3 | 2 | 4 | 5 | 6 | 1, 3 |
| | | | | | | | |
| **Probing Target** | **Digestion Enzyme** | **Expected Band Sizes on Each Southern Blot** | | | | | |
| **Plasmid PV-BNHT2672** | *Bam* HI and *Sca* I | ~2.5 kb<br>~7.2 kb | ~2.5 kb | ~7.2 kb | ~7.2 kb | ~7.2 kb | ~2.5 kb<br>~7.2 kb |
| **Probe Template Spikes**[1] | N/A | ~2.3 kb<br>~1.3 kb | ~~[2] | ~~[2] | ~~[2] | ~~[2] | ~2.3 kb<br>~1.3 kb |
| | | | | | | | |
| **MON 88302** | *Ase* I | ~3.8 kb<br>~1.4 kb | ~3.8 kb | No band | No band | No band | ~3.8 kb<br>~1.4 kb |
| | *Sal* I and *Sca* I | >1.8 kb<br>~4.3 kb | ~4.3 kb | No band | No band | No band | --[3] |

1 probe template spikes were used as positive hybridization controls in Southern blot analyses when multiple probes were hybridized to the Southern blot simultaneously.
2 '~~' indicates that probe template spikes were not used.
3 '--' indicates that the combination of the restriction enzymes was not used in the analysis.

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## Table V-2. Summary of Genetic Elements in MON 88302

| Genetic Element | Location in Sequence | Function (Reference) |
|---|---|---|
| 5' Flanking Sequence | 1-839 | DNA sequence adjacent to the 5' end of the insertion site |
| B[1]-Right Border Region [r1] | 840-882 | DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982) |
| Intervening Sequence | 883-952 | Sequence used in DNA cloning |
| P[2]-*FMV/Tsf1* | 953-1992 | Chimeric promoter consisting of the promoter of the *Tsf1* gene from the *Arabidopsis thaliana* encoding elongation factor EF-1α (Axelos et al., 1989) and enhancer sequences from the 35S promoter from the figwort mosaic virus (Richins et al., 1987) |
| L[3]-*Tsf1* | 1993-2038 | 5' untranslated leader (exon 1) from the *Arabidopsis thaliana Tsf1* gene encoding elongation factor EF-1 α (Axelos et al., 1989) |
| I[4]-*Tsf1* | 2039-2660 | Intron from the *Arabidopsis thaliana Tsf1* gene encoding elongation factor EF-1α (Axelos et al., 1989) |
| Intervening Sequence | 2661-2669 | Sequence used in DNA cloning |
| TS[5]-*CTP2* | 2670-2897 | Targeting sequence from the *shkG* gene encoding the chloroplast transit peptide region of *Arabidopsis thaliana* EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast |
| CS[6]-*cp4 epsps* | 2898-4265 | Codon optimized coding sequence of the *aroA* gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgette et al., 1996) |
| Intervening Sequence | 4266-4307 | Sequence used in DNA cloning |
| T[7]-*E9* | 4308-4950 | 3' untranslated sequence from the *rbcS2* gene of *Pisum sativum* encoding the Rubisco small subunit (Coruzzi et al., 1984) |
| Intervening Sequence | 4951-4993 | Sequence used in DNA cloning |
| B-Left Border Region [r1] | 4994-5267 | DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983; Zambryski et al., 1982) |
| 3' Flanking Sequence | 5268-6174 | DNA sequence adjacent to the 3' end of the insertion site |

[1] B, Border
[2] P, Promoter
[3] L, Leader
[4] I, Intron
[5] TS, Targeting Sequence
[6] CS, Coding Sequence
[7] T, Transcription Termination Sequence
[r1] Superscripts in Left and Right Border Regions indicate that the sequences in MON 88302 were truncated compared to the sequences in PV-BNHT2672.

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## V.A. Insert and Copy Number of T-DNA in MON 88302

The numbers of copies and insertion sites of the T-DNA sequences in the canola genome were evaluated by digesting MON 88302 and conventional control genomic DNA samples with the restriction enzyme *Ase* I or the combination of restriction enzymes *Sal* I and *Sca* I and hybridizing Southern blots with probes that span the T-DNA (Figure IV-1). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table V-1). Any additional copies and/or integration sites would be detected as additional bands on the blots.

The restriction enzyme *Ase* I cleaves once within the inserted T-DNA and within the known genomic DNA flanking the 5' and 3' ends of the insert (Figure V-1). Therefore, if T-DNA sequences were present as a single copy at a single integration site in MON 88302, the digestion with *Ase* I was expected to generate two border segments with expected sizes of ~3.8 kb and ~1.4 kb (Figure V-1 and Table V-1). The combination of restriction enzymes *Sal* I and *Sca* I cleaves once within the inserted T-DNA and within the known genomic DNA flanking the 3' end of the insert (Figure V-1). If T-DNA sequences were present as a single copy at a single integration site in MON 88302, the digestion with *Sal* I and *Sca* I was expected to generate two border segments with expected sizes of >1.8 kb and ~4.3 kb (Figure V-1 and Table V-1).

The Southern blots were hybridized with T-DNA probes that collectively span the entire inserted DNA sequence (Figures IV-1 and V-1, Probe 1, Probe 2, and Probe 3). Conventional control genomic DNA digested with the restriction enzyme *Ase* I and spiked with either probe templates and/or digested PV-BNHT2672 DNA served as positive hybridization controls. The positive hybridization control was spiked at approximately 0.1 and 1 genome equivalents to demonstrate sufficient sensitivity of the Southern blot. Conventional control genomic DNA digested with the appropriate restriction enzymes was used as a negative control. The results of these analyses are shown in Figure V-2 and Figure V-3.

### V.A.1. T-DNA Probes 1 and 3

Conventional control genomic DNA digested with *Ase* I (Figure V-2, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure V-2, Lane 3 and Lane 7) and simultaneously hybridized with Probe 1 and Probe 3 (Figures IV-1 and V-1) produced no detectable hybridization bands as expected for the negative control. Conventional control genomic DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure IV-1), produced two bands at ~7.2 kb and ~2.5 kb (Figure V-2, Lane 10), as expected. Conventional control genomic DNA digested with *Ase* I and spiked with probe templates of Probe 1 and Probe 3 (Figure IV-1) produced the expected bands at ~2.3 kb and ~1.3 kb (Figure V-2, Lane 11 and Lane 12). Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 88302 DNA digested with *Ase* I and simultaneously hybridized with Probe 1 and Probe 3 (Figures IV-1 and V-1) produced the expected bands at ~3.8 kb and ~1.4 kb

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(Figure V-2, Lane 2 and Lane 6). MON 88302 DNA digested with the combination of restriction enzymes *Sal* I and *Sca* I and hybridized with Probe 1 and Probe 3 (Figures IV-1 and V-1) produced two bands at ~2.7 kb and ~4.3 kb (Figure V-2, Lane 4 and Lane 8), which is consistent with the expected >1.8 kb and ~4.3 kb bands (Figure V-1 and Table V-1), respectively.

The results presented in Figure V-2 indicate that the sequences covered by Probe 1 and Probe 3 reside at a single detectable locus of integration in MON 88302.

### V.A.2. T-DNA Probe 2

Conventional control DNA digested with *Ase* I (Figure V-3, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure V-3, Lane 3 and Lane 7) and hybridized with Probe 2 (Figures IV-1 and V-1) produced no detectable hybridization bands as expected for the negative control. Conventional control genomic DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure IV-1), produced a unique band at ~2.5 kb (Figure V-3, Lane 10 and Lane 11), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88302 DNA digested with *Ase* I and hybridized with Probe 2 (Figures IV-1 and V-1) produced the expected band at ~3.8 kb (Figure V-3, Lane 2 and Lane 6). MON 88302 DNA digested with the combination of restriction enzymes *Sal* I and *Sca* I and hybridized with Probe 2 (Figures IV-1 and V-1) produced the expected band at ~4.3 kb (Figure V-3, Lane 4 and Lane 8, Figure V-1, and Table V-1).

The results presented in Figure V-3 indicate that the sequence covered by Probe 2 resides at a single detectable locus of integration in MON 88302.

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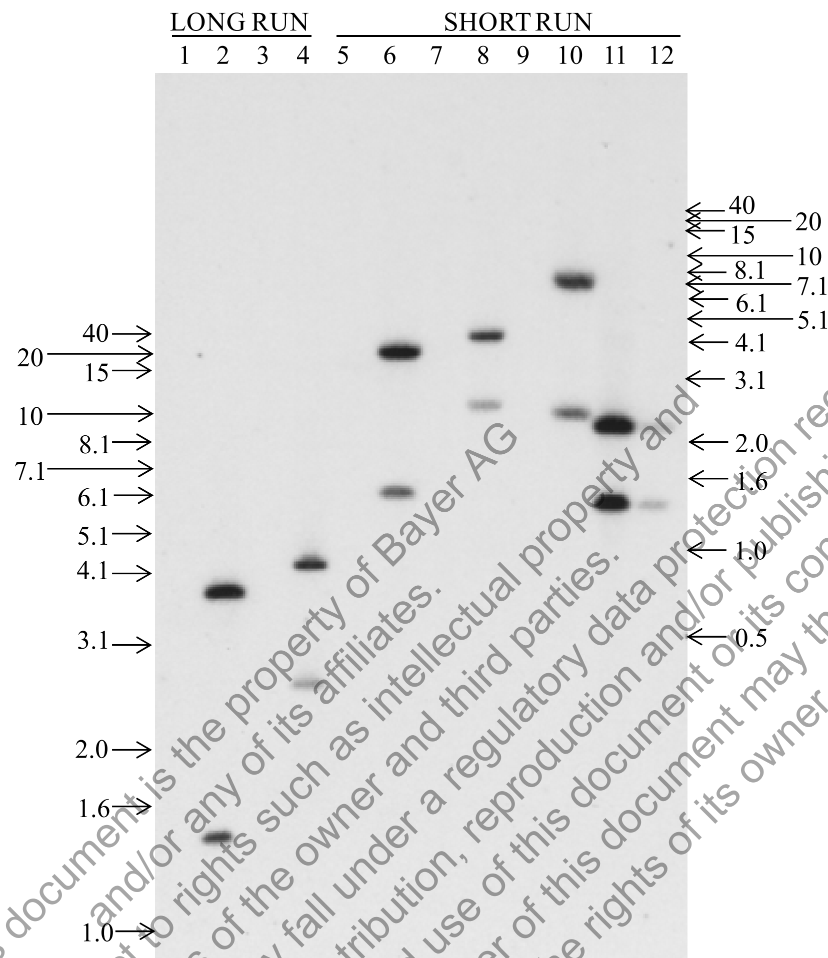


**Figure V-2. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88302: Probes 1 and 3**

The blot was simultaneously hybridized with two $^{32}$P-labeled probes that span a portion of the T-DNA sequence (Figure IV-1, Probe 1 and Probe 3). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

| **Lane** | |
|---|---|
| 1 | Conventional control (*Ase* I) |
| 2 | MON 88302 (*Ase* I) |
| 3 | Conventional control (*Sal* I/*Sca* I) |
| 4 | MON 88302 (*Sal* I/*Sca* I) |
| 5 | Conventional control (*Ase* I) |
| 6 | MON 88302 (*Ase* I) |
| 7 | Conventional control (*Sal* I/*Sca* I) |
| 8 | MON 88302 (*Sal* I/*Sca* I) |
| 9 | Blank |
| 10 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent] |
| 11 | Conventional control (*Ase* I) spiked with Probe 1 and Probe 3 [~1 genome equivalent] |
| 12 | Conventional control (*Ase* I) spiked with Probe 1 and Probe 3 [~0.1 genome equivalent] |

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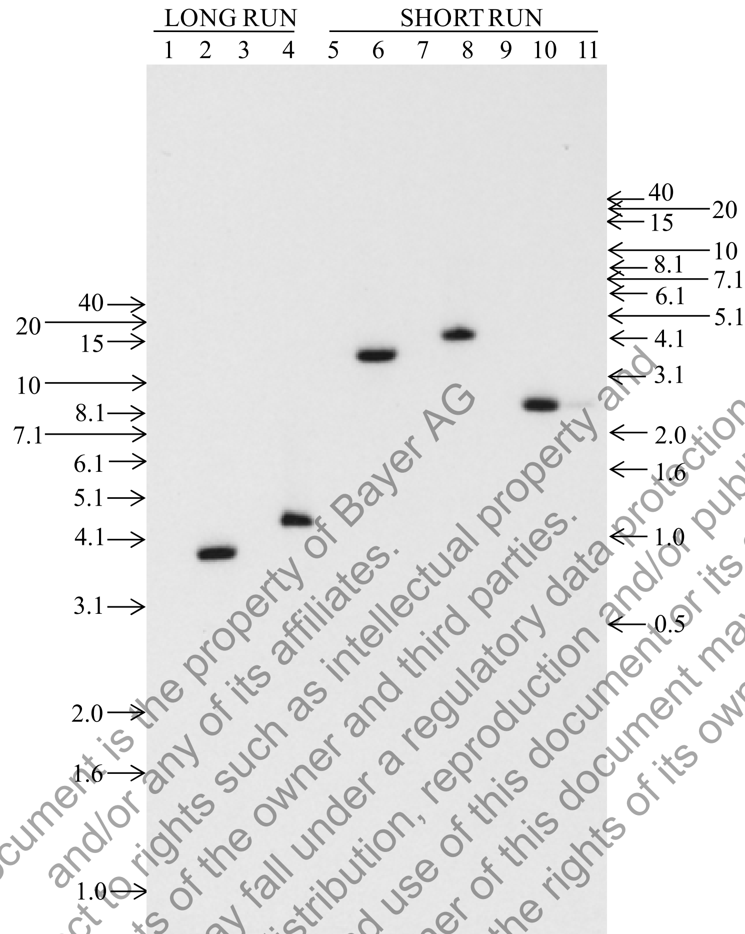


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**Figure V-3. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88302: Probe 2**

The blot was hybridized with a $^{32}$P-labeled probe that spans a portion of the T-DNA sequence (Figure IV-1, Probe 2). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

| **Lane** | |
|---|---|
| 1 | Conventional control (*Ase* I) |
| 2 | MON 88302 (*Ase* I) |
| 3 | Conventional control (*Sal* I/*Sca* I) |
| 4 | MON 88302 (*Sal* I/*Sca* I) |
| 5 | Conventional control (*Ase* I) |
| 6 | MON 88302 (*Ase* I) |
| 7 | Conventional control (*Sal* I/*Sca* I) |
| 8 | MON 88302 (*Sal* I/*Sca* I) |
| 9 | Blank |
| 10 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent] |
| 11 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~0.1 genome equivalent] |

## V.B. Southern Blot Analysis to Determine the Presence or Absence of the PV-BNHT2672 Backbone Sequences in MON 88302

To determine the presence or absence of the PV-BNHT2672 backbone sequences, MON 88302 and conventional control genomic DNA were digested with the restriction enzyme *Ase* I or the combination of restriction enzymes *Sal* I and *Sca* I, and hybridized with one of the three backbone probes that collectively span the entire backbone sequences (Figure IV-1, Probe 4, Probe 5, and Probe 6). If backbone sequences are present in MON 88302, then probing with backbone probes should result in hybridizing bands. Conventional control genomic DNA digested with the restriction enzyme *Ase* I and spiked with digested PV-BNHT2672 DNA served as positive hybridization controls. The positive hybridization control was spiked at approximately 0.1 and 1 genome equivalents to demonstrate sufficient sensitivity of the Southern blot. Conventional control genomic DNA digested with the appropriate restriction enzymes was used as a negative control. The results of these analyses are shown in Figures V-4, V-5, and V-6.

### V.B.1. Backbone Probe 4

Conventional control DNA digested with *Ase* I (Figure V-4, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure V-4, Lane 3 and Lane 7) and hybridized with Probe 4 (Figure IV-1) produced no detectable hybridization bands as expected for the negative control. Conventional control DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure IV-1), produced a unique band at ~7.2 kb (Figure V-4, Lane 10 and Lane 11), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88302 DNA digested with *Ase* I (Figure V-4, Lane 2 and Lane 6) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure V-4, Lane 4 and Lane 8) and hybridized with Probe 4 produced no detectable bands.

The results presented in Figure V-4 indicate that MON 88302 contains no detectable backbone sequences covered by Probe 4.

### V.B.2. Backbone Probe 5

Conventional control DNA digested with *Ase* I (Figure V-5, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure V-5, Lane 3 and Lane 7) and hybridized with Probe 5 (Figure IV-1) produced no detectable hybridization bands as expected for the negative control. Conventional control DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure IV-1), produced a unique band at ~7.2 kb (Figure V-5, Lane 10 and Lane 11), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88302 DNA digested with *Ase* I (Figure V-5, Lane 2 and Lane 6) or the combination of restriction enzymes *Sal* I and Sca I (Figure V-5, Lane 4 and Lane 8) and hybridized with Probe 5 produced no detectable bands.

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The results presented in Figure V-5 indicate that MON 88302 contains no detectable backbone sequences covered by Probe 5.

### V.B.3. Backbone Probe 6

Conventional control DNA digested with *Ase* I (Figure V-6, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure V-6, Lane 3 and Lane 7) and hybridized with Probe 6 (Figure IV-1) produced no detectable hybridization bands as expected for the negative control. Conventional control DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure IV-1), produced a unique band at ~7.2 kb (Figure V-6, Lane 10 and Lane 11), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88302 DNA digested with *Ase* I (Figure V-6, Lane 2 and Lane 6) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure V-6, Lane 4 and Lane 8) and hybridized with Probe 6 produced no detectable bands.

The results presented in Figure V-6 indicate that MON 88302 contains no detectable backbone sequences covered by Probe 6.

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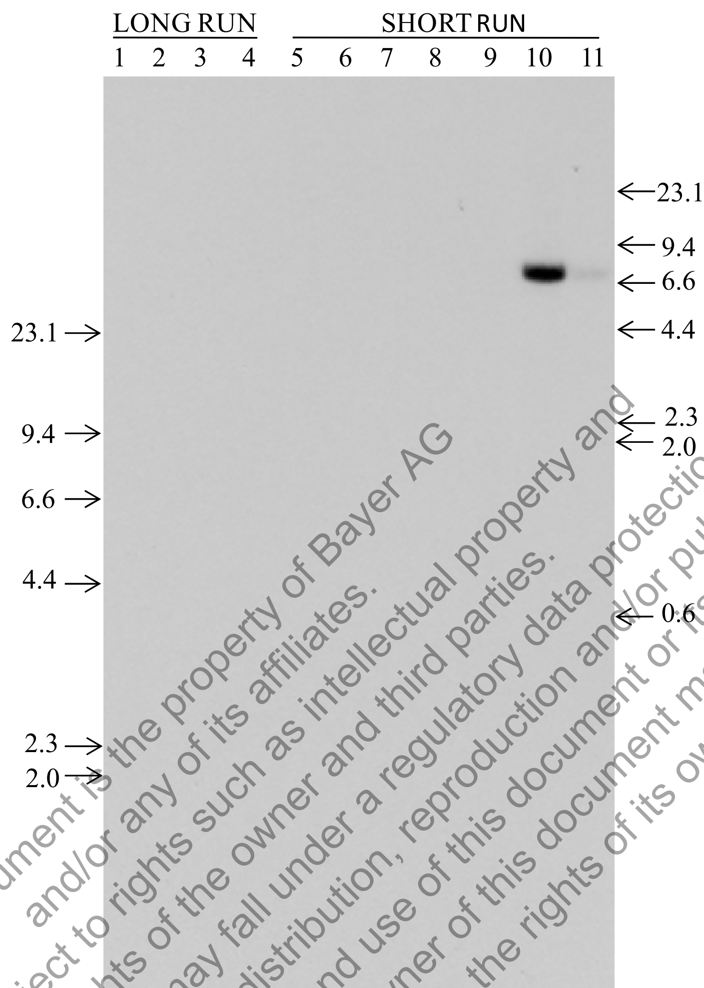


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**Figure V-4. Southern Blot Analysis to Determine the Presence or Absence of the PV-BNHT2672 Backbone Sequences in MON 88302: Probe 4**

The blot was hybridized with a $^{32}$P-labeled probe that spans a portion of the plasmid vector backbone sequences (Figure IV-1, Probe 4). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from λ DNA/*Hind* III Fragments on the ethidium bromide stained gel. Lane designations are as follows:

| Lane | |
|---|---|
| 1 | Conventional control (*Ase* I) |
| 2 | MON 88302 (*Ase* I) |
| 3 | Conventional control (*Sal* I/*Sca* I) |
| 4 | MON 88302 (*Sal* I/*Sca* I) |
| 5 | Conventional control (*Ase* I) |
| 6 | MON 88302 (*Ase* I) |
| 7 | Conventional control (*Sal* I/*Sca* I) |
| 8 | MON 88302 (*Sal* I/*Sca* I) |
| 9 | Blank |
| 10 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent] |
| 11 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~0.1 genome equivalent] |

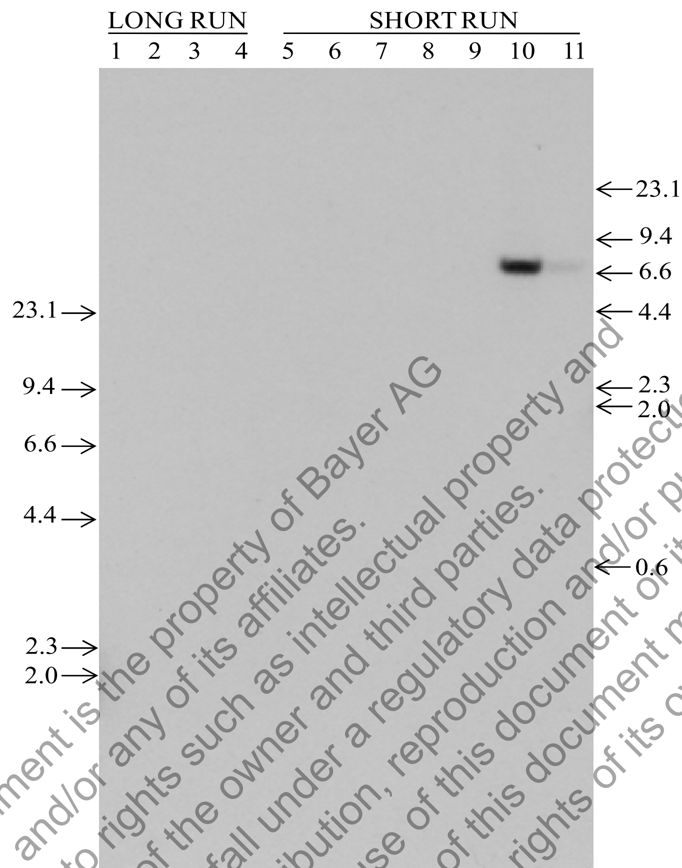


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**Figure V-5. Southern Blot Analysis to Determine the Presence or Absence of the PV-BNHT2672 Backbone Sequences in MON 88302: Probe 5**

The blot was hybridized with a $^{32}$P-labeled probe that spans a portion of the plasmid vector backbone sequences (Figure IV-1, Probe 5). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from λ DNA/*Hind* III Fragments ethidium bromide stained gel. Fragments on the Lane designations are as follows:

| **Lane** | |
|---|---|
| 1 | Conventional control (*Ase* I) |
| 2 | MON 88302 (*Ase* I) |
| 3 | Conventional control (*Sal* I/*Sca* I) |
| 4 | MON 88302 (*Sal* I/*Sca* I) |
| 5 | Conventional control (*Ase* I) |
| 6 | MON 88302 (*Ase* I) |
| 7 | Conventional control (*Sal* I/*Sca* I) |
| 8 | MON 88302 (*Sal* I/*Sca* I) |
| 9 | Blank |
| 10 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent] |
| 11 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~0.1 genome equivalent] |

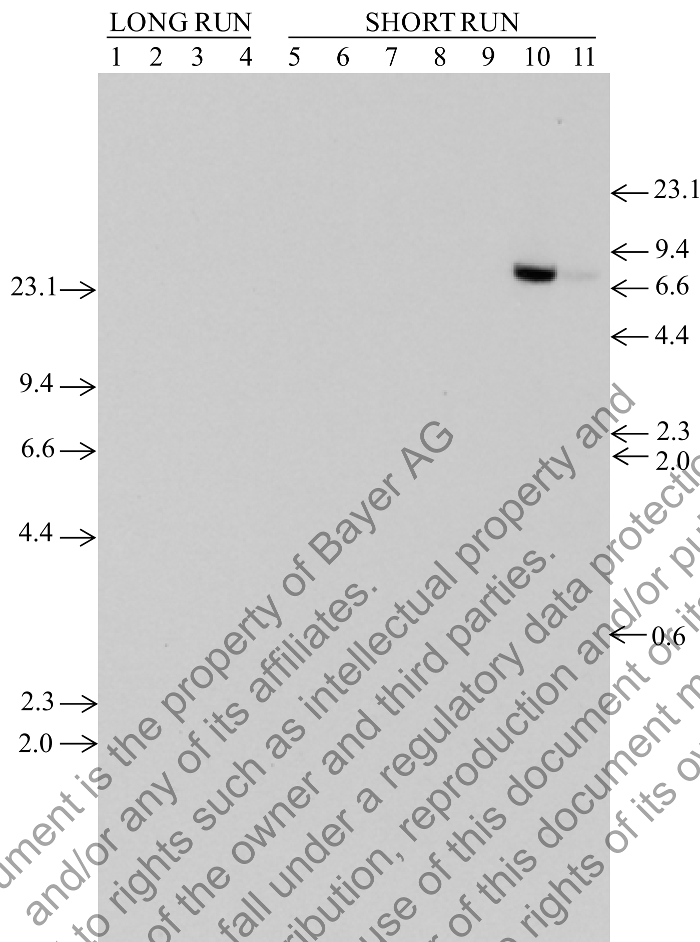

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**Figure V-6. Southern Blot Analysis to Determine the Presence or Absence of the PV-BNHT2672 Backbone Sequences in MON 88302: Probe 6**

The blot was hybridized with a $^{32}$P-labeled probe that spans a portion of the plasmid vector backbone sequences (Figure IV-1, Probe 6). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from λ DNA/*Hind* III Fragments on the ethidium bromide stained gel. Lane designations are as follows:

| **Lane** | |
|---|---|
| 1 | Conventional control (*Ase* I) |
| 2 | MON 88302 (*Ase* I) |
| 3 | Conventional control (*Sal* I/*Sca* I) |
| 4 | MON 88302 (*Sal* I/*Sca* I) |
| 5 | Conventional control (*Ase* I) |
| 6 | MON 88302 (*Ase* I) |
| 7 | Conventional control (*Sal* I/*Sca* I) |
| 8 | MON 88302 (*Sal* I/*Sca* I) |
| 9 | Blank |
| 10 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent] |
| 11 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~0.1 genome equivalent] |

## V.C. Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 88302

The organization and sequence of the elements within the MON 88302 insert was confirmed by DNA sequence analysis. PCR primers were designed with the intent to amplify two overlapping DNA amplicons that span the entire length of the insert and the associated DNA flanking the 5' and 3' ends of the insert (Figure V-7). The amplified PCR products were subjected to DNA sequence analyses. This analysis determined that the DNA sequence of the MON 88302 insert is 4428 bp long (Table V-2) and is identical to the corresponding T-DNA sequence of PV-BNHT2672 as described in Table IV-1.

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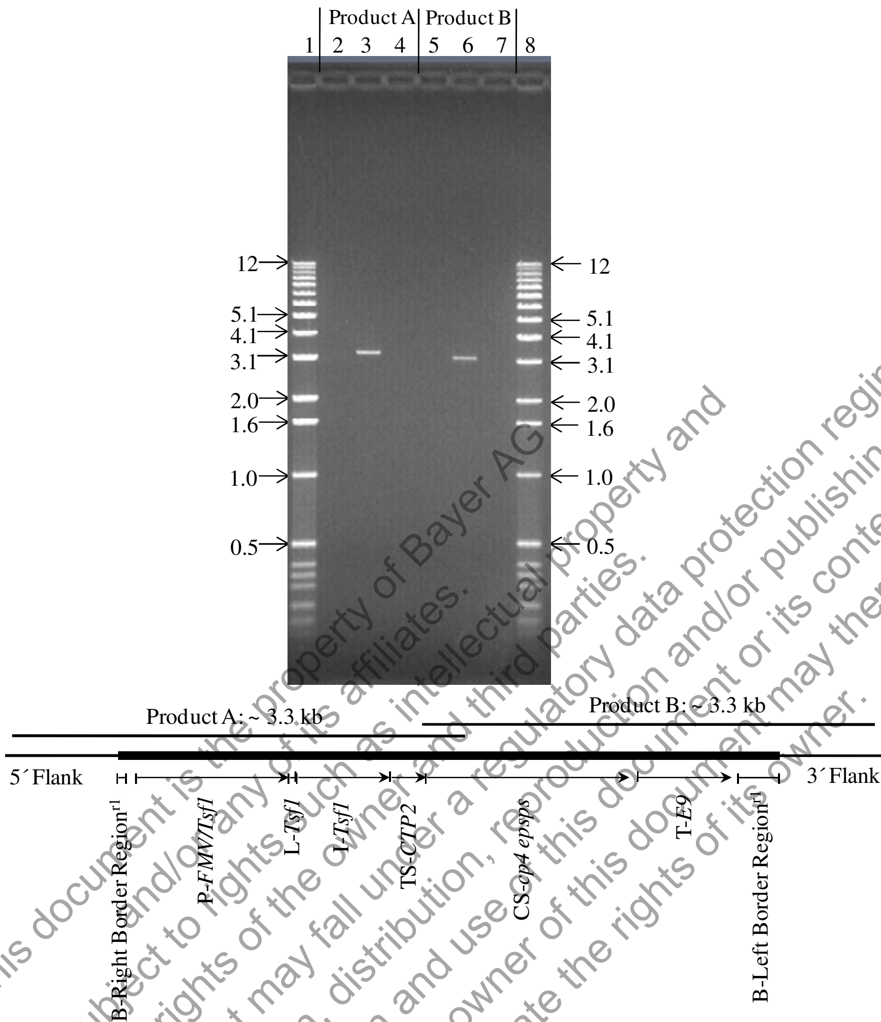


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**Figure V-7. Overlapping PCR Analysis across the Insert in MON 88302**

PCR was performed on both conventional control genomic DNA and MON 88302 genomic DNA using two pairs of primers to generate overlapping PCR fragments from MON 88302 for sequence analysis. Five microliters of each of the PCR reactions was loaded on the gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 88302 that appears at the bottom of the figure. Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Ladder on the ethidium bromide stained gel. Lane designations are as follows:

| Lane | |
|---|---|
| 1 | 1 Kb DNA Ladder |
| 2 | Conventional control |
| 3 | MON 88302 |
| 4 | No template DNA control |
| 5 | Conventional control |
| 6 | MON 88302 |
| 7 | No template DNA control |
| 8 | 1 Kb DNA Ladder |

### V.D. PCR and DNA Sequence Analyses to Examine the MON 88302 Insertion Site

PCR and sequence analyses were performed on genomic DNA extracted from MON 88302 and the conventional control to examine the MON 88302 insertion site. The PCR was performed with a forward primer specific to the genomic DNA sequence flanking the 5' end of the insert paired with a reverse primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure V-8). The amplified PCR product from the conventional control was subjected to DNA sequence analysis. Alignments between the conventional control sequence obtained from this analysis and the sequences immediately flanking the 5' and 3' end of the MON 88302 insert were separately performed to determine the integrity and genomic organization of the insertion site in MON 88302. From these alignment analyses, a 9 base pair insertion immediately adjacent to the 3' end of the MON 88302 insert and a 29 base pair deletion from the conventional genomic DNA were identified. Such changes are quite common during plant transformation; these changes presumably resulted from double-stranded break repair mechanisms in the plant during the *Agrobacterium*-mediated transformation process (Salomon and Puchta, 1998). A single nucleotide difference between the conventional control sequence and the genomic DNA sequence flanking the 3' end of the MON 88302 insert was also identified. The difference was most likely caused by a single nucleotide polymorphism (SNP) segregating in the canola population (Trick et al., 2009).

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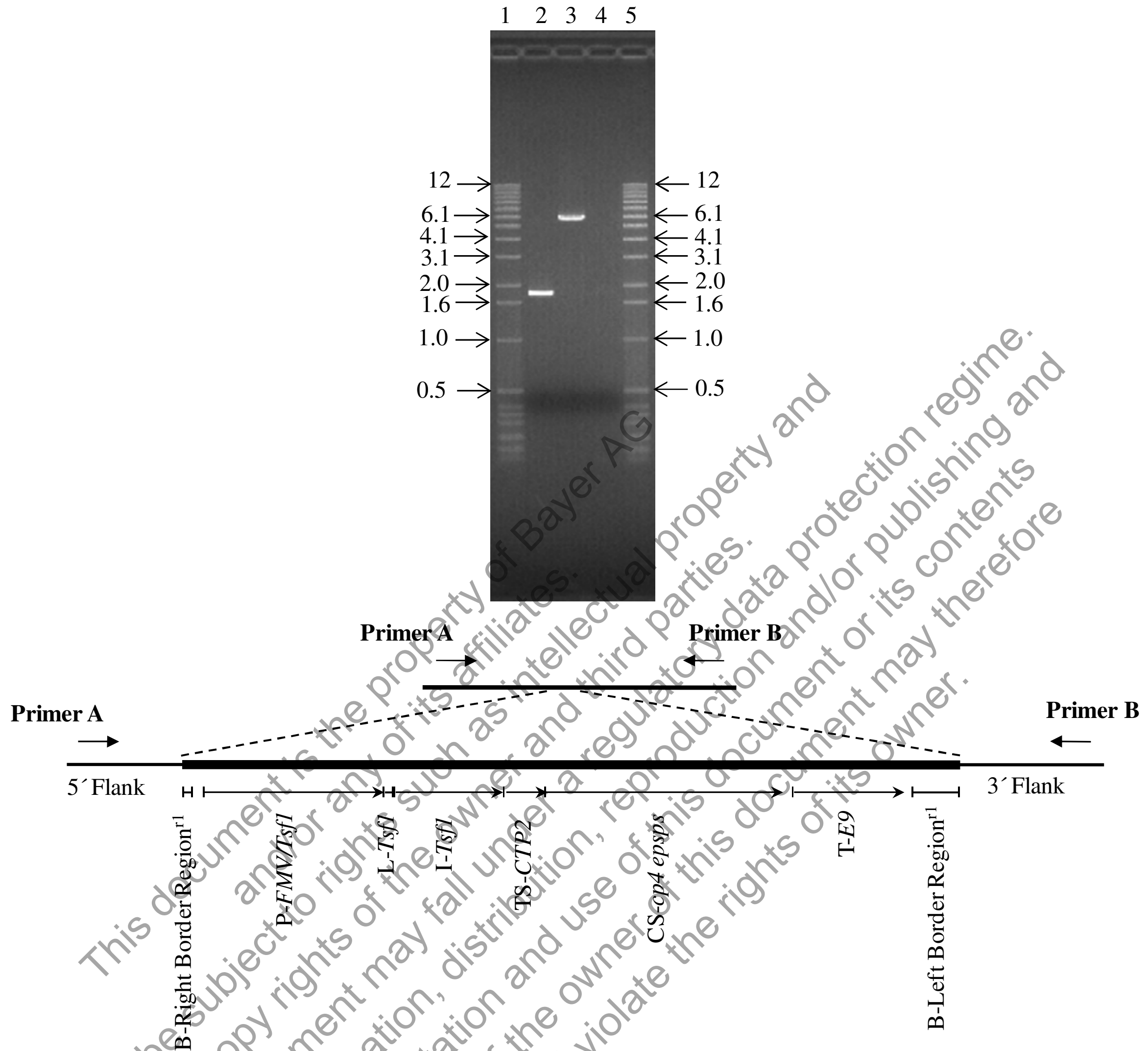


**Figure V-8. PCR Amplification of the MON 88302 Insertion Site in Conventional Control**

PCR was performed on both conventional control genomic DNA and MON 88302 genomic DNA, using Primer A specific to the 5' flanking sequence and Primer B specific to the 3' flanking sequence of the insert in MON 88302, to generate DNA fragments for sequence analysis. The insertion site in conventional control (top) and MON 88302 (bottom) are illustrated at the bottom of the figure. Five microliters of each of the PCR reactions were loaded on the gel. Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from 1Kb DNA Ladder on the ethidium bromide stained gel. Lane designations are as follows:

| **Lane** | |
|---|---|
| 1 | 1 Kb DNA Ladder |
| 2 | Conventional control |
| 3 | MON 88302 |
| 4 | No template DNA control |
| 5 | 1 Kb DNA Ladder |

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## V.E. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 88302

In order to demonstrate the stability of the insert in MON 88302, Southern blot analysis was performed using genomic DNA extracted from leaf tissues from four breeding generations of MON 88302. For reference, the breeding history of MON 88302 is presented in Figure V-9. The specific generations tested are indicated in the legend of Figure V-9. The $R_3$ generation was used for the molecular characterization analyses shown in Figure V-2 through Figure V-6. To analyze insert stability, four samples from three additional generations of MON 88302 were evaluated by Southern blot analysis and compared to the $R_3$ generation. Genomic DNA, isolated from each of the selected generations of MON 88302, was digested with the restriction enzyme *Ase* I and simultaneously hybridized with Probe 1 and Probe 3 (Figures IV-1 and V-1), which was designed to detect both fragments generated by the *Ase* I digest. Any instability associated with the insert would be detected as extra bands within the fingerprint on the Southern blot. The Southern blot has the same controls as described in Section V.A.1.

### V.E.1. T-DNA Probes 1 and 3

Conventional control genomic DNA digested with restriction enzyme *Ase* I and simultaneously hybridized with Probe 1 and Probe 3 (Figures IV-1 and V-1) produced no hybridization signals (Figure V-10, Lane 1) as expected for the negative control. Conventional control genomic DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure IV-1 and Table V-1), produced the expected bands at ~2.5 kb and ~7.2 kb (Figure V-10, Lane 8). Conventional control genomic DNA digested with *Ase* I and spiked with probe templates of Probe 1 and Probe 3 produced the expected bands at ~2.3 kb and ~1.3 kb (Figure V-10, Lane 9 and Lane 10). Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 88302 genomic DNA digested with *Ase* I and hybridized with Probe 1 and Probe 3 (Figures IV-1 and V-1) is expected to produce a Southern fingerprint with two bands at ~3.8 kb and ~1.4 kb (Figure V-1 and Table V-1). Southern fingerprints produced from multiple generations (Figure V-10, Lane 2, Lane 4, Lane 5, and Lane 6), of MON 88302 are consistent with the one produced from the fully characterized generation $R_3$ (Figure V-2, Lane 2 and Lane 6, and Figure V-10, Lane 3), indicating that MON 88302 contains one copy of the T-DNA insert that is stable across multiple generations.

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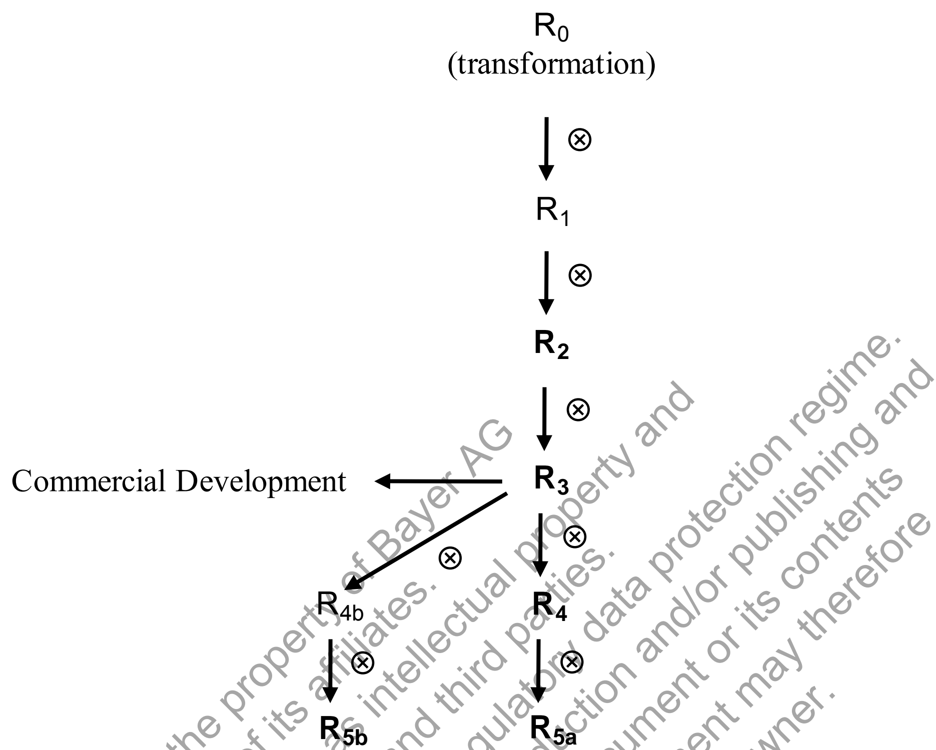


$R_0$- Originally transformed plant ; ⊗-self pollination

**Figure V-9. Breeding History of MON 88302**

$R_0$ corresponds to the transformed canola plant. All generations were self pollinated. ⊗ designates self-pollination. The $R_3$ generation was used for the molecular characterization and commercial development of MON 88302. The $R_2$, $R_3$, $R_4$, $R_{5a}$, and $R_{5b}$ (bolded in the breeding tree) generations of MON 88302 were used for analyzing the stability of the insert across generations. $R_{5b}$ was propagated independently of $R_{5a}$ beginning with the $R_3$ generation.

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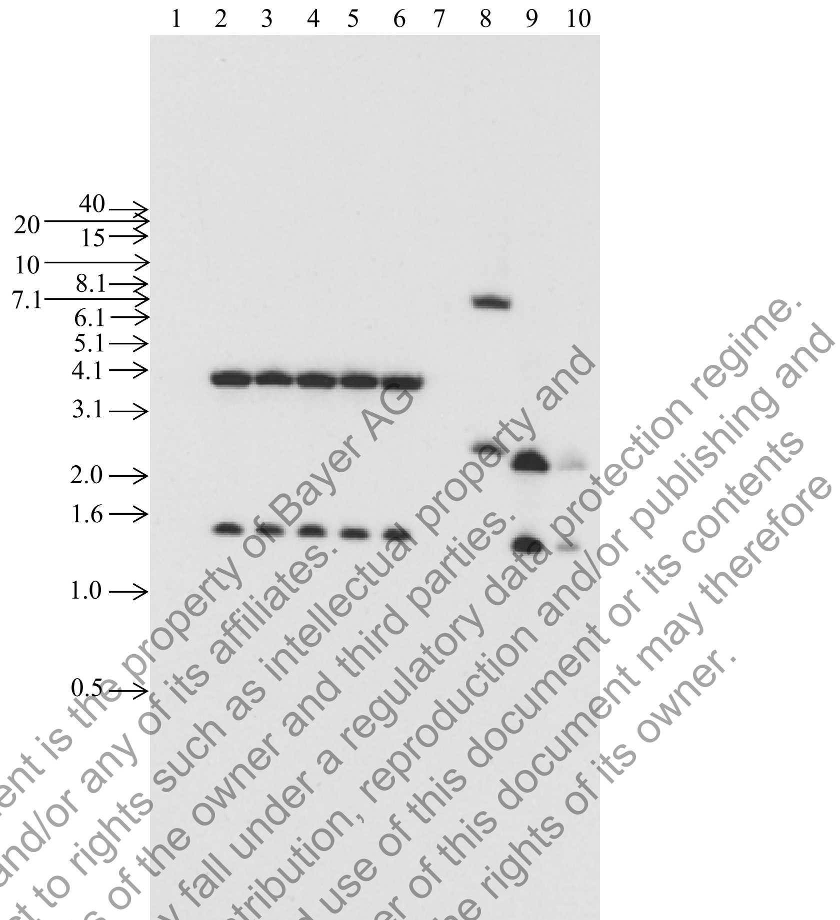


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**Figure V-10. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 88302: Probes 1 and 3**

The blot was simultaneously hybridized with two $^{32}$P-labeled probes that span a portion of the T-DNA sequence (Figure IV-1, Probe 1 and Probe 3). Each lane contains ~10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

| **Lane** | |
|---|---|
| 1 | Conventional control (*Ase* I) |
| 2 | $R_2$ generation of MON 88302 (*Ase* I) |
| 3 | $R_3$ generation of MON 88302 (*Ase* I) |
| 4 | $R_4$ generation of MON 88302 (*Ase* I) |
| 5 | $R_{5a}$ generation of MON 88302 (*Ase* I) |
| 6 | $R_{5b}$ generation of MON 88302 (*Ase* I) |
| 7 | Blank |
| 8 | Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent] |
| 9 | Conventional control (*Ase* I) spiked with probe templates Probe 1 and Probe 3 [~1 genome equivalent] |
| 10 | Conventional control (*Ase* I) spiked with probe templates Probe 1 and Probe 3 [~0.1 genome equivalent] |

### V.F. Inheritance of the Genetic Insert in MON 88302

During development of MON 88302, segregation data were recorded to assess the inheritance and stability of the coding sequence present in MON 88302. Chi-square ($\chi^2$) analysis was performed over several generations to confirm the segregation and stability of the MON 88302 insert. The $\chi^2$ analysis is based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 88302 breeding path for generating segregation data is described in Figure V-11. The transformed $R_0$ plant was self-pollinated to generate $R_1$ seed. From the $R_1$ segregating population, an individual plant homozygous for the *cp4 epsps* coding sequence (subsequently designated MON 88302) was identified via TaqMan PCR copy number assay and Southern blot copy number analysis. The *cp4 epsps* homozygous $R_1$ plant was self-pollinated to give rise to $R_2$ plants that were self-pollinated to produce $R_3$ seed. At each generation, the homozygous plants were tested for the expected segregation pattern of 1:0 (positive: negative) for the *cp4 epsps* gene using a glyphosate spray test and/or TaqMan PCR assay.

An individual *cp4 epsps* positive $R_3$ plant, which was confirmed by Endpoint TaqMan PCR assay, was crossed to a Monsanto proprietary canola inbred, which does not contain the MON 88302 insert, via traditional breeding techniques to produce hemizygous $F_1$ seed. The resulting $F_1$ plant was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR, and was then self-pollinated to produce $F_2$ seed. A *cp4 epsps* hemizygous $F_2$ plant from the $F_2$ population was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR and was then self-pollinated to produce the $F_3$ population. A *cp4 epsps* hemizygous $F_3$ plant from the $F_3$ population was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR and was self-pollinated to produce the $F_4$ population. The copy number of the *cp4 epsps* gene in the $F_2$, $F_3$, and $F_4$ populations was then assessed using a real-time TaqMan PCR assay.

A $\chi^2$ analysis was performed on each of the $F_2$, $F_3$, and $F_4$ populations using the statistical program R (Version 2.10.1) to compare the observed segregation ratio of *cp4 epsps* coding sequence to the expected ratio according to Mendelian principles of inheritance. The Chi-square was calculated as:

$$\chi^2 = \sum [(o - e)^2 / e]$$

where o = observed frequency of the genotype or phenotype and e = expected frequency of the genotype or phenotype. The level of statistical significance was predetermined to be 5% ($\alpha = 0.05$).

The results of the $\chi^2$ analysis of the MON 88302 segregating progeny are presented in Table V-3. The $\chi^2$ value in the $F_2$, $F_3$, and $F_4$ populations indicated no statistically significant difference between the observed and expected 1:2:1 segregation ratio

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(homozygous positive: hemizygous: homozygous negative) of *cp4 epsps* coding sequence. These results support the conclusion that the *cp4 epsps* expression cassette in MON 88302 resides at a single locus within the canola genome and is inherited according to Mendelian principles of inheritance. These results are also consistent with the molecular characterization data indicating that MON 88302 contains a single, intact copy of the *cp4 epsps* expression cassette inserted at a single locus in the canola genome.

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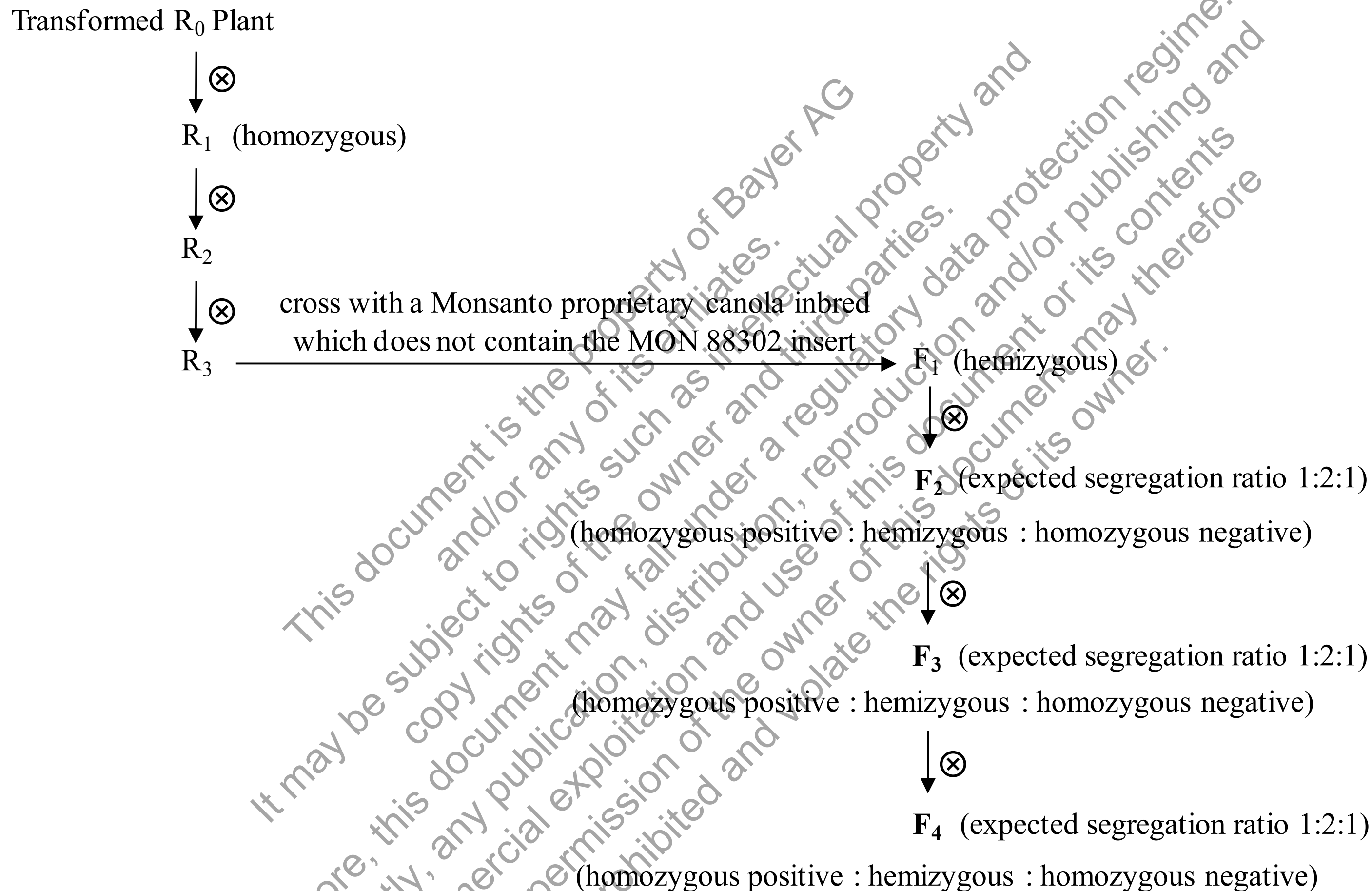


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**Figure V-11. Breeding Path for Generating Segregation Data for MON 88302**

An individual hemizygous plant from each of the $F_1$, $F_2$, and $F_3$ populations was self-pollinated to produce the population of the next generation. Chi-square analyses were conducted on segregation data from the $F_2$, $F_3$, and $F_4$ populations.

**Table V-3. Segregation of the *cp4 epsps* Gene During the Development of MON 88302**

| | | | | | 1:2:1 Segregation | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Generation | Total Plants* | Observed # Plants Homozygous Positive | Observed # Plants Hemizygous | Observed # Plants Homozygous Negative | Expected # Plants Homozygous Positive | Expected # Plants Hemizygous | Expected # Plants Homozygous Negative | $\chi^2$ | Probability |
| $F_2$ | 220 | 51 | 122 | 47 | 55.00 | 110.00 | 55.00 | 2.76 | 0.2511 |
| $F_3$ | 166 | 39 | 94 | 33 | 41.50 | 83.00 | 41.50 | 3.35 | 0.1874 |
| $F_4$ | 198 | 53 | 97 | 48 | 49.50 | 99.00 | 49.50 | 0.33 | 0.8465 |

*Plants were evaluated for the copy number of the *cp4 epsps* gene using a real-time TaqMan PCR assay.

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## V.G. Characterization of the Genetic Modification Summary and Conclusion

Molecular characterization of MON 88302 by Southern blot analyses demonstrated that the T-DNA was inserted into the canola genome at a single locus containing one copy of the *cp4 epsps* expression cassette. No additional elements were detected other than those associated with the insert. Moreover, no plasmid backbone sequences were detected in the genome of MON 88302.

DNA sequence analyses performed on MON 88302 determined the complete DNA sequence of the insert in MON 88302, confirmed the predicted organization of the genetic elements within the insert, determined the sequences flanking the insert, and examined the MON 88302 insertion site. Sequence analysis of the T-DNA insertion site indicated that a 9 base pair insertion immediately adjacent to the 3' end of the MON 88302 insert and a 29 base pair deletion from the conventional genomic DNA occurred during the insertion of the T-DNA into the conventional canola to form MON 88302. In addition, a single nucleotide difference between the conventional control sequence and the known DNA sequence flanking the 3' end of the MON 88302 insert was also identified. This single nucleotide difference was most likely caused by single nucleotide polymorphism (SNP) segregating in the canola population (Trick et al., 2009).

Southern blot analysis of multiple MON 88302 generations demonstrated that the inserted DNA has been stably maintained through multiple generations of breeding, thereby, confirming the stability of the insert. Results from segregation analyses show inheritance and stability of the insert was as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA in MON 88302 at a single chromosomal locus.

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## VI. SAFETY ASSESSMENT OF EXPRESSED PRODUCT

Numerous Roundup Ready crops including Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready corn 2, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton and Roundup Ready alfalfa that produce the CP4 EPSPS protein have been reviewed by the FDA. The CP4 EPSPS protein expressed in MON 88302 is identical to the CP4 EPSPS in other Roundup Ready crops. Further, Harrison et al. (1996) has published on the safety of CP4 EPSPS proteins present in biotechnology-derived crops, and concluded that CP4 EPSPS does not pose any important concerns from an allergenicity or toxicity perspective.

A multistep approach to the safety assessment was conducted according to guidelines established by the Codex Alimentarius Commission and OECD and which embody the principles and guidance of the FDA's 1992 policy on foods from new plant varieties. This approach was used to characterize the CP4 EPSPS protein present in MON 88302 as a result of the genetic modification. These steps include: 1) documentation of the history of safe use of the CP4 EPSPS protein and its homology with proteins that lack adverse effects on human or animal health; 2) characterization of the physicochemical and functional properties of CP4 EPSPS; 3) quantification of CP4 EPSPS expression in plant tissues; 4) examination of the similarity of CP4 EPSPS to known allergens; 5) evaluation of the digestibility of CP4 EPSPS in simulated gastrointestinal fluids; 6) evaluation of the stability of the CP4 EPSPS protein in response to typical food/feed preparation conditions such as heat treatment; 7) examination of the similarity of CP4 EPSPS to known toxins or other biologically active proteins known to have adverse effects on mammals; 8) investigation of potential mammalian toxicity through an animal assay and calculating margins of exposure; and 9) examination of the similarity of putative polypeptides encoded by the insert and flanking sequences to known allergens, toxins, or other biologically active proteins known to have adverse effects on mammals. Additionally, this Section includes a stepwise approach to assess the potential allergenicity for the newly expressed protein (Codex Alimentarius, 2009). The safety assessment supports the conclusion that dietary exposure to CP4 EPSPS protein derived from MON 88302 poses no meaningful risk to human or animal health.

The purified CP4 EPSPS protein produced in MON 88302 was characterized to demonstrate the equivalence between MON 88302- and *E. coli*-produced CP4 EPSPS proteins. The MON 88302-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein purified from both sources was shown to be biochemically, structurally, and functionally equivalent. As the *E. coli*-produced CP4 EPSPS protein has been used previously in a number of safety assessment studies, including the simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and acute mouse gavage studies (Harrison et al., 1996), demonstration of protein equivalence between *E. coli*- and MON 88302-produced CP4 EPSPS proteins allows utilization of the existing data to confirm the safety of the CP4 EPSPS protein in MON 88302.

The information provided in this section also addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 34, 35, 36, 37, 38, 39, and 40 for assessment of

possible toxicity and paragraphs 41, 42, and 43 and Annex 1 for assessment of possible allergenicity (Codex Alimentarius, 2009).

## VI.A. Mode-of-Action of CP4 EPSPS Protein

The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) family of enzymes is found in plants and microorganisms and their properties have been extensively studied (Klee et al., 1987; Schönbrunn et al., 2001; Steinrücken and Amrhein, 1984). EPSPS enzymes generally have a molecular weight of 44-51 kDa and are mono-functional (Franz et al., 1997; Kishore et al., 1988). They catalyze one of the steps in the shikimate pathway for the biosynthesis of aromatic amino acids (phenylalanine, tryptophan and tyrosine) as well as other aromatic molecules and are the target of the broad spectrum herbicide, glyphosate. Specifically, EPSPS enzymes catalyze the transfer of the enolpyruvyl group from phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (EPSP) (Alibhai and Stallings, 2001.

The EPSPS transgene (*cp4 epsps*) in MON 88302 is derived from *Agrobacterium* sp. strain CP4. It encodes a 47.6 kDa EPSPS protein that consists of a single polypeptide of 455 amino acids and is functionally identical to endogenous plant EPSPS enzymes, but has a reduced affinity for glyphosate relative to endogenous plant EPSPS (Padgette et al., 1996). In conventional plants, glyphosate binds to the endogenous EPSPS enzyme and blocks the biosynthesis of EPSP thereby depriving the plant of essential amino acids (Steinrücken and Amrhein, 1980). In Roundup Ready plants, requirements for aromatic amino acids and other metabolites are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette et al., 1996).

## VI.B. History of Safe Use of CP4 EPSPS Protein

EPSPS enzymes are ubiquitous in plants and microorganisms and have been isolated from both sources (Harrison et al., 1996; Haslam, 1993; Klee et al., 1987; Schönbrunn et al., 2001; Steinrücken and Amrhein, 1984). While the shikimate pathway and the EPSPS enzyme are absent in mammals, fish, birds, reptiles, and insects (Alibhai and Stallings, 2001), the EPSPS enzyme and its activity are found widely in food and feed derived from plant and microbial sources. Genes for EPSPSs from numerous sources have been cloned (Padgette et al., 1996), and the expressed catalytic domains of this group of proteins are highly conserved. Bacterial EPSPS enzymes have been well characterized with respect to their three dimensional crystal structures (Stallings et al., 1991) as well as their kinetic and chemical mechanisms (Anderson and Johnson, 1990). The CP4 EPSPS protein thus represents one of many different EPSPSs found in nature; the CP4 EPSPS and native plant EPSPS enzymes are functionally equivalent except for their tolerance to glyphosate (Padgette et al., 1996).

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by the FDA including Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton, and Roundup Ready alfalfa. The CP4 EPSPS protein expressed in

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MON 88302 is identical to the CP4 EPSPS proteins in other Roundup Ready crops. Results from the protein characterization studies included in this petition confirmed the identity of the MON 88302-produced CP4 EPSPS protein and established the equivalence of MON 88302-produced protein to the *E. coli*-produced CP4 EPSPS protein (Section VI.C.) used previously to demonstrate the safety of the CP4 EPSPS protein produced in other Roundup Ready crops. The Environmental Protection Agency (EPA) also reviewed the safety of the CP4 EPSPS protein and has established a tolerance exemption for the protein and the genetic material necessary for its production either in or on all raw agricultural commodities (U.S. EPA, 1996). This exemption was based on a safety assessment that included rapid digestion in simulated gastric fluids, lack of significant homology to known toxins and known allergens, and lack of toxicity in an acute oral mouse gavage study. The history of safe use of CP4 EPSPS is supported by the lack of any documented reports of adverse effects of this protein since the introduction of Roundup Ready crops in 1996.

## VI.C. Characterization of the CP4 EPSPS Protein from MON 88302

### VI.C.1. CP4 EPSPS Protein Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of the protein(s) produced from the inserted DNA, and confirmation of the safety of the protein(s). The safety of *E. coli*-produced CP4 EPSPS protein has been assessed previously and the results are summarized by Harrison et al. (1996). For the existing CP4 EPSPS safety data set to be applied to CP4 EPSPS protein produced in MON 88302, the equivalence of the plant- and *E. coli*-produced protein was established. The equivalence of the plant- and *E. coli*-produced CP4 EPSPS proteins has been established previously for Roundup Ready crops such as soybean, corn, canola, sugar beet, alfalfa and cotton. To assess the equivalence between MON 88302-produced and *E. coli*-produced CP4 EPSPS protein, a small quantity of the CP4 EPSPS protein was purified from MON 88302 seed. The MON 88302-produced CP4 EPSPS protein was characterized and the equivalence of the physicochemical characteristics and functional activity between the MON 88302-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein was assessed using a panel of analytical tests, including: 1) N-terminal sequence analysis of the MON 88302-produced CP4 EPSPS protein to establish identity, 2) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of peptides derived from tryptic digested MON 88302-produced CP4 EPSPS protein to establish identity, 3) western blot analysis using anti-CP4 EPSPS polyclonal antibodies to establish identity and immunoreactive equivalence between MON 88302-produced protein and the *E. coli*-produced protein, 4) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to establish equivalence of the apparent molecular weight between MON 88302-produced protein and the *E. coli*-produced protein, 5) glycosylation analysis of the MON 88302-produced CP4 EPSPS protein to establish the equivalence between the MON 88302-produced and *E. coli*-produced CP4 EPSPS proteins, and 6) CP4 EPSPS enzymatic activity analysis to demonstrate functional equivalence between MON 88302-produced and the *E. coli*-produced protein. The details of the materials and methods for the panel of analytical tests used to evaluate and compare the

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properties of the MON 88302- and *E. coli*-produced proteins are described in Appendix B. A summary of the data obtained to support a conclusion of protein equivalence is below.

A comparison of the MON 88302-produced CP4 EPSPS to the *E. coli*-produced CP4 EPSPS protein confirmed the identity of the MON 88302-produced CP4 EPSPS protein and established the equivalence of the two proteins. The identity of the CP4 EPSPS protein isolated from the seed of MON 88302 was confirmed by N-terminal sequencing, MALDI-TOF MS analysis of peptides produced after trypsin digestion, and by western blot analysis using anti-CP4 EPSPS polyclonal antibodies. The N-terminus of the MON 88302-produced CP4 EPSPS protein matched the predicted amino acid sequence translated from the *cp4 epsps* coding sequence. The MALDI-TOF MS analysis yielded peptide masses consistent with the expected peptide masses from the translated *cp4 epsps* coding sequence. The MON 88302-produced CP4 EPSPS protein was detected on a western blot probed with antibodies specific for CP4 EPSPS protein. Furthermore, the immunoreactive properties and electrophoretic mobility of the MON 88302-produced CP4 EPSPS protein were shown to be equivalent to those of the *E. coli*-produced CP4 EPSPS protein by immunoblot. The apparent molecular weight, glycosylation status, and functional activity of the MON 88302-produced CP4 EPSPS protein and *E. coli*-produced CP4 EPSPS protein were also all found to be equivalent. Taken together, these data provide a detailed characterization of the CP4 EPSPS protein isolated from MON 88302 and establish its equivalence to the *E. coli*-produced CP4 EPSPS protein. Furthermore, because CP4 EPSPS proteins isolated from other Roundup Ready crops have been demonstrated previously to be equivalent to the *E. coli*-produced CP4 EPSPS protein, by inference, the MON 88302-produced CP4 EPSPS protein is equivalent to the CP4 EPSPS proteins expressed in other Roundup Ready crops, all of which have been the subject of consultations with the FDA.

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### VI.C.1.1 Results of the N-terminal Sequence Analysis

N-terminal sequencing of the first 15 amino acids was performed on MON 88302-produced CP4 EPSPS protein. The expected sequence for the CP4 EPSPS protein deduced from the *cp4 epsps* gene present in MON 88302 was observed. The data obtained correspond to the deduced CP4 EPSPS protein beginning at amino acid positions 2 and 4 (Figure VI-1, Experimental Sequence 1 and 2, respectively). Hence, the sequence information confirms the identity of the CP4 EPSPS protein isolated from the seed of MON 88302.

| Amino acid residue # from the N-terminus | → | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Expected Sequence | → | M | L | H | G | A | S | S | R | P | A | T | A | R | K | S | S | G | L |
| Experimental Sequence 1 | → | - | L | H | G | A | X | X | X | P | A | T | X | X | X | X | X | ^ | ^ |
| Experimental Sequence 2 | → | - | - | - | G | A | S | X | R | P | A | T | A | X | K | S | X | G | X |

**Figure VI-1. N-Terminal Sequence of the MON 88302 CP4 EPSPS Protein**
The expected amino acid sequence of the N-terminus of CP4 EPSPS protein was deduced from the *cp4 epsps* coding region present in MON 88302. The experimental sequences obtained from the MON 88302-produced CP4 EPSPS protein were compared to the expected sequence. The single letter IUPAC-IUB amino acid code is M, methionine; L, leucine; H, histidine; G, glycine; A, alanine, S, serine; R, arginine; P, proline; T, threonine; K. lysine; (X) indicates that the residue was not identifiable; (-) indicates the residue was not observed; (^) indicates not done, i.e., sequencing cycle was not conducted.

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### VI.C.1.2. Results of MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 88302-produced CP4 EPSPS protein was also confirmed by MALDI-TOF MS analysis of peptide fragments produced from tryptic digestion of the MON 88302-produced CP4 EPSPS protein. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen et al., 1997).

There were 34 unique peptides identified that corresponded to the masses (Table VI-1) expected to be produced by tryptic digestion of the CP4 EPSPS protein. The identified masses were used to assemble a coverage map of the entire CP4 EPSPS protein (Figure VI-2). The experimentally determined mass coverage of the CP4 EPSPS protein was 85.5% (389 out of 455 amino acids). This analysis serves as additional identity confirmation for the MON 88302-produced CP4 EPSPS protein.

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**Table VI-1. Summary of the Tryptic Masses Identified for MON 88302-Produced CP4 EPSPS Protein Using MALDI-TOF MS**

| α-Cyano | α-Cyano | DHB | DHB | Sinapinic acid | Sinapinic acid | Expected Mass[1] | Diff[2] | Position[3] | Sequence |
|---|---|---|---|---|---|---|---|---|---|
| Extract 1 | Extract 2 | Extract 1 | Extract 2 | Extract 1 | Extract 2 | | | | |
| | | 389.28 | | | | 389.25 | 0.03 | 225-227 | TIR |
| | | 474.32 | | | | 474.27 | 0.05 | 228-231 | LEGR |
| 506.29 | | | | | | 506.22 | 0.07 | 354-357 | ESDR |
| | | 529.36 | | | | 529.30 | 0.06 | 24-28 | IPGDK |
| 599.43 | 599.51 | 599.41 | | | | 599.33 | 0.10 | 29-33 | SISHR |
| 616.44 | 616.48 | 616.41 | | 616.24 | | 616.34 | 0.10 | 128-132 | RPMGR |
| 629.44 | | 629.45 | | | | 629.29 | 0.15 | 201-205 | DHTEK |
| 629.44 | | 629.45 | | | | 629.34 | 0.10 | 383-388 | GRPDGK |
| 711.57 | 711.62 | 711.56 | 711.61 | | | 711.45 | 0.12 | 133-138 | VLNPLR |
| | | 790.55 | | | | 790.48 | 0.07 | 306-312 | VRSSTLK |
| | | 790.55 | | | | 790.41 | 0.14 | 139-145 | EMGVQVK |
| | | 805.54 | | | | 805.43 | 0.11 | 447-453 | IELSDTK |
| 835.54 | 835.58 | 835.53 | | | | 835.39 | 0.15 | 62-69 | AMQAMGAR |
| 863.61 | 863.68 | 863.60 | | | | 863.46 | 0.15 | 15-23 | SSGLSGTVR |
| 872.61 | 872.66 | 872.61 | 872.67 | 872.53 | | 872.45 | 0.16 | 313-320 | GVTVPEDR |
| 872.61 | 872.66 | 872.61 | 872.67 | 872.53 | | 872.52 | 0.09 | 358-366 | LSAVANGLK |
| | | 930.66 | | | | 930.51 | 0.15 | 169-177 | VPMASAQVK |
| 948.68 | 948.74 | 948.68 | 948.75 | | | 948.52 | 0.16 | 161-168 | TPTPITYR |
| 991.72 | | 991.71 | | | | 991.55 | 0.17 | 14-23 | KSSGLSGTVR |
| 1115.75 | 1115.83 | 1115.77 | 1115.86 | 1115.69 | | 1115.57 | 0.18 | 295-305 | LAGGEDVADLR |
| 1357.94 | 1358.01 | 1357.97 | 1358.05 | 1357.89 | | 1357.71 | 0.23 | 146-157 | SEDGDRLPVTLR |
| 1359.88 | 1359.96 | 1359.91 | 1360.00 | 1359.81 | 1359.87 | 1359.72 | 0.16 | 354-366 | ESDRLSAVANGLK |
| 1359.88 | 1359.96 | 1359.91 | 1360.00 | 1359.81 | 1359.87 | 1359.64 | 0.24 | 34-46 | SFMFGGLASGETR |
| 1559.11 | 1559.18 | 1559.13 | 1559.01 | | | 1558.83 | 0.28 | 47-61 | ITGLLEGEDVINTGK |
| 1647.10 | 1647.24 | 1647.16 | 1647.24 | | | 1646.84 | 0.26 | 389-405 | GLGNASGAAVATHLDHR |
| 1764.10 | 1764.26 | 1764.16 | | 1764.06 | | 1763.81 | 0.29 | 367-382 | LNGVDCDEGETSLVVR |
| 1994.31 | 1994.43 | 1994.35 | 1994.55 | 1994.21 | 1994.35 | 1993.97 | 0.34 | 206-224 | MLQGFGANLTVETDADGVR |
| 2183.54 | 2183.67 | 2183.57 | 2183.80 | 2183.45 | 2183.53 | 2183.17 | 0.37 | 275-294 | TGLILTLQEMGADIEVINPR |
| 2367.73 | 2367.87 | 2367.77 | 2367.85 | 2367.65 | 2367.8 | 2367.33 | 0.40 | 178-200 | SAVLLAGLNTPGITTVIEPIMTR |
| 2450.65 | 2450.83 | 2450.80 | | 2450.51 | 2450.6 | 2450.23 | 0.42 | 24-46 | IPGDKSISHRSFMFGGLASGETR |
| 2450.65 | 2450.83 | 2450.80 | | 2450.51 | 2450.6 | 2450.22 | 0.43 | 105-127 | LTMGLVGVYDFDSTFIGDASLTK |
| 3247.10 (Ave) | 3247.05 (Ave) | | | 3246.89 (Ave) | 3246.97 (Ave) | 3246.54 (Ave) | 0.56 | 73-104 | EGDTWIIDGVGNGGLLAPEAPLDFGNAATGCR |
| 3251.94 (Ave) | 3252.18 (Ave) | 3252.06 (Ave) | 3253.42 (Ave) | 3252.58 (Ave) | 3252.04 (Ave) | 3251.75 (Ave) | 0.19 | 321-351 | APSMIDEYPILAVAAAFAEGATVMNGLEELR |
| | | 4191.34 (Ave) | 4191.48 (Ave) | 4191.89 (Ave) | 4191.63 (Ave) | 4190.89 (Ave) | 0.37 | 234-274 | LTGQVIDVPGDPSSTAFPLVAALLVPGSDVTILNVLMNPTR |

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The difference between the expected mass and the first column mass. Other masses shown within a row are also within 1 Da of the expected mass.
[3]Position refers to amino acid residues within the predicted CP4 EPSPS sequence as depicted in Figure VI-2.
DHB = 5-dihydroxybenzoic acid matrix, α-cyano = α-cyano-4-hydroxycinnamic acid matrix; Sinapinic acid = 3, 5-dimethoxy-4-hydroxycinnamic acid matrix; Ave = experimental mass average (for large peptides the monoisotopic mass is poorly resolved, therefore the mass average value is used for comparison), see Appendix B for details.

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```
001  MLHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
051  LEGEDVINTG KAMQAMGARI RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101  TGCRLTMGLV GVYDFDSTFI GDASLTKRPM GRVLNPLREM GVQVKSEDGD
151  RLPVTLRGPK TPTPITYRVP MASAQVKSAV LLAGLNTPGI TTVIEPIMTR
201  DHTEKMLQGF GANLTVETDA DGVRTIRLEG RGKLTGQVID VPGDPSSTAF
251  PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED
301  VADLRVRSST LKGVTVPEDR APSMIDEYPI LAVAAAFAEG ATVMNGLEEL
351  RVKESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401  HLDHRIAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451  DTKAA
```

**Figure VI-2. MALDI-TOF MS Coverage Map of the MON 88302-produced CP4 EPSPS Protein**

The amino acid sequence of the mature CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in MON 88302. Boxed regions correspond to regions covered by tryptic peptides that were identified from the MON 88302-produced CP4 EPSPS protein sample using MALDI-TOF MS. In total, 85.5% (389 of 455 total amino acids) of the expected protein sequence was covered by the identified peptides.

### VI.C.1.3. Results of Western Blot Analysis of the CP4 EPSPS Protein Isolated from the Seed of MON 88302 and Immunoreactivity Comparison to *E. coli*-produced CP4 EPSPS

Western blot analysis was conducted using goat anti-CP4 EPSPS polyclonal antibody to 1) confirm the identity of the CP4 EPSPS protein isolated from the seed of MON 88302 and 2) to determine the relative immunoreactivity of the MON 88302- and the *E. coli*-produced CP4 EPSPS proteins. The results demonstrated that the anti-CP4 EPSPS antibody recognized the MON 88302-produced CP4 EPSPS protein that migrated to an identical position as the *E. coli*-produced CP4 EPSPS protein (Figure VI-3). Furthermore, the immunoreactive signal increased with increasing amounts of CP4 EPSPS protein loaded.

Densitometric analysis was conducted to compare the immunoreactivity of MON 88302- and *E. coli*-produced CP4 EPSPS proteins. The average signal intensity (OD x $mm^2$) from the MON 88302-produced CP4 EPSPS bands and the *E. coli*-produced CP4 EPSPS bands at each amount of protein loaded are shown in Table VI-2. The percent differences in the average signal intensity from the MON 88302-produced CP4 EPSPS bands and from the *E. coli*-produced CP4 EPSPS bands for each amount analyzed was calculated. These values as well as the overall average percent difference (24.1%) are also shown in

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Table VI-2. The acceptance criterion for equivalence of immunoreactivity (±35%) of the MON 88302-produced CP4 EPSPS bands and *E. coli*-produced CP4 EPSPS bands was met. Thus, the western blot analysis established identity of the MON 88302-produced CP4 EPSPS and demonstrated that the MON 88302- and *E. coli*-produced CP4 EPSPS proteins have equivalent immunoreactivity with a CP4 EPSPS-specific antibody.

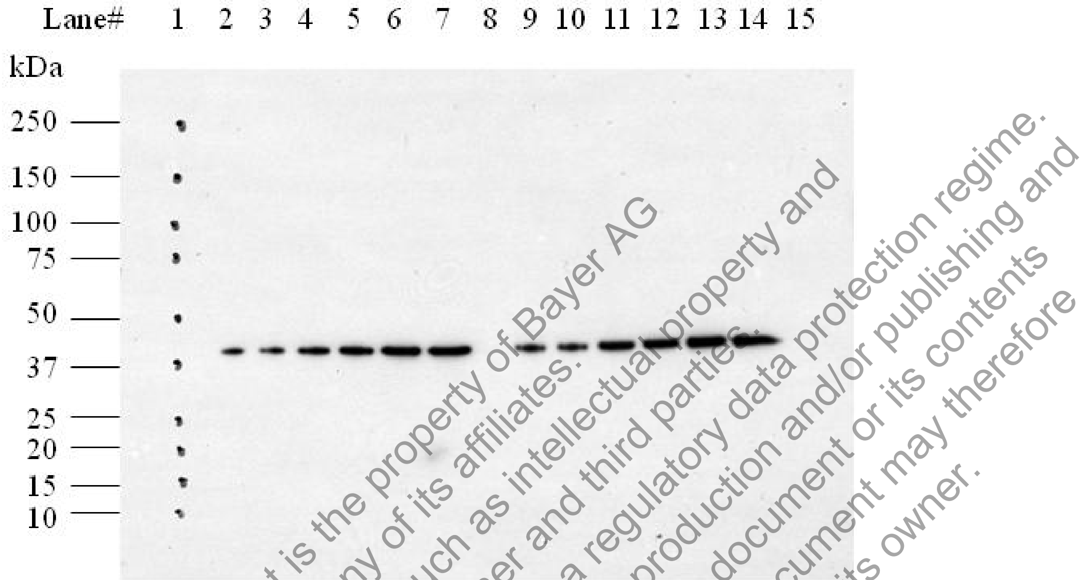


**Figure VI-3. Western Blot Analysis of the MON 88302- and *E. coli*-produced CP4 EPSPS Protein**

Aliquots of the MON 88302-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with anti-CP4 EPSPS antibodies and immunoreactive bands were visualized using an ECL system (GE Healthcare, Piscataway, NJ). Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lane 1. The 1 min exposure is shown.

| Lane | Sample | Amount (ng) |
|---|---|---|
| 1 | Precision Plus Protein Standards Dual color | - |
| 2 | *E. coli*-produced CP4 EPSPS protein | 1 |
| 3 | *E. coli*-produced CP4 EPSPS protein | 1 |
| 4 | *E. coli*-produced CP4 EPSPS protein | 2 |
| 5 | *E. coli*-produced CP4 EPSPS protein | 2 |
| 6 | *E. coli*-produced CP4 EPSPS protein | 3 |
| 7 | *E. coli*-produced CP4 EPSPS protein | 3 |
| 8 | Empty | - |
| 9 | MON 88302-produced CP4 EPSPS protein | 1 |
| 10 | MON 88302-produced CP4 EPSPS protein | 1 |
| 11 | MON 88302-produced CP4 EPSPS protein | 2 |
| 12 | MON 88302-produced CP4 EPSPS protein | 2 |
| 13 | MON 88302-produced CP4 EPSPS protein | 3 |
| 14 | MON 88302-produced CP4 EPSPS protein | 3 |
| 15 | Empty | - |

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**Table VI-2. Comparison of Immunoreactive Signals from the MON 88302- and *E. coli*-produced CP4 EPSPS Proteins**

| Sample | Gel lane | Amount (ng) | Contour Qty (OD × mm²) | Average Contour Qty[1] | Percent Difference[2] (%) | Average Difference[3] (%) |
|---|---|---|---|---|---|---|
| *E. coli* CP4 EPSPS | 2 | 1 | 1.257 | 1.408 | 30.8 | 24.1 |
| *E. coli* CP4 EPSPS | 3 | 1 | 1.558 | | | |
| MON 88302 CP4 EPSPS | 9 | 1 | 2.064 | 2.033 | | |
| MON 88302 CP4 EPSPS | 10 | 1 | 2.002 | | | |
| *E. coli* CP4 EPSPS | 4 | 2 | 3.296 | 3.748 | 26.5 | |
| *E. coli* CP4 EPSPS | 5 | 2 | 4.199 | | | |
| MON 88302 CP4 EPSPS | 11 | 2 | 4.979 | 5.101 | | |
| MON 88302 CP4 EPSPS | 12 | 2 | 5.222 | | | |
| *E. coli* CP4 EPSPS | 6 | 3 | 6.264 | 6.407 | 14.9 | |
| *E. coli* CP4 EPSPS | 7 | 3 | 6.549 | | | |
| MON 88302 CP4 EPSPS | 13 | 3 | 7.737 | 7.527 | | |
| MON 88302 CP4 EPSPS | 14 | 3 | 7.317 | | | |

[1]Average Contour Quantity = ∑(Contour Quantity)/2; contour quantity is average pixel density × band area.
[2]Percent Difference (%) = ((|Average Contour Quantity MON 88302–Average Contour Quantity *E. coli*|)/(Average Contour Quantity MON 88302)) × 100% .
[3]Average difference (%) = ∑ 〚% difference〛/3.

### VI.C.1.4. Results of the MON 88302 CP4 EPSPS Protein Molecular Weights and Purity Analysis

For molecular weight and purity analysis, the MON 88302-produced CP4 EPSPS protein was separated using SDS-PAGE. The gel was stained with Brilliant Blue G Colloidal stain and analyzed by densitometry (Figure VI-4). The MON 88302-produced CP4 EPSPS protein (Figure VI-4, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced CP4 EPSPS protein (Figure VI-4, lane 2) and had an apparent molecular weight of 43.1 kDa (Table VI-3). The apparent molecular weight of the *E. coli*-produced CP4 EPSPS protein as reported on its Certificate of Analysis was 43.8 kDa (Table VI-3). The apparent molecular weights of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were considered equivalent if they were within 10% of one another. Because the experimentally determined apparent molecular weight of the MON 88302-produced CP4 EPSPS protein was within 10% of the *E. coli*-produced CP4 EPSPS protein (Table VI-3), the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were determined to have equivalent apparent molecular weights.

The purity of the MON 88302-produced CP4 EPSPS protein was calculated based on the six loads on the gel (Figure VI-4, lanes 3 to 8). The average purity was determined to be 99%.

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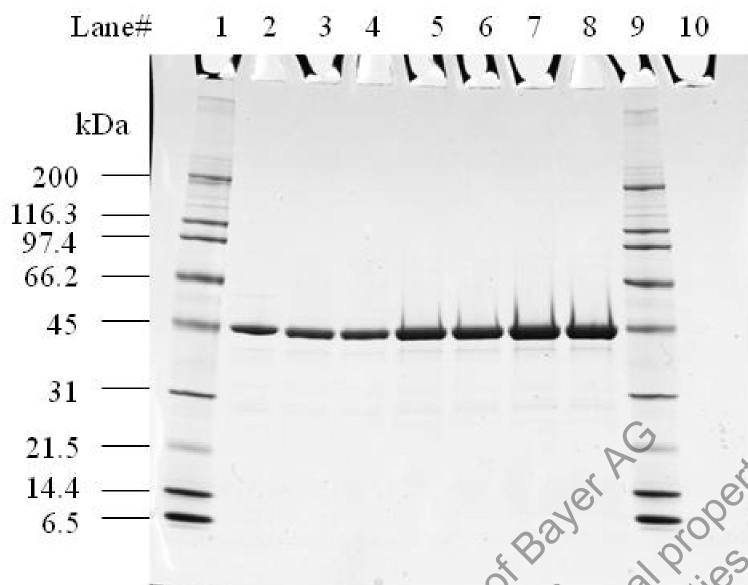


**Figure VI-4. Molecular Weight and Purity Analysis of the MON 88302-produced CP4 EPSPS Protein**

Aliquots of the MON 88302- and the *E. coli*-produced CP4 EPSPS proteins were separated on a 4-20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G-Colloidal stain. Approximate molecular weights are shown on the left and correspond to the markers loaded in Lanes 1 and 9.

| Lane | Sample | Amount (µg) |
|---|---|---|
| 1 | Broad Range Molecular Weight Markers | 4.5 |
| 2 | *E. coli*-produced CP4 EPSPS protein | 1 |
| 3 | MON 88302-produced CP4 EPSPS protein | 1 |
| 4 | MON 88302-produced CP4 EPSPS protein | 1 |
| 5 | MON 88302-produced CP4 EPSPS protein | 2 |
| 6 | MON 88302-produced CP4 EPSPS protein | 2 |
| 7 | MON 88302-produced CP4 EPSPS protein | 3 |
| 8 | MON 88302-produced CP4 EPSPS protein | 3 |
| 9 | Broad Range Molecular Weight markers | 4.5 |
| 10 | Empty | - |

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**Table VI-3. Molecular Weight Comparison Between the MON 88302-Produced and *E. coli*-Produced CP4 EPSPS Proteins Based on SDS-PAGE**

| Molecular Weight of MON 88302-Produced CP4 EPSPS Protein | Molecular Weight of *E. coli*-Produced CP4 EPSPS Protein[1] | % Difference from *E. coli*-Produced CP4 EPSPS Protein |
|---|---|---|
| 43.1 kDa | 43.8 kDa | 1.6% |

[1]The molecular weight of the *E. coli*-produced CP4 EPSPS protein as reported on its Certificate of Analysis.

### VI.C.1.5. CP4 EPSPS Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher et al., 1988). To test whether CP4 EPSPS protein was glycosylated when expressed in the seed of MON 88302, the MON 88302-produced CP4 EPSPS protein was analyzed using an ECL Glycoprotein Detection Module (GE, Healthcare, Piscataway, NJ). Transferrin, a glycosylated protein, was used as a positive control in the assay. To assess equivalence of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins, the *E. coli*-produced CP4 EPSPS protein, previously been shown to be free of glycosylation (Harrison et al., 1996), was also analyzed. The positive control was clearly detected at expected molecular weight (~76 kDa) and the band intensity increased with increasing concentration (Figure VI-5, Panel A, lanes 2-5). In contrast, signals were not observed in the lanes containing the MON 88302- or *E. coli*- produced protein at the expected molecular weight for the CP4 EPSPS protein (Figure VI-5 panel A, lanes 6-9). To confirm that sufficient MON 88302- and *E. coli*-produced CP4 EPSPS proteins were present for glycosylation analysis, a second membrane (with identical loadings and transfer times) was stained with Coomassie Blue R250 for protein detection (Figure VI-5 Panel B). Both the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were clearly detected (Figure VI-5, Panel B, Lanes 6-9). These data indicate that the glycosylation status of MON 88302-produced CP4 EPSPS protein is equivalent to that of the *E. coli*-produced CP4 EPSPS protein and that neither is glycosylated.

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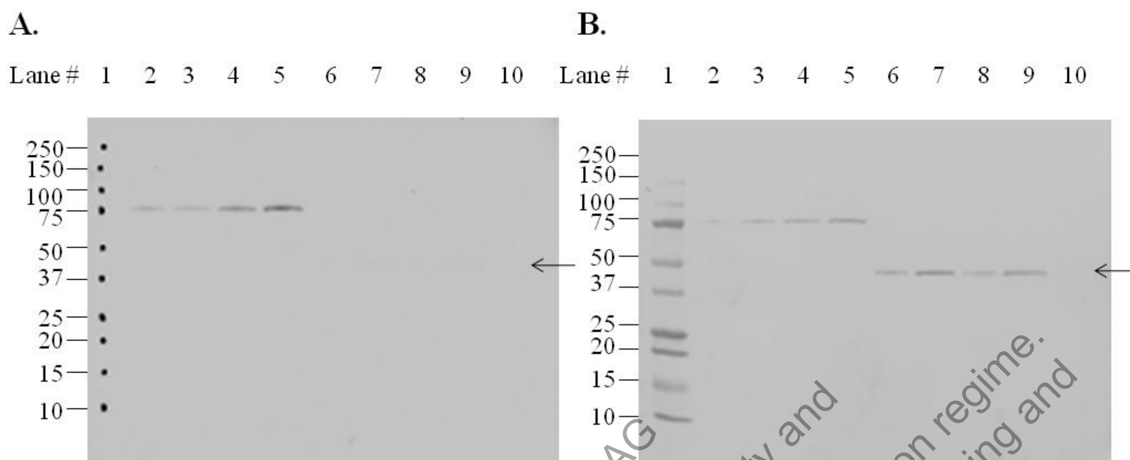


**Figure VI-5. Glycosylation Analysis of the MON 88302-produced CP4 EPSPS Protein**

Aliquots of the transferrin (positive control), *E. coli*-produced CP4 EPSPS protein and MON 88302-produced CP4 EPSPS protein were separated by SDS-PAGE (4-20%) and electrotransferred to PVDF membranes. (A) Where present, the labeled carbohydrate moieties were detected using the ECL-based system with exposure to Hyperfilm. A 2 min exposure is shown. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. The signal was captured using a Bio-Rad GS-800 with Quantity One software (version 4.4.0). Approximate molecular weights (kDa) correspond to the Precision Plus, dual color markers (used to verify transfer and MW) in Lane 1. Arrows indicate the band corresponding to CP4 EPSPS protein.

| Lane | Sample | Amount (ng) |
|---|---|---|
| 1 | Precision Plus, dual color MW markers | - |
| 2 | Transferrin (positive control) | 50 |
| 3 | Transferrin (positive control) | 100 |
| 4 | Transferrin (positive control) | 150 |
| 5 | Transferrin (positive control) | 200 |
| 6 | *E. coli*-produced CP4 EPSPS (negative control) | 100 |
| 7 | *E. coli*-produced CP4 EPSPS (negative control) | 200 |
| 8 | MON 88302-produced CP4 EPSPS | 100 |
| 9 | MON 88302-produced CP4 EPSPS | 200 |
| 10 | Empty | - |

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### VI.C.1.6. CP4 EPSPS Functional Activity

The functional activities of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were determined using a colorimetric assay that measures formation of inorganic phosphate (Pi) from the EPSPS-catalyzed reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP). In this assay, protein-specific activity is expressed as units per milligram of protein (U/mg), where a unit is defined as one µmole of inorganic phosphate released from PEP per minute at 25 °C. The MON 88302- and *E. coli*-produced CP4 EPSPS proteins were considered to have equivalent functional activity if the specific activities were within 2-fold of one another.

The experimentally determined specific activities for the MON 88302- and *E. coli*-produced CP4 EPSPS proteins are presented in Table VI-4. The specific activities of MON 88302- and *E. coli*-produced CP4 EPSPS proteins were 4.93 U/mg and 2.79 U/mg of CP4 EPSPS protein, respectively. Because the specific activity of the MON 88302-produced CP4 EPSPS protein falls within the preset acceptance criterion (Table VI-4), the MON 88302-produced CP4 EPSPS protein was considered to have equivalent functional activity to that of the *E. coli*-produced CP4 EPSPS protein.

**Table VI-4. CP4 EPSPS Functional Activity**

| MON 88302-produced CP4 EPSPS Protein[1] (U/mg) | *E. coli*-produced CP4 EPSPS Protein[1] (U/mg) | Previously set acceptance limits[2] (U/mg) |
|---|---|---|
| 4.93 ± 0.36 | 2.79 ± 0.26 | 1.40 – 5.58 |

[1]Value refers to mean and standard deviation calculated based on n = 6 which includes three replicate assays spectrophotometrically.
[2]Within 2-fold of the *E.coli*-produced CP4 EPSPS specific activity (2.79 ÷ 2 U/mg to 2.79 x 2 U/mg)

### VI.C.2. CP4 EPSPS Protein Identity and Equivalence Conclusion

A panel of analytical techniques was used to characterize the MON 88302-produced CP4 EPSPS protein purified from seed of MON 88302. Identity of the MON 88302-produced CP4 EPSPS was confirmed by N-terminal sequencing, mapping of tryptic peptides that yielded a 85.5% overall coverage of the expected protein sequence and recognition with anti-CP4 EPSPS antibodies. The purity and apparent molecular weight of the MON 88302-produced CP4 EPSPS was 99% and 43.1 kDa, respectively. The MON 88302-produced CP4 EPSPS protein was not glycosylated and had a specific activity of 4.93 U/mg of CP4 EPSPS.

The equivalence of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins was evaluated by comparing their immunoreactivity with anti-CP4 EPSPS antibodies, the apparent molecular weight, glycosylation status, and functional activity. The results obtained demonstrate that the MON 88302-produced CP4 EPSPS protein is equivalent to

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the *E. coli*-produced CP4 EPSPS protein. This equivalence justifies the use of protein safety studies conducted previously in which the *E. coli*-produced CP4 EPSPS protein was used as a test substance.

**VI.D. Expression Levels of CP4 EPSPS Protein in MON 88302**

CP4 EPSPS protein levels in various tissues of MON 88302 relevant to the risk assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA). Tissues of MON 88302 were collected from four replicate plots planted in a randomized complete block field design (PE) during the 2009 growing season from the following three field sites in the U.S.: Power County, Idaho; Wilkin County, Minnesota; and McHenry County, North Dakota, and the following three field sites in Canada: Portage la Prairie, Manitoba; Newton, Manitoba; and Saskatoon, Saskatchewan. These field sites were representative of canola producing regions suitable for commercial production. Forage, seed, over-season leaf (OSL-1 through OSL-4), and root (Root-1 and Root-2) tissue samples were collected from each replicated plot at all field sites.

CP4 EPSPS protein levels were determined in all eight tissue types. The results obtained from ELISA are summarized in Table VI-5 and the details of the materials and methods are described in Appendix C. CP4 EPSPS protein levels in MON 88302 across tissue types ranged from 22 to 500 µg/g dw. The mean CP4 EPSPS protein levels were determined across six sites with the exception of seed (5 sites), OSL-1 (5 sites), OSL-2 (3 sites), and Root-2 (4 sites). The mean CP4 EPSPS protein levels were determined across six sites with the exception of seed (5 sites), OSL-1 (5 sites), OSL-2 (3 sites), and Root-2 (4 sites). Sample collections are detailed in Appendix C. The mean CP4 EPSPS protein levels were highest in leaf (ranging from OSL-1 at 180 µg/g dw to OSL-3 at 230 µg/g dw), followed by forage (170 µg/g dw), root (ranging from Root-2 at 38 µg/g dw to Root-1 at 82 µg/g dw), and seed (27 µg/g dw).

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**Table VI-5. Summary of CP4 EPSPS Protein Levels in Canola Tissues from MON 88302 Grown in 2009 U.S. and Canadian Field Trials**

| Tissue[1] | Development Stage[2] | Days After Planting (DAP) | CP4 EPSPS Mean (SD) Range (µg/g fw)[3] | CP4 EPSPS Mean (SD) Range (µg/g dw)[4] | LOQ/LOD[5] (µg/g fw) |
|---|---|---|---|---|---|
| Forage | 30 BBCH | 37 - 57 | 18 (4.4)<br>14 - 28 | 170 (22)<br>120 - 210 | 0.91/0.28 |
| Seed | 99 BBCH | 118 - 132 | 25 (5.2)<br>21 - 43 | 27 (5.6)<br>22 - 46 | 0.91/0.81 |
| OSL-1 | 13-14 BBCH | 23 - 40 | 23 (10)<br>10 - 45 | 180 (40)<br>110 - 250 | 0.91/0.098 |
| OSL-2 | 17-19 BBCH | 32 - 54 | 22 (5.9)<br>18 - 37 | 180 (41)<br>120 - 250 | 0.91/0.098 |
| OSL-3 | 30 BBCH | 37 - 57 | 31 (6.3)<br>20 - 41 | 230 (50)<br>130 - 300 | 0.91/0.098 |
| OSL-4 | 60-62 BBCH | 51 - 61 | 36 (14)<br>20 - 85 | 210 (80)<br>110 - 500 | 0.91/0.098 |
| Root-1 | 30 BBCH | 37 - 57 | 19 (4.1)<br>11 - 25 | 82 (17)<br>46 - 100 | 0.91/0.60 |
| Root-2 | 71-73 BBCH | 63 - 81 | 10 (3.3)<br>7.0 - 17 | 38 (14)<br>24 - 62 | 0.91/0.60 |

[1]OSL = over-season leaf.
[2]The development stage each tissue was collected. The canola growth stages are based on the Bayer, BASF, Ciba-Geigy and Hoechst Cereal Grain Growth Scale (BBCH) (BBCH, 2001).
[3]Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites. The numbers of samples (n) figured into the calculations are as follows: forage n = 20, seed n = 16, OSL-1 n = 16, OSL-2 n = 9, OSL-3 n = 20, OSL-4 n = 2 0, Root-1 n = 19, and Root-2 n = 11. Sample collections are detailed in Appendix C.
[4]Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data.
[5]LOQ = limit of quantitation; LOD = limit of detection.

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## VI.E. Generational Stability of CP4 EPSPS Protein Expression in MON 88302

In order to confirm the presence of the CP4 EPSPS protein in MON 88302 across multiple generations, western blot analysis of MON 88302 was conducted on leaf tissue collected from generations $R_2$, $R_3$, $R_4$, $R_{5a}$, and $R_{5b}$ (Figure V-9) of MON 88302, and on leaf tissue of the conventional control (Ebony). Materials and methods are detailed in Appendix D. The presence of the CP4 EPSPS protein in harvested leaf tissue of the $R_2$, $R_3$, $R_4$, $R_{5a}$, and $R_{5b}$ generations of MON 88302 was demonstrated (Figure VI-6). An *E. coli*-produced CP4 EPSPS standard (2 ng) was used as a reference for the identification of the CP4 EPSPS protein. The presence of CP4 EPSPS protein in MON 88302 leaf tissue samples was determined by visual comparison of the bands produced in the multiple breeding generations (Figure VI-6, lanes 4 through 8) to the CP4 EPSPS reference standard (Figure VI 6, lane 2). As shown in Figure VI-6, CP4 EPSPS protein was present in multiple generations of MON 88302 tissue samples and migrated with a mobility indistinguishable from that of the *E. coli*-produced protein standard analyzed on the same western blot. As expected, the CP4 EPSPS protein was not detected in the conventional control extract (Figure VI-6, lane 3). Two additional faint bands were observed at approximately 30 kDa and 60 kDa in the MON 88302 samples (Figure VI-6, lanes 4 through 8). These bands are likely the result of non-specific binding of either the primary or secondary antibody to endogenous canola leaf proteins.

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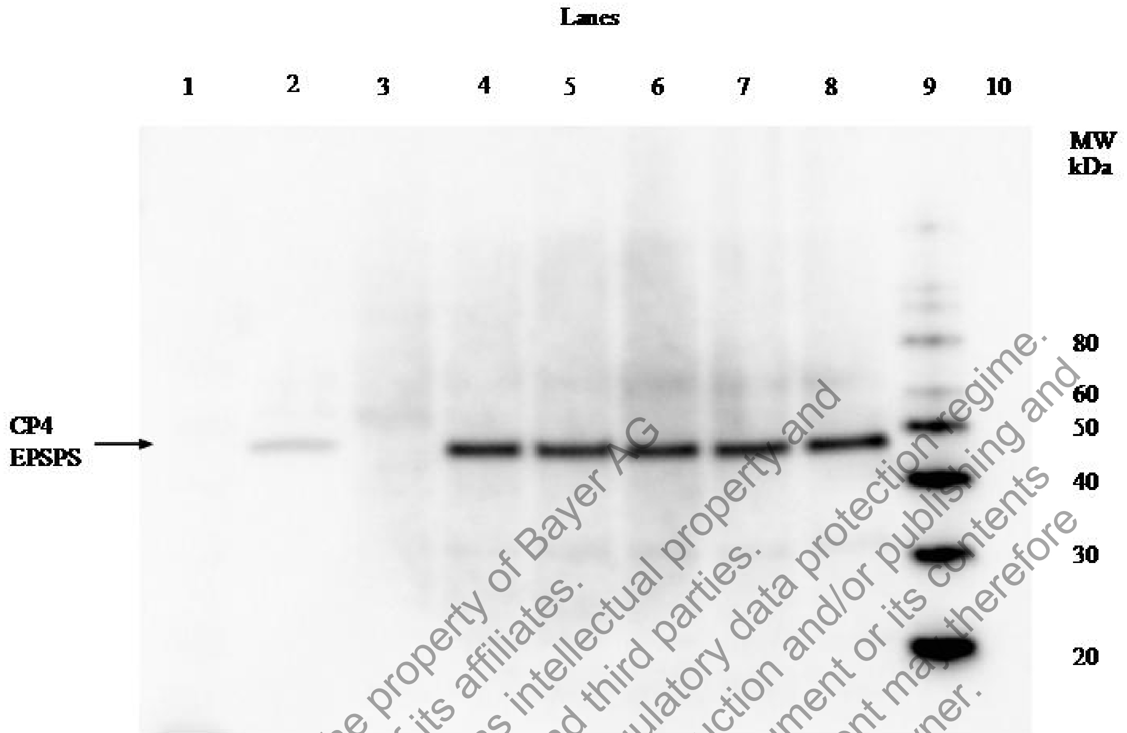


**Figure VI-6. Presence of CP4 EPSPS Protein in Multiple Generations of MON 88302**

Extracts from multiple generations of MON 88302 leaf tissues and molecular weight markers were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with goat anti-CP4 EPSPS antibody. The image represents a 30 second exposure.

| Lane | Sample Description | Amount Loaded |
|---|---|---|
| 1 | Precision Plus Molecular Weight Marker | 10 µl |
| 2 | *E. coli*-produced CP4 EPSPS protein (2 ng) (Molecular weight 43.8 kDa) | 20 µl |
| 3 | Conventional Control | 20 µl |
| 4 | $R_2$ Generation | 20 µl |
| 5 | $R_3$ Generation | 20 µl |
| 6 | $R_4$ Generation | 20 µl |
| 7 | $R_{5a}$ Generation | 20 µl |
| 8 | $R_{5b}$ Generation | 20 µl |
| 9 | Magic Marker Molecular Weight Marker | 0.5 µl |
| 10 | Blank | N/A |

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## VI.F. Assessment of the Potential Allergenicity, Toxicity, and Dietary Safety of the CP4 EPSPS Protein

History of safe use of the introduced protein is a key consideration in the potential for allergenicity and toxicity and for assessment of dietary safety (Section VI.B).

Additionally, following the guidelines adopted by the Codex Alimentarius Commission (Codex Alimentarius, 2009), an assessment of potential allergenicity of introduced proteins has been conducted, by comparing the characteristics of the introduced protein to characteristics of known allergens (Codex Alimentarius, 2009). A protein is not likely to be associated with allergenicity if: 1) the protein is from a nonallergenic source; 2) the protein represents only a very small portion of the total plant protein; 3) the protein does not share structural similarities to known allergens based on the amino acid sequence; 4) the protein is rapidly digested in mammalian gastrointestinal systems; and 5) the protein is not stable to heat treatment. The CP4 EPSPS protein in MON 88302 has been assessed for its potential allergenicity according to these safety assessment guidelines.

The assessment of the potential toxicity of an introduced protein is based on comparing the biochemical characteristics of the introduced protein to characteristics of known toxins. These biochemical characteristics are assessed by determining: 1) if the protein has structural similarity to known toxins or other biologically-active proteins that could cause adverse effects in humans or animals; 2) if the protein is rapidly digested in mammalian gastrointestinal systems; 3) if the protein is stable to heat treatment; 4) if the protein exerts any acute toxic effects in mammals; and 5) the anticipated exposure levels for humans and animals. The CP4 EPSPS protein in MON 88302 has been assessed for its potential toxicity based on these criteria.

### VI.F.1. Assessment of Potential Allergenicity of CP4 EPSPS Protein

#### VI.F.1.1 Safety of the Donor Organism

The donor organism, *Agrobacterium* sp. strain CP4, was isolated based on its tolerance to glyphosate brought about by the production of a naturally glyphosate-tolerant EPSPS protein (Padgette et al., 1996). The bacterial isolate, CP4, was identified by the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are not commonly known for human or animal pathogenicity or allergenicity. According to a report of a joint FAO/WHO Expert Consultation (FAO/WHO, 2001), there is no known population of individuals sensitized to bacterial proteins. Furthermore, *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready corn 2, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton, and Roundup Ready alfalfa.

#### VI.F.1.2. The CP4 EPSPS Protein as a Proportion of Total Protein

The CP4 EPSPS protein was detected in all plant tissues assayed, at a number of time points during the growing season (Table VI-5). Among tested tissues of MON 88302,

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seed is the most relevant to the assessment of food allergenicity since seed is the source of canola oil. The mean level of CP4 EPSPS protein in seed of MON 88302 is 27 µg/g dw. The mean percent dry weight of total protein in seed of MON 88302 is 23% (or 230,000 µg/g; Table VII-2). The percentage of CP4 EPSPS protein in MON 88302 seed is calculated as follows:

(27 µg/g ÷ 230,000 µg/g) × 100% ≈ 0.01% or 100 ppm of total canola seed protein

Therefore, the CP4 EPSPS protein represents a very small portion of the total protein in harvested seed of MON 88302. Additionally, the total protein content in oil extracted from canola seed is very low (<0.00002% or < 0.2 ppm, (Martín-Hernández et al., 2008). Canola oil is the predominant seed fraction that is used for foods, therefore the levels of CP4 EPSPS in oil from MON 88302 seed would be estimated to be 0.01% of <0.2 ppm total protein in the oil, essentially present in the oil.

### VI.F.1.3. Structural Similarity of CP4 EPSPS Protein to Known Allergens

The Codex guidelines for the evaluation of the allergenicity potential of introduced proteins (Codex Alimentarius, 2009) are based on the comparison of amino acid sequences between introduced proteins and allergens, where allergenic cross-reactivity may exist if the introduced protein is found to have at least 35% amino acid identity with an allergen over any segment of at least 80 amino acids. The Codex guideline also recommends that a sliding window search with a scientifically justified peptide size could be used to identify immunologically relevant peptides in otherwise unrelated proteins. Therefore, the extent of sequence similarities between the CP4 EPSPS protein present in MON 88302 and known allergens, gliadins, and glutenins was assessed using the FASTA sequence alignment tool and an eight-amino acid sliding window search (Codex Alimentarius, 2009; Thomas et al., 2005). The methods used are summarized below and detailed in Appendix E. The data generated from these analyses confirm that the CP4 EPSPS protein does not share amino acid sequence similarities with known allergens, gliadins, or glutenins.

The FASTA program directly compares amino acid sequences (i.e. primary, linear protein structure). This alignment data may be used to infer shared higher order structural similarities between two sequences (i.e., secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. By definition, homologous proteins have common secondary structures, and three-dimensional configuration, and, consequently, may share similar functions. The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from Food Allergy Research and Resource Program Database (FARRP_2010) and was used for the evaluation of sequence similarities shared between the CP4 EPSPS protein and all proteins. The AD_2010 database contains 1,471 sequences. When used to align the sequence of the introduced protein to each protein in the database, the FASTA algorithm produces an *E*-score (expectation score) for each alignment. The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a low degree of similarity between the query sequence and the sequence from the database. Typically, alignments

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between two sequences which have an *E*-score of less than or equal to $1 \times 10^{-5}$ are considered to have significant homology. Results indicate that the CP4 EPSPS protein sequence does not share significant similarity with sequences in the allergen database. No alignment met nor exceeded the threshold of 35% identity over 80 amino acids recommended by Codex Alimentarius (2009) or had an *E*-score of less than or equal to $1 \times 10^{-5}$.

A second bioinformatic tool, an eight-amino acid sliding window search, was used to specifically identify short linear polypeptide matches to known allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may contain smaller immunologically significant epitopes. An amino acid sequence may have allergenic potential if it has an exact sequence identity of at least eight linearly contiguous amino acids with a potential allergen epitope (Hileman et al., 2002; Metcalfe et al., 1996). Using a sliding window of less than eight amino acids can produce matches containing significant uncertainty depending on the length of the query sequence (Silvanovich et al., 2006) and are not useful to the allergy assessment process (Thomas et al., 2005). No eight contiguous amino acid identities were detected when the CP4 EPSPS protein sequence was compared to the proteins in the AD_2010 sequence database.

Results show there were no similarities to allergens when the CP4 EPSPS protein sequence was used as a query for a FASTA search of the AD_2010 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the CP4 EPSPS protein sequence and proteins in the allergen database. These data show that the CP4 EPSPS protein sequence lacks both structurally and immunologically relevant sequence similarities to known allergens, gliadins, and glutenins.

**VI.F.1.4. Digestive Fate of the CP4 EPSPS Protein**

A correlation between digestive stability in simulated gastric fluid (SGF) and the allergenicity of a protein has been previously reported (Astwood et al., 1996), but this correlation is not absolute (Fu et al., 2002). The SGF assay serves as a tool to compare the relative susceptibility of proteins to digestion in pepsin. The SGF assay protocol has been standardized based on results obtained from an international, multi-laboratory study (Thomas et al., 2004). This study showed that the standardized protocol provides reproducibility and consistency for determining the digestive stability of a protein. Using this standardized protocol, the digestive stability of CP4 EPSPS protein was analyzed (Appendix F) and a summary of the results is reported below.

Harrison et al. (1996) demonstrated that the *E. coli*-produced CP4 EPSPS protein is rapidly degraded in simulated digestive fluids. Based on Western blot analysis, CP4 EPSPS protein was undetectable within 15 seconds under simulated gastric conditions greatly minimizing the potential for this protein to be absorbed in the intestinal mucosa. In addition, when digested in simulated intestinal fluid (SIF), the half life of CP4 EPSPS protein was less than 10 minutes (Harrison et al. 1996). Therefore, if any of the CP4 EPSPS protein were to survive in the gastric system, it is expected that it would be rapidly degraded in the intestine. Based on this information, CP4 EPSPS protein is expected to degrade rapidly in the mammalian digestive tract.

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Subsequent experiments using the standardized method published by the International Life Science Institute (ILSI) (Thomas et al., 2004), confirmed the *in vitro* digestibility of the *E. coli*-produced CP4 EPSPS protein in SGF. *E. coli*-produced CP4 EPSPS protein, shown to be physiochemically and functionally equivalent to the CP4 EPSPS protein produced in MON 88302 (Section VI.C), was used in these experiments. Similar to the results reported by Harrison et al. (1996), greater than 98% of the CP4 EPSPS protein was digested within 15 sec, based on the results of visual inspection of colloidal blue stained SDS-PAGE gels (Figure VI-7). Western blot analysis confirmed that greater than 95% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 sec (Figure VI-8). In summary, the results of these experiments confirmed that the *E. coli*-produced CP4 EPSPS protein was rapidly digested after incubation in SGF and is therefore unlikely to pose a human health concern.

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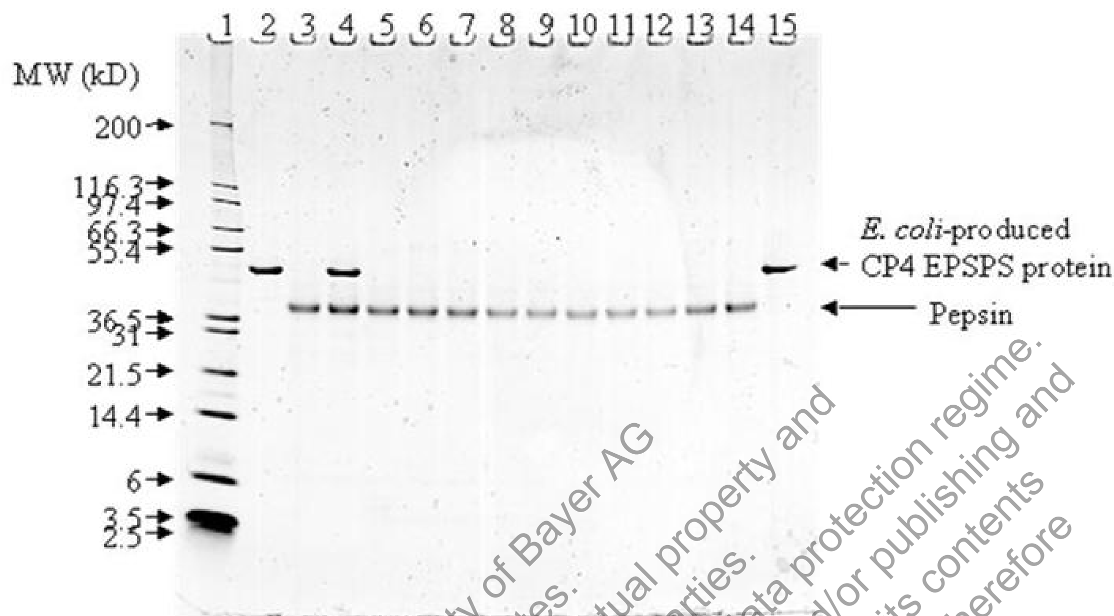


**Figure VI-7. Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid**

Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G Colloidal stain. *E. coli*-produced CP4 EPSPS protein was loaded at 500 ng per lane based on pre-digestion concentrations.

| Lane | Description | Incubation Time |
|---|---|---|
| 1 | Molecular weight markers (Invitrogen P/N LC 5677) | |
| 2 | Experimental control without pepsin | 0 |
| 3 | Experimental control without CP4 EPSPS | 0 |
| 4 | CP4 EPSPS protein in SGF | 0 |
| 5 | CP4 EPSPS protein in SGF | 15 sec |
| 6 | CP4 EPSPS protein in SGF | 30 sec |
| 7 | CP4 EPSPS protein in SGF | 1 min |
| 8 | CP4 EPSPS protein in SGF | 2 min |
| 9 | CP4 EPSPS protein in SGF | 4 min |
| 10 | CP4 EPSPS protein in SGF | 8 min |
| 11 | CP4 EPSPS protein in SGF | 15 min |
| 12 | CP4 EPSPS protein in SGF | 30 min |
| 13 | CP4 EPSPS protein in SGF | 60 min |
| 14 | Experimental control without CP4 EPSPS | 60 min |
| 15 | Experimental control without pepsin | 60 min |

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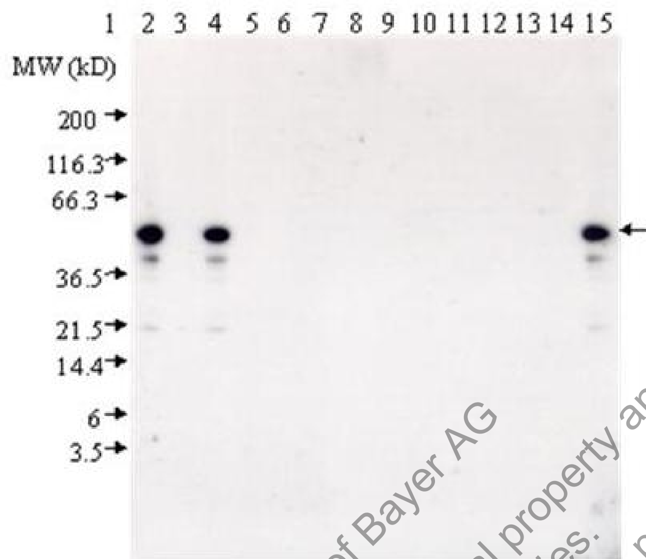


**Figure VI-8. Western Blot Analysis of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid**

Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a tricine buffered gel, electroblotted, and probed with anti-CP4 EPSPS goat serum. *E. coli*-produced CP4 EPSPS protein was loaded at 1 ng per lane based on 90% purity and pre-digestion concentrations. Lane 1 containing the molecular weight markers was cropped and the arrow on the right side of the image indicates the band corresponding to CP4 EPSPS protein.

| Lane | Description | Incubation Time |
|---|---|---|
| 1 | Molecular weight markers (Invitrogen P/N LC5677) | |
| 2 | Experimental control without pepsin | 0 |
| 3 | Experimental control without CP4 EPSPS | 0 |
| 4 | CP4 EPSPS protein in SGF | 0 |
| 5 | CP4 EPSPS protein in SGF | 15 sec |
| 6 | CP4 EPSPS protein in SGF | 30 sec |
| 7 | CP4 EPSPS protein in SGF | 1 min |
| 8 | CP4 EPSPS protein in SGF | 2 min |
| 9 | CP4 EPSPS protein in SGF | 4 min |
| 10 | CP4 EPSPS protein in SGF | 8 min |
| 11 | CP4 EPSPS protein in SGF | 15 min |
| 12 | CP4 EPSPS protein in SGF | 30 min |
| 13 | CP4 EPSPS protein in SGF | 60 min |
| 14 | Experimental control without CP4 EPSPS | 60 min |
| 15 | Experimental control without pepsin | 60 min |

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### VI.F.1.5. Heat Stability of the Purified CP4 EPSPS Protein

Heat treatment is used during processing of canola seed into oil and in canola oil refinement (Booth, 2004). The effect of heat treatment on the activity of *E. coli*-produced CP4 EPSPS protein was evaluated using purified protein. CP4 EPSPS protein was heated to 25, 37, 55, 75, and 95 °C for either 15 min or 30 min. The method for evaluating heat stability is described in Appendix G. Heat-treated samples and an unheated control sample of CP4 EPSPS protein were analyzed: 1) using a functional assay to assess the impact of temperature on the enzymatic activity of CP4 EPSPS protein and 2) using SDS-PAGE to assess the impact of temperature on protein integrity.

Canola seed processing involves treatment with different temperature regimes, some of which are higher than 55 °C and of variable duration (Booth, 2004). Additionally, some steps, especially oil refinement and deodorization, are carried out at considerably higher temperatures, (e.g., 70 °C for 20 minutes and 240 °C for 20 minutes, respectively) (Booth, 2004). The effect of heating on the functional activity of the *E. coli*-produced CP4 EPSPS protein for 15 min and 30 min is presented in Tables VI-6 and VI-7 respectively. After treatment at temperatures of 75 ºC and higher CP4 EPSPS functional activity was below the limit of detection. There was no effect on band intensity, as measured by SDS-PAGE, of heat-treated samples after incubation for 15 or 30 minutes at all temperatures tested (Figures VI-9 and VI-10 respectively). These data demonstrate that CP4 EPSPS behaves with a predictable tendency toward enzyme denaturation at elevated temperatures. Therefore, in the unlikely event that canola oil contains protein, it is reasonable to conclude that CP4 EPSPS protein would not be consumed as an active protein in food products.

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**Table VI-6. Activity of CP4 EPSPS after 15 Minutes at Elevated Temperatures**

| Temperature | Functional Activity CP4 EPSPS (U/mg) (Mean[1] ± SD[2]) | Relative activity[3] |
|---|---|---|
| Unheated Control (0 ºC) | 6.03 ± 0.29 | 100% |
| 25 ºC | 4.88 ± 0.24 | 81% |
| 37 ºC | 5.08 ± 0.33 | 84% |
| 55 ºC | 4.22 ± 0.12 | 70% |
| 75 ºC | < LOD[4] | < 3%[5] |
| 95 ºC | < LOD[4] | < 3%[5] |

[1] Mean specific activity determined from n = 3.
[2] SD = standard deviation
[3] CP4 EPSPS activity of unheated control was assigned 100 %.
[4] LOD is defined as the value that is three standard deviations above the mean of the assay blank.
[5] Calculated from the LOD of the CP4 EPSPS activity assay.

**Table VI-7. Activity of CP4 EPSPS after 30 Minutes at Elevated Temperatures**

| Temperature | Functional Activity CP4 EPSPS (U/mg) (Mean[1] ± SD[2]) | Relative activity[3] |
|---|---|---|
| Unheated Control (0 ºC) | 2.8 ± 0.26 | 100% |
| 25 ºC | 3.1 ± 0.23 | 110% |
| 37 ºC | 2.5 ± 0.05 | 88% |
| 55 ºC | 0.70 ± 0.09 | 25% |
| 75 ºC | < LOD[4] | < 8%[5] |
| 95 ºC | < LOD[4] | < 8%[5] |

[1] Mean specific activity determined from n = 3.
[2] SD = standard deviation
[3] CP4 EPSPS activity of unheated control was assigned 100 %.
[4] LOD is defined as the value that is three standard deviations above the mean of the assay blank.
[5] Calculated from the LOD of the CP4 EPSPS activity assay.

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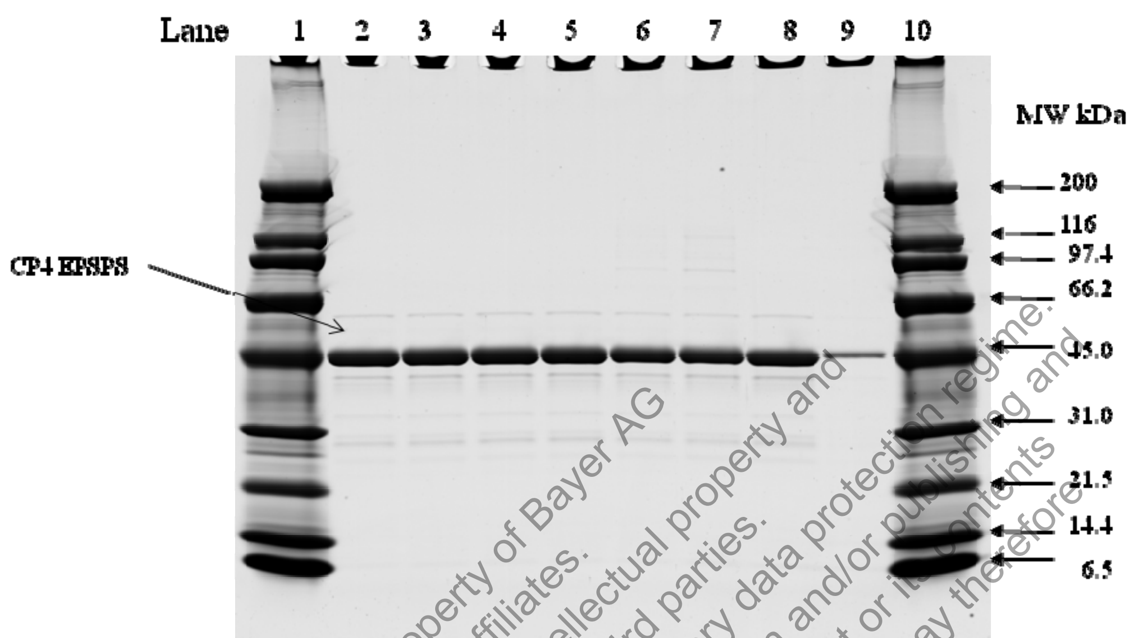


**Figure VI-9. SDS-PAGE of CP4 EPSPS Following Heat Treatment for 15 Minutes**

Heated-treated samples of CP4 EPSPS (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

| Lane | Description | Amount (µg) |
|---|---|---|
| 1 | Broad Range Molecular Weight Markers | 4.5 |
| 2 | CP4 EPSPS Temperature Unheated Control (0 ºC) | 3.2 |
| 3 | CP4 EPSPS 25 ºC | 3.2 |
| 4 | CP4 EPSPS 37 ºC | 3.2 |
| 5 | CP4 EPSPS 55 ºC | 3.2 |
| 6 | CP4 EPSPS 75 ºC | 3.2 |
| 7 | CP4 EPSPS 95 ºC | 3.2 |
| 8 | CP4 EPSPS Reference | 3.2 |
| 9 | CP4 EPSPS Reference | 0.32 |
| 10 | Broad Range Molecular Weight Markers | 4.5 |

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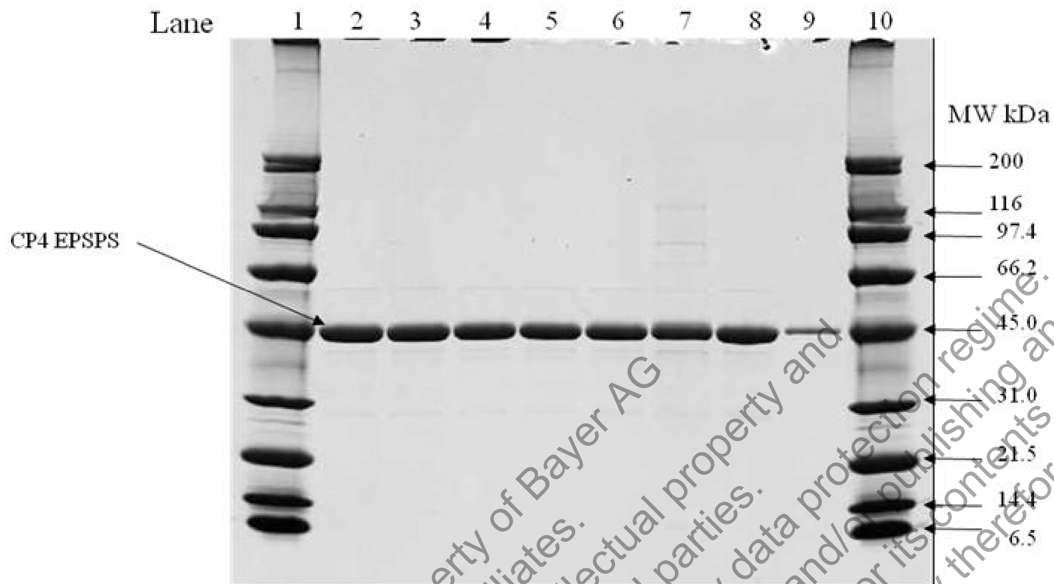


**Figure VI-10. SDS-PAGE of CP4 EPSPS Following Heat Treatment for 30 Minutes**

Heated samples of CP4 EPSPS protein (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

| Lane | Description | Amount (µg) |
|---|---|---|
| 1 | Broad Range Molecular Weight Markers | 4.5 |
| 2 | CP4 EPSPS 25 ºC | 3.2 |
| 3 | CP4 EPSPS 37 ºC | 3.2 |
| 4 | CP4 EPSPS 55 ºC | 3.2 |
| 5 | CP4 EPSPS 75 ºC | 3.2 |
| 6 | CP4 EPSPS 95 ºC | 3.2 |
| 7 | CP4 EPSPS Unheated Control (0 ºC) | 3.2 |
| 8 | CP4 EPSPS Reference | 3.2 |
| 9 | CP4 EPSPS Reference | 0.32 |
| 10 | Broad Range Molecular Weight Markers | 4.5 |

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### VI.F.2. Assessment for the Potential for Toxicity of the CP4 EPSPS Protein

### VI.F.2.1. Structural Similarity of CP4 EPSPS Protein to Known Toxins

The assessment of the potential for protein toxicity includes bioinformatic analysis of the amino acid sequence of the introduced protein. The goal of the bioinformatic analysis is to ensure that the introduced protein does not share homology to known toxins or anti-nutritional proteins associated with adverse health effects.

Potential structural similarities shared between the CP4 EPSPS protein and sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (i.e., primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (i.e. secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. By definition, homologous proteins have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions.

FASTA bioinformatic alignment searches using the CP4 EPSPS amino acid sequence were performed with the toxin database to identify possible homology with proteins that may be harmful to human and animal health. The toxin database, TOX_2010, is a subset of sequences derived from the PRT_2010 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. The TOX_2010 database contains 8,448 sequences.

An *E*-score acceptance criteria of $1\text{x}10^{-5}$ or less for any alignment was used to identify proteins from the TOX_2010 database with potential for significant shared structural similarity and function with CP4 EPSPS protein. As described above, the *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences require an *E*-score of $1\times10^{-5}$ or less to be considered to have sufficient sequence similarity to infer homology. The results of the search comparisons showed that no relevant alignments were observed against proteins in the TOX_2010 database.

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the CP4 EPSPS protein and any known toxic or other biologically active proteins that would be harmful to human or animal health.

### VI.F.2.2. Heat Stability and Digestibility of the CP4 EPSPS Protein

The stability of a protein to heat or its degradation in simulated mammalian gastrointestinal fluids is a key consideration in the assessment of its potential toxicity. Exposure to heat during food processing or cooking, and to digestive fluids is likely to have a profound effect on the structure and function of proteins. The effect of heat treatment on the activity of CP4 EPSPS protein was evaluated using a functional assay to

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assess the impact of temperature on enzymatic activity, and using SDS-PAGE to assess the impact of temperature on protein integrity. The results show that CP4 EPSPS protein was completely deactivated by heating at temperatures above 75ºC (Section VI.F.1.5.). The digestibility of CP4 EPSPS protein was evaluated by incubation with simulated gastric fluid, and the results show that CP4 EPSPS protein was readily digested (Section VI.F.1.4.). Therefore, it is anticipated that exposure to functionally active CP4 EPSPS protein from the consumption of MON 88302 or foods derived from MON 88302 will be negligible.

### VI.F.2.3. Acute Oral Toxicity Study with the CP4 EPSPS Protein

Most known protein toxins act through acute mechanisms to exert toxicity (Hammond and Fuchs, 1998; Pariza and Johnson, 2001; Sjoblad et al., 1992). The primary exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, which manifest toxicity in a short term (few weeks) feeding study (Liener, 1994). The amino acid sequence of the CP4 EPSPS protein produced in MON 88302 is not similar to any of these anti-nutritional proteins or to any other known protein toxin. Therefore, an acute oral mouse toxicity study was considered sufficient to evaluate the toxicity of the CP4 EPSPS protein.

*E. coli*-produced CP4 EPSPS protein was administered as a single dose by gavage to three groups of 10 male and 10 female CD-1 mice at dose levels up to 572 mg/kg body wt (bw) (Harrison et al., 1996). Additional groups of mice were administered comparable volume of the buffer or a comparable amount (mg/kg bw) of bovine serum albumin (BSA) to serve as vehicle or protein controls, respectively. Following dosing, all mice were observed twice daily for mortality or signs of toxicity. Food consumption was measured daily. Body weights were measured prior to dosing and at study day 7. All animals were sacrificed on day 8 or 9 and subjected to a gross necropsy. There were no treatment-related effects on survival, clinical observations, body weight gain, food consumption or gross pathology. Therefore, the No Observable Adverse Effect Level (NOAEL) for CP4 EPSPS was considered to be 572 mg/kg bw, the highest dose tested.

## VI.F.3. Dietary Risk Assessment of the CP4 EPSPS Protein

### VI.F.3.1. Estimated Human Exposure to the CP4 EPSPS Protein from MON 88302

The primary human food currently produced from canola is refined, bleached, and deodorized (RBD) oil. Because RBD oil contains negligible amounts of protein (Martín-Hernández et al., 2008), oil produced from MON 88302 will contain negligible levels of CP4 EPSPS protein. Therefore, there is minimal, if any, dietary exposure to CP4 EPSPS protein from consumption of foods derived from MON 88302 at this time.

#### VI.F.3.1.1. Dietary Exposure Assessment: Margin of Exposure for the CP4 EPSPS Protein Derived from MON 88302

A common approach used to assess potential health risks from chemicals or other potentially toxic products is to calculate a Margin of Exposure (MOE) between the lowest NOAEL from an appropriate animal toxicity study and an estimate of human

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exposure. No adverse health effects were observed when male or female mice were administered doses up to 572 mg/kg bw of CP4 EPSPS protein (Harrison et al., 1996). Therefore, based on an apparent absence of hazard, a dietary risk assessment for this protein would normally not be considered necessary. In addition, the safety of CP4 EPSPS has been extensively assessed (Harrison et al., 1996). Several Roundup Ready crops produce a CP4 EPSPS protein that is identical to the protein produced in MON 88302 have been reviewed by the FDA and other regulatory agencies.

### VI.F.3.2. Estimated Animal Exposure to MON 88302

Together, canola meal and rapeseed meal are the second most widely traded protein ingredients after soybean meal (CCC, 2009). In the U.S. 2.6 million tons of canola meal were used primarily as animal feed (USDA-ERS, 2010b). A four-week old broiler consumes 16.7 g/kg bw/day when the inclusion rate of canola meal is 20% of the total diet (NRC, 1994). Canola meal intake would be 4.7 g/kg bw/day, assuming 10% dietary inclusion rate, for the young pig and 4.9 g/kg bw/day for the finishing pig, assuming 18% dietary inclusion rate (NRC, 1998). Lactating dairy cows (680 kg bw) producing 45 kg/day of milk consumes 10.3 g/kg bw/day of canola meal assuming a 25% inclusion rate (NRC, 2001).

The exposure of poultry and livestock to MON 88302 would result primarily from feeding canola meal and whole canola seed. Since livestock diets typically contain a much higher level of protein from canola meal than from the whole seeds, estimates of dietary intake will assume consumption as canola meal only. Canola meal from MON 88302 bought for animal feed would be expected to have gone through a series of commingling steps with canola meal from non-MON 88302 sources as it makes its way through commerce. However, for this assessment it will be assumed that canola from MON 88302 is the only canola in the animal diet, as a conservative estimate of its dietary intake as a feed.

#### VI.F.3.2.1. Animal Dietary Intake of CP4 EPSPS from MON 88302

Animals will be exposed to the CP4 EPSPS protein through dietary intake of feed derived from MON 88302. The quantity of canola consumed on a daily basis by poultry and livestock, as well as the levels of CP4 EPSPS protein in MON 88302 are necessary to derive an estimate of daily dietary intake (DDI). DDI is computed as follows:

DDI (μg CP4 EPSPS protein/kg bw/day) = Daily consumption of canola meal (g/kg bw/day) × CP4 EPSPS protein concentration (μg/g) × $10^{-6}$ (g/μg)

For the purpose of this dietary intake calculation, which is to characterize a highly conservative scenario for exposure of animals to CP4 EPSPS protein from the consumption of canola from MON 88302, the mean and maximum protein concentrations of CP4 EPSPS protein reported for canola seed were used. The mean and maximum values of CP4 EPSPS protein in canola seed used in this assessment were from MON 88302 grown in the U.S. and Canada in 2009 (Section VI.D.). Also, to calculate the highest animal exposure to CP EPSPS protein, several assumptions were made. First,

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canola meal bought for animal feed would be expected to have gone through a series of commingling steps as it makes its way through commerce, and therefore be mixed with canola meal derived from canola seeds that were not MON 88302. However, this assessment will use the assumption that canola meal from MON 88302 would be the only source of canola in the diet and would not be commingled with non-MON 88302 sources. Second, statistics from Statistics Canada for 2010 indicate that during the crushing process 2.7 million metric tons (M MT) of canola meal were produced from 4.8 M MT of canola resulting in a crushing yield of 56% (Statistics Canada, 2010). The mean level of CP4 EPSPS protein in canola seed from MON 88302 is 27 µg/g dry weight (dw) with a maximum of 46 µg/g dw. Therefore, assuming a crushing yield of 56% and no loss of CP4 EPSPS protein during crushing, the calculated mean and maximum levels of CP4 EPSPS protein in canola meal derived from MON 88302 canola seed would be 48 µg/g dw and 82 µg/g dw, respectively.

The estimated mean and maximum poultry and livestock dietary exposures to CP4 EPSPS protein from MON 88302 are shown in Table VI-8.

The broiler chicken, young pig, finishing pig, and lactating dairy cow would typically consume 18 g dietary protein/kg bw (NRC, 1994), 14 g dietary protein/kg bw (NRC, 1998), 4 g dietary protein/kg bw (NRC, 1998), and 6 g dietary protein/kg bw (NRC, 2001), respectively. The highest percentage of CP4 EPSPS protein (g/kg bw) per total protein consumed was for the lactating dairy cow at 0.0141% (g/g) of the total dietary protein intake (0.00084 g CP4 EPSPS/kg bw divided by 6 g/kg bw of dietary protein).

In the most conservative scenario, poultry, swine and lactating dairy cattle would be consuming less than 0.015% (g/g) of their total protein intake as CP4 EPSPS protein from MON 88302.

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**Table VI-8. Mean and Maximum Daily Intake of the CP4 EPSPS Protein by Poultry, Swine, and Cattle (g/kg body weight/day)**

| Species | Total Consumption of Canola Meal g/kg bw/day[2] | CP4 EPSPS Protein Intake[1] Mean Level | Maximum Level |
|---|---|---|---|
| | | g/kg bw/day | |
| Chicken broiler | 16.7 | 0.00080 | 0.00137 |
| Young pig | 4.7 | 0.00023 | 0.00039 |
| Finishing pig | 4.9 | 0.00024 | 0.00040 |
| Lactating dairy cow | 10.3 | 0.00050 | 0.00084 |

[1] Canola meal consumed × estimated mean or maximum concentration of CP4 EPSPS protein in MON 88302-derived meal.
[2] Dry weight basis.

### VI.F.4. Potential Allergenicity or Toxicity of CP4 EPSPS Protein Produced in MON 88302 Summary and Conclusion

MON 88302-produced CP4 EPSPS protein possesses a strong safety profile. Its donor organism, *Agrobacterium* sp. strain CP4, is ubiquitous in the environment, and is not commonly known for human or animal pathogenicity or allergenicity. Furthermore, *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding other approved Roundup Ready crops. MON 88302-produced CP4 EPSPS protein is present at a very low level in the harvested seed of MON 88302, therefore, constitutes a very small portion of the total protein present in food and feed derived from MON 88302. MON 88302-produced CP4 EPSPS protein lacks structural similarity to allergens or toxins or other proteins known to have adverse effects on mammals. The CP4 EPSPS protein was rapidly digested in SGF and SIF, lost activity upon heating, and demonstrated no oral toxicity in mice at the level tested. In addition, no consumption of the CP4 EPSPS protein derived from MON 88302 is expected for the U.S. general population at the present time. Finally, the overall animal exposure as a percent of total protein is demonstrated to be small.

Based on the above information, the consumption of the CP4 EPSPS protein from seed of MON 88302 or products derived from MON 88302 is considered safe for humans and animals.

## VI.G. Bioinformatic Assessment of Putative Open Reading Frames (ORFs) of MON 88302 Insert and Flanking Sequences

The 2009 Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2009) includes an assessment element on the identification and evaluation of "open reading frames within the inserted DNA or created by the insertion with contiguous plant genomic DNA". These assessments examine the potential homology of any putative polypeptides or proteins that could be produced from open reading frames (ORFs) in the insert or at the plant-insert junction to known toxins or allergens. These analyses are conducted even if there is no evidence that such ORFs at the plant-insert junction or alternative reading frames in the insert are capable of being transcribed or translated into a protein. Results from these bioinformatic analyses demonstrate that any putative polypeptides in MON 88302 are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

In addition to the bioinformatic analysis conducted on MON 88302-produced CP4 EPSPS protein (see Sections VI.F.1.3 and VI.F.2.1) bioinformatic analyses were also performed on the MON 88302 insert and flanking genomic DNA sequences to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by all six reading frames present in the MON 88302 insert DNA (Table V-2), as well as ORFs present in the 5' and 3' inserted DNA-5' and 3' flanking sequence junctions. These various bioinformatic evaluations are depicted in Figure VI-11. ORFs spanning the 5' flanking sequence DNA-inserted DNA junctions, and 3' flanking sequence DNA-inserted DNA junctions were translated from stop codon to stop codon in all six reading

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frames (three forward reading frames and three reading frames in reverse complement orientation). Putative peptides/polypeptides from each reading frame were then compared to toxin, allergen, and all protein databases using bioinformatic tools. Similarly, the entire MON 88302 insert DNA sequence was translated in all six reading frames (three forward reading frames and three reading frames in reverse complement orientation) and the resulting amino acid sequence was subjected to bioinformatic analyses. There are no analytical data that indicate any putative polypeptides/proteins subjected to bioinformatic evaluation other than the MON 88302-produced CP4 EPSPS protein which is part of the insert DNA sequence analysis are produced. Moreover, the data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than MON 88302-produced CP4 EPSPS protein was derived from frames 1 to 6 of the insert DNA, or the ORFs spanning the insert junctions; they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Therefore, there is no evidence for concern regarding the putative polypeptides for MON 88302 relatedness to known toxins and allergens, or biologically active putative peptides.

### VI.G.1. Bioinformatics Assessment of Insert DNA Reading Frames

Bioinformatic analyses were performed to assess the potential of toxicity, allergenicity or biological activity of any putative peptides encoded by translation of reading frames 1 through 6 of the inserted DNA in MON 88302 (Figure VI-11).

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2010, TOX_2010, and PRT_2010 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity and alignment length as 35% or greater identity in 80 or greater amino acids (to ascertain if alignments exceeded Codex (Codex Alimentarius, 2009) thresholds for FASTA searches of the AD_2010 database), and the *E*-score. Alignments having *E*-score less than $1 \times 10^{-5}$ are deemed significant because they may reflect shared structure and function among sequences. In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope (Silvanovich et al., 2006) and evaluated against the AD_2010 database.

The results of the search comparisons showed that no relevant structural similarity to known allergens and toxins were observed for any of the putative polypeptides when compared to proteins in the allergen (AD_2010) or toxin (TOX_2010) databases. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database.

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When used to search the PRT_2010 database, translations of frames 1 to 5 yielded alignments with *E*-scores less than or equal to a $1\times10^{-5}$ threshold. Translation of frame 1 yielded numerous alignments with *E*-scores less than or equal to $1\times10^{-5}$ when used to search the PRT_2010 database. The top alignment yielding the most significant *E*-score positively identified CP4 EPSPS in the MON 88302 T-DNA insert. Translation of frame 2 yielded two alignments with *E*-scores less than or equal to $1\times10^{-5}$. The top alignment yielding the most significant *E*-score was with an unknown protein product derived from Figwort Mosaic Virus. This result is not unexpected as the translated DNA sequence yielding this alignment was derived from the promoter for CP4 EPSPS which is partially derived from Figwort Mosaic Virus. While this alignment reflects conserved structure, there is no indication that it reflects the potential for adverse biological activity.

Translation of frames 3 and 5 each yielded two alignments with *E*-scores less than or equal to $1\times10^{-5}$. Inspection of the alignments for both the frame 3 and 5 translations revealed that the query sequences were punctuated with numerous stop codons and required numerous gaps to optimize the alignment. As a result, it is unlikely these alignments reflect conserved structure. Translation of frame 4 yielded an alignment displaying an *E*-score of $4\times10^{-7}$ with an unknown amino acid sequence found in a patent submission. The alignment which displayed only 27.7% identity in a 173 amino acid overlap did not provide any indication of the potential for adverse effects human or animal health if it were to be produced. Taken together, these data demonstrate the lack of relevant similarities between known allergens and toxins for putative peptides derived from all six reading frames from the inserted DNA sequence of MON 88302. As a result, in the unlikely event that any translation products other than MON 88302-produced CP4 EPSPS protein were derived from reading frames 1 to 6, then such putative polypeptides would not be expected to be cross-reactive allergens, toxins, or display adverse biological activity.

### VI.G.2. Insert Junction Open Reading Frame Bioinformatics Analysis

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' genomic junctions of the MON 88302 inserted DNA were performed using a bioinformatic comparison strategy. The purpose of the assessment is to evaluate the potential for novel open reading frames (ORFs) that may have homology to known allergens, toxins, or proteins that display adverse biological activity. Sequences spanning the 5' genomic DNA-T-DNA and the 3' genomic DNA-intervening DNA and/or intervening DNA-T-DNA junctions, (Figure VI-11) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. The resulting putative polypeptides from each reading frame, that were eight amino acids or greater in length, were compared to AD_2010, TOX_2010, and PRT_2010 databases using FASTA and to the AD_2010 database using an eight amino acid sliding window search.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2010, TOX_2010, and PRT_2010 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent

identity, and alignment length as 35% or greater identity in 80 or greater amino acids to ascertain if alignments exceeded Codex (Codex Alimentarius, 2009) (thresholds for FASTA searches of the AD_2010 database), and the *E*-score. Alignments having an *E*-score less than $1\times10^{-5}$ are deemed significant because they may reflect shared structure and function among sequences. In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope (Silvanovich et al., 2006) and evaluated against the AD_2010 database.

No biologically relevant structural similarity to known allergens and toxins was observed for any of the putative polypeptides. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. As a result, in the unlikely event that any translation products were derived from DNA spanning the 5' or 3' genomic DNA insert DNA junctions of MON 88302, then such putative polypeptides would not be expected to be cross-reactive allergens, toxins, or display adverse biological activity.

### VI.G.3. Bioinformatic Assessment of Allergenicity, Toxicity, and Adverse Biological Activity Potential of MON 88302 Polypeptides Putatively Encoded by the Insert and Flanking Sequences Summary and Conclusions

A conservative bioinformatic assessment of potential allergenicity, toxicity and adverse biological activity for putative polypeptides that span the 5' and 3' insert junctions or were derived from different reading frames of the entire insert was conducted for MON 88302. The data generated from these analyses confirm that even in the highly unlikely occurrence that translation products other than MON 88302-produced CP4 EPSPS protein were derived from frames 1 to 6 of the insert DNA, or the insert junctions, they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. Therefore, there is no evidence for concern regarding health implications of putative polypeptides for MON 88302.

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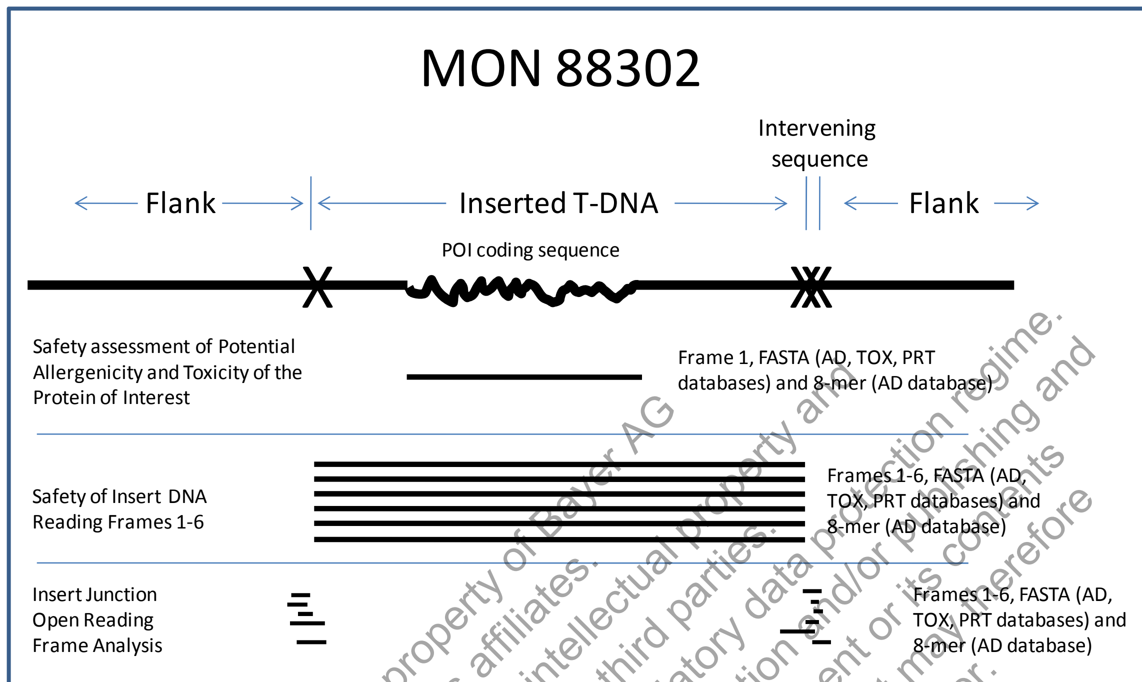


AD = AD_2010; TOX = TOX_2010 and PRT = PRT_2010 (GenBank release 175); 8-mer = the eight amino acid sliding window search

**Figure VI-11. Schematic Summary of MON 88302 Bioinformatic Analyses**

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## VI.H. Safety Assessment of Expressed Products Summary and Conclusion

The data and information provided in this section address the questions important to the food and feed safety of the CP4 EPSPS protein in MON 88302 including its potential allergenicity and toxicity. To summarize, the physicochemical characteristics of the CP4 EPSPS protein were determined and shown to be equivalent to those of an *E. coli*-produced CP4 EPSPS protein. The expression levels of the CP4 EPSPS protein in selected tissues of MON 88302 were determined. The donor organism for the CP4 EPSPS coding sequence, *Agrobacterium* sp. strain CP4, is ubiquitous in the environment, is not commonly known for human or animal pathogenicity, or allergenicity. The CP4 EPSPS protein is present at a very low level in the harvested seed of MON 88302 and, therefore, constitutes a very small portion of the total protein present in food and feed derived from MON 88302. A bioinformatic analysis confirmed that the CP4 EPSPS protein lacks structural similarity to known allergens and toxins, or other proteins known to have adverse effects on mammals. The CP4 EPSPS protein was rapidly digested in simulated digestive fluids. The CP4 EPSPS protein lost activity upon heating, therefore, it is reasonable to conclude that it would not be consumed as an active protein in food products. The CP4 EPSPS protein demonstrated no oral toxicity in mice at the level tested. Based on the above information, the consumption of the CP4 EPSPS protein from MON 88302 or its progeny, and the consumption of food and feed products derived from MON 88302 or its progeny are considered safe for humans and animals. Finally, bioinformatics analyses demonstrate the lack of relevant similarities between known allergens and toxins and all putative peptides derived from all six reading frames from the entire inserted DNA sequence of MON 88302 or its flanking sequences.

Taken together, this safety assessment reaffirms the earlier conclusion that the CP4 EPSPS protein expressed in MON 88302, as in other Roundup Ready crops, does not pose a significant health risk. Finally, in the unlikely event that translation products other than the CP4 EPSPS protein were to be produced, they would pose no allergenic or toxic risk.

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## VII. COMPOSITIONAL ASSESSMENT OF MON 88302

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by the FDA. The CP4 EPSPS protein expressed in MON 88302 is identical to the CP4 EPSPS protein in other Roundup Ready crops and the mode of action of CP4 EPSPS protein is well understood. Previous Roundup Ready crops reviewed by the FDA have had no biologically relevant compositional changes identified, and there is no reason to expect the CP4 EPSPS protein in MON 88302 would affect nutritionally important nutrients, toxicants, and anti-nutrients present in seed from this new product.

Safety assessments of biotechnology-derived crops typically include comparisons of the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop to that of conventional counterparts (Codex Alimentarius, 2009). Compositional assessments are performed using the principles and analytes outlined in the OECD consensus document for canola composition (OECD, 2001).

A recent review of compositional assessments conducted according to OECD guidelines that encompassed a total of seven biotechnology-derived crop varieties, nine countries and eleven growing seasons concluded that incorporation of biotechnology-derived agronomic traits has had little impact on natural variation in crop composition. Most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan et al., 2010). Compositional quality, therefore, implies a very broad range of endogenous levels of individual constituents. Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients and anti-nutrients and secondary metabolites that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan et al., 2010; Mailer and Pratley, 1990; Marwede et al., 2004; Naczk et al., 1998; OECD, 2001; Pritchard et al., 2000; Reynolds et al., 2005; Ridley et al., 2004; Werteker et al., 2010).

Compositional equivalence between biotechnology-derived and conventional crops provides an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2002). OECD consensus documents on compositional considerations for new crop varieties emphasize quantitative measurements of essential nutrients and known anti-nutrients. This is based on the premise that such comprehensive and detailed analyses will most effectively discern any compositional changes that imply potential nutritional or safety (*e.g.*, anti-nutritional) concerns. Levels of the components in seed and forage of the biotechnology-derived crop are compared to: 1) corresponding levels in a conventional comparator, the genetically similar conventional line, grown concurrently, under field conditions, and 2) natural ranges generated from an evaluation of commercial reference varieties grown concurrently and from data published in the scientific literature. The comparison to data published in the literature places any potential differences between the assessed crop and its comparator in the context of the well-documented variation in the concentrations of crop nutrients, toxicants, and anti-nutrients.

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This section provides analyses of concentrations of key nutrients, toxicants, and anti-nutrients of MON 88302 compared with equivalent analyses of a conventional counterpart grown and harvested under the same conditions, as appropriate. In addition, commercial canola reference varieties were included in the composition analyses to establish a range of natural variability for each analyte, defined by a 99% tolerance interval. The production of materials for the compositional analyses used field designs to allow accurate assessments of compositional characteristics over a range of environmental conditions under which MON 88302 is expected to be grown. Design parameters included a sufficient number of trial sites to allow adequate exposure to the variety of conditions met in nature. Field sites were replicated with an adequate number of plants samples, and the methods of analysis were sufficiently sensitive and specific to detect variations in the components measured and to allow statistically rigorous analyses. The information provided in this section also addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45 for compositional analyses (Codex Alimentarius, 2009).

## VII.A. Compositional Equivalence of MON 88302 Seed to Conventional Canola

Compositional analysis comparing MON 88302 to the conventional control variety (Ebony) and commercial reference varieties demonstrated that MON 88302 is compositionally equivalent to conventional canola. Seed samples were collected from MON 88302 and the conventional control grown in a 2009 North American field production. Canola forage is rarely consumed by animals and is not a source of nutrition for humans. Therefore, the OECD consensus document on compositional considerations for canola (OECD, 2001) does not recommend analysis of canola forage, and forage samples were not collected. The background genetics of the conventional control were similar to that of MON 88302, but did not contain the *cp4 epsps* expression cassette. Seven different commercial reference varieties were included across all sites of the field production to provide data on natural variability of each compositional component analyzed. The samples utilized for compositional analysis were obtained from two U.S. sites [Wilkin County, MN (MNCA) and McHenry County, North Dakota (NDVA)] and three Canadian sites [Portage la Prairie, Manitoba (MBPL); Newton, Manitoba (MBNW); and Saskatoon, Saskatchewan (SKSA)]. The sites were planted in a randomized complete block design with four replicates per site. MON 88302, the conventional control, and commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition to the conventional weed control programs, MON 88302 plots were treated at the 5-6 leaf stage with a glyphosate application at a target rate of 1.6 lb acid equivalents per acre (1800 g a.e./ha).

Compositional analyses were conducted as recommended for canola seed (OECD, 2001) to assess whether levels of key nutrients, toxicants and anti-nutrients in MON 88302 were equivalent to levels in the conventional control and to the composition of commercial reference varieties. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and total fat), fibers (acid detergent fiber [ADF], neutral detergent fiber [NDF], and total dietary fiber [TDF]), amino acids (18 components), fatty acids (FA; C8-C24,), vitamin E (α-tocopherol), and minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and

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zinc) in seed. The toxicants assessed in seed included erucic acid and glucosinolates (alkyl glucosinolates [including 3-butenyl, 4-pentenyl, 2-hydroxy-3-butenyl, and 2-hydroxy-4-pentenyl glucosinolates], indolyl glucosinolates [including 3-indolylmethyl and 4-hydroxy-3-indoylmethyl], and total glucosinolates). The anti-nutrients assessed in seed included phytic acid and sinapine (as sinapic acid). Methods used in the assessments of nutrients, toxicants and anit-nutrients are found in Appendix H. The toxicant and anti-nutrient results are discussed together under the general heading of anti-nutrients. In all, 70 different components were measured. Of those 70 components, 18 nutrients and one toxicant (18 fatty acids, including erucic acid, and one mineral) had more than 50% of the observations below the assay limit of quantitation (LOQ) and, as a result, were excluded from the statistical analyses. Therefore, 51 components were statistically assessed using a mixed model analysis of variance method. Values for all components were expressed on a dry weight basis with the exception of moisture, expressed as percent fresh weight, and fatty acids, expressed as percent of total FA.

For MON 88302, six statistical comparisons to the conventional control were conducted for each compositional component. One comparison was based on compositional data combined across all five field sites (combined-site analysis) and five separate comparisons were conducted on data from each of the individual field sites. Statistically significant differences were identified at the 5% level ($\alpha = 0.05$). Data from the commercial reference varieties were combined across all sites and used to calculate a 99% tolerance interval for each compositional component to define the natural variability of each component in canola varieties that have a history of safe consumption, and that were grown concurrently with MON 88302 and the conventional control in the same trial.

For the combined-site analysis, significant differences in nutrient, toxicant, and anti-nutrient components were further evaluated using considerations relevant to the safety and nutritional quality of MON 88302 when compared to the conventional control, which is the conventional counterpart with a history of safe consumption. Considerations used to assess the relevance of each combined-site statistically significant difference included: 1) the relative magnitude of the difference in the mean values of nutrient, toxicant, and anti-nutrient components of MON 88302 and the conventional control; 2) whether the MON 88302 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of the commercial reference varieties grown concurrently in the same trial; 3) evaluation of the reproducibility of the statistically significant ($\alpha = 0.05$) combined-site component differences at individual sites; and 4) an assessment of the differences within the context of natural variability of commercial canola composition published in the scientific literature. If statistically significant differences detected in the individual site analyses were not observed in the combined-site analysis, they were not considered further for the compositional assessment of safety. Statisitical summaries of nutrients, toxicants and anti-nutrients for individual sites are found in Appendix H.

This analysis provides a comprehensive comparative assessment of the levels of key nutrients, toxicants, and anti-nutrients in seed of MON 88302 and the conventional control, discussed in the context of natural variability in composition of commercial canola. Results of the comparison indicate that the composition of the seed of

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MON 88302 is equivalent to that of the conventional control and within the natural variability of commercial reference varieties.

### VII.A.1. Nutrient Levels in Seed

In the combined-site analysis of nutrient levels in seed, the following components showed no significant differences in mean values between MON 88302 and the conventional control: proximates, two types of fiber (ADF and NDF), 18 amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine), four fatty acids (16:0 palmitic acid, 20:1 eicosenoic acid, 24:0 lignoceric acid, and 24:1 nervonic acid), eight minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, and zinc), and vitamin E (Table VII-2).

The components that showed significant differences in mean values between MON 88302 and the conventional control in the combined-site analysis were: total dietary fiber (TDF) and seven fatty acids (16:1 palmitoleic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic acid, 20:0 arachidic acid, and 22:0 behenic acid) (Tables VII-1 and VII-2).

1) The statistically significant differences in nutrients were evaluated using considerations relevant to the nutritional quality of MON 88302 when compared to the conventional control: eight combined-site nutrient significant differences ($\alpha = 0.05$) between MON 88302 and the conventional control were attributable to TDF (expressed as % dry weight) and seven fatty acids (expressed as % total FA). The relative magnitudes of differences between the combined-site mean values for MON 88302 and the conventional control showed an increase for TDF, 18:2 linoleic acid, and 18:3 linolenic acid, (13.81%, 8.98%, and 20.01%, respectively) and a decrease for 16:1 palmitoleic acid, 18:0 stearic acid, 18:1 oleic acid, 20:0 arachidic acid, and 22:0 behenic acid (7.56%, 15.06%, 4.52%, 10.68%, and 6.01%, respectively). The relative differences in these components in the across-site analysis and at individual sites were between 3.48% and 28.69% (Table VII-2 and Tables H-3, H-5, H-7, H-9, and H-11). The magnitudes of differences observed between MON 88302 and the conventional control were small relative to the natural variability of these components as determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption as presented in the tables referenced above.

2) Mean values for all of the nutrient components found to be significantly different ($\alpha = 0.05$) from the combined-site analysis of MON 88302 were within the 99% tolerance interval established from the commercial references grown concurrently and were, therefore, within the range of natural variability of that component in commercial canola varieties with a history of safe consumption (Table VII-1).

3) Assessment of the reproducibility of the combined-site differences at the five individual sites demonstrated no significant differences for TDF; however, significant differences ($\alpha = 0.05$) were observed for 18:0 stearic acid, 18:1 oleic

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acid, and 18:2 linoleic acid at all five sites; significant differences for 16:1 palmitoleic acid and 18:3 linolenic acid at four sites, significant differences for 20:0 arachidic acid at three sites, and significant differences for 22:0 behenic acid at two sites (Table VII-1). The magnitudes of differences between the mean fatty acid values for MON 88032 and the conventional control were small relative to the variability of these components as determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption, and relative to the variability of fatty acid components in canola due to environment (Pritchard et al., 2000). Individual site mean values of MON 88302 for all nutrient components with significant differences fell within the 99% tolerance interval established from the commercial reference varieties grown concurrently and were, therefore, within the range of natural variability of that component in commercial canola varieties with a history of safe consumption (Table VII-2).

4) With the exception of TDF, for which no commercial reference values have been published, all of the compositional components identified as significantly different from the conventional control were within the natural variability of these components in commercial canola composition as published in the scientific literature (Table VII-4).

In summary, the combined-site statistical analysis identified eight significant differences ($\alpha = 0.05$) that were small in magnitude relative to their natural variability as determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption.

Of these significant differences, only 18:0 stearic acid, 18:1 oleic acid, and 18:2 linoleic acid were observed consistently at all of the individual sites. All of the components identified as significantly different in the combined-site analysis and corresponding individual site analyses, were within the natural variability of commercial canola defined by the 99% tolerance interval established by the concurrently grown commercial reference varieties, and were within the published literature ranges (TDF does not have published reference data). Therefore, these significant differences are not meaningful to food and feed safety and nutrition. These findings support the conclusion that nutrients in seed from MON 88302 are compositionally equivalent to those in conventional canola varieties with a history of safe usage.

### VII.A.2. Anti-Nutrient Levels in Seed

According to OECD (2001), canola seed contains toxicants including erucic acid and glucosinolates, and anti-nutrients, including phytic acid and sinapine. Erucic acid has been shown to have cardiopathic potential resulting in a weakening of the heart muscle in experimental animals (Bozcali et al., 2009; Chien et al., 1983). Glucosinolates in canola seed can be characterized into two main chemical groups, alkyl and indolyl, with alkyl being the most common (CCC, 2009). Upon enzymatic hydrolysis with myrosinase, certain glucosinolates form compounds that can depress growth and thyroid function (Bell, 1984). The standard for glucosinolates in canola seed is <18 µmoles/g

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(Szmigielska et al., 2000). Phytic acid is present in canola seed. Phytic acid chelates mineral nutrients, including calcium, magnesium, potassium, iron, and zinc, rendering them biologically unavailable to monogastric animals consuming the seed (Liener, 2000). Sinapine is the choline ester of sinapic acid, the primary phenolic component in canola seed. Sinapine imparts a bitter taste and reduces palatability of the seed (OECD, 2001). Sinapine levels were determined based on quantitation of the hydrolysis product, sinapic acid.

MON 88302 levels of 22:1 erucic acid were below the level of detection (0.04% total FA) in canola seed, and therefore, 22:0 erucic acid was excluded from statistical analysis. In the combined-site analysis, no significant difference ($\alpha = 0.05$) was observed between MON 88302 and the conventional control (Tables VII-1 and VII-3) for indolyl glucosinolates, total glucosinolates, phytic acid, and sinapine. One statistically significant difference was identified for alkyl glucosinolates, and the net effect was a slight reduction of this anti-nutrient in MON 88302. The following considerations show that this difference is not a meaningful concern from a food/feed nutritional or safety perspective:

1) The magnitude of the difference between the combined-site mean value for alkyl glucosinolates in MON 88302 and the conventional control showed a 27.59% decrease. This magnitude of difference was small relative to the natural variability of these components as determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption.

2) The MON 88302 mean alkyl glucosinolates value from the combined-site analysis was within the 99% tolerance interval established from the commercial reference varieties grown concurrently. The mean value was, therefore, within the range of natural variability for alkyl glucosinolates in commercial canola varieties with a history of safe consumption (Tables VII-1 and VII-3).

3) Assessment of the reproducibility of the combined-site difference at the five individual sites was not consistent across sites. A significant difference for alkyl glucosinolates was observed at one of the individual sites. However, the mean value for alkyl glucosinolates in MON 88302 at this individual site was within the 99% tolerance interval established from the concurrently grown commercial reference varieties.

4) An assessment based on of the natural variability of alkyl glucosinolates in commercial canola varieties could not be made because a range was not available in the scientific literature.

In summary, the statistical analyses found a combined-site significant difference in alkyl glucosinolates that was lower than the conventional mean value, and not consistently observed at the individual sites. The mean alkyl glucosinolates value for MON 88302 was within the natural variability of commercial canola defined by the 99% tolerance interval established from the concurrently grown commercial reference varieties with a

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history of safe consumption, and the value was within the safety threshold for canola. Total glucosinolate levels in seed from MON 88302 ranged from 1.73 to 11.42 µmoles/g (Table VII-3 and Tables H-4, H-6, H-8, H-10, and H-12), within the standard for canola. Thus, an evaluation of anti-nutrient components in seed supports the conclusion that MON 88302 is as safe as and compositionally equivalent to conventional canola.

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**Table VII-1. Summary of Differences ($p < 0.05$) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (MON 88302 minus Control): Mean Difference (% of Control) | Mean Difference (MON 88302 minus Control): Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
|---|---|---|---|---|---|---|
| **Statistical Differences Observed in Combined-Site Analysis** | | | | | | |
| **Seed Fiber (% dw)** | | | | | | |
| Total Dietary Fiber | 20.90 | 18.37 | 13.81 | 0.004 | 16.91 - 27.81 | 13.97, 24.85 |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 16:1 Palmitoleic | 0.22 | 0.24 | -7.56 | 0.008 | 0.20 - 0.26 | 0.17, 0.30 |
| 18:0 Stearic | 1.68 | 1.98 | -15.06 | <0.001 | 1.54 - 1.87 | 0.90, 3.05 |
| 18:1 Oleic | 62.82 | 65.79 | -4.52 | <0.001 | 60.51 - 65.20 | 56.13, 70.69 |
| 18:2 Linoleic | 19.26 | 17.67 | 8.98 | <0.001 | 17.78 - 20.66 | 12.60, 24.49 |
| 18:3 Linolenic | 9.58 | 7.98 | 20.01 | <0.001 | 8.71 - 11.23 | 6.96, 11.73 |
| 20:0 Arachidic | 0.54 | 0.60 | -10.68 | <0.001 | 0.50 - 0.57 | 0.45, 0.80 |
| 22:0 Behenic | 0.27 | 0.28 | -6.01 | 0.016 | 0.24 - 0.29 | 0.19, 0.43 |

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**Table VII-1. Summary of Differences ($p<0.05$) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (MON 88302 minus Control): Mean Difference (% of Control) | Mean Difference (MON 88302 minus Control): Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
|---|---|---|---|---|---|---|
| **Statistical Differences Observed in Combined-Site Analysis** | | | | | | |
| **Seed Anti-nutrient** | | | | | | |
| Alkyl Glucosinolate (µmole/g dw) | 3.68 | 5.08 | -27.59 | 0.035 | 1.19 - 5.87 | 0, 29.02 |
| **Statistical Differences Observed in More than One Individual Site** | | | | | | |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 18:0 Stearic Site MBNW | 1.73 | 1.97 | -12.23 | 0.028 | 1.64 - 1.87 | 0.90, 3.05 |
| 18:0 Stearic Site MBPL | 1.58 | 1.87 | -15.64 | <0.001 | 1.55 - 1.59 | 0.90, 3.05 |
| 18:0 Stearic Site MNCA | 1.67 | 1.86 | -10.01 | 0.022 | 1.65 - 1.71 | 0.90, 3.05 |
| 18:0 Stearic Site NDVA | 1.77 | 2.11 | -16.06 | 0.004 | 1.71 - 1.84 | 0.90, 3.05 |
| 18:0 Stearic Site SKSA | 1.66 | 2.08 | -20.14 | 0.001 | 1.54 - 1.72 | 0.90, 3.05 |
| 18:1 Oleic Site MBNW | 63.40 | 65.71 | -3.51 | 0.004 | 62.94 - 64.03 | 56.13, 70.69 |

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**Table VII-1. Summary of Differences ($p<0.05$) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (MON 88302 minus Control) Mean Difference (% of Control) | Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
|---|---|---|---|---|---|---|
| **Statistical Differences Observed in More than One Individual Site** | | | | | | |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 18:1 Oleic Site MBPL | 62.06 | 64.30 | -3.48 | <0.001 | 61.82 - 62.35 | 56.13, 70.69 |
| 18:1 Oleic Site MNCA | 61.67 | 64.86 | -4.92 | 0.005 | 61.70 - 61.87 | 56.13, 70.69 |
| 18:1 Oleic Site NDVA | 65.14 | 68.38 | -4.74 | 0.003 | 64.90 - 65.20 | 56.13, 70.69 |
| 18:1 Oleic Site SKSA | 61.91 | 65.69 | -5.75 | 0.001 | 60.51 - 62.29 | 56.13, 70.69 |
| 18:2 Linoleic Site MBNW | 19.27 | 17.89 | 7.71 | 0.011 | 18.82 - 19.66 | 12.60, 24.49 |
| 18:2 Linoleic Site MBPL | 20.43 | 19.18 | 6.50 | <0.001 | 20.13 - 20.66 | 12.60, 24.49 |
| 18:2 Linoleic Site MNCA | 20.20 | 18.35 | 10.07 | 0.001 | 20.00 - 20.32 | 12.60, 24.49 |
| 18:2 Linoleic Site NDVA | 17.86 | 15.71 | 13.67 | 0.009 | 17.78 - 18.02 | 12.60, 24.49 |

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**Table VII-1. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (MON 88302 minus Control) Mean Difference (% of Control) | Mean Difference (MON 88302 minus Control) Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
|---|---|---|---|---|---|---|
| **Statistical Differences Observed in More than One Individual Site** | | | | | | |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 18:2 Linoleic Site SKSA | 18.49 | 17.22 | 7.36 | 0.019 | 18.08 - 19.48 | 12.60, 24.49 |
| **Seed Vitamin (mg/100g dw)** | | | | | | |
| Vitamin E (a-tocopherol) Site MBNW | 13.06 | 9.36 | 39.51 | 0.004 | 12.22 - 13.47 | 3.88, 17.28 |
| Vitamin E (α-tocopherol) Site MBPL | 11.50 | 7.63 | 50.83 | <0.001 | 10.70 - 12.20 | 3.88, 17.28 |
| Vitamin E (α-tocopherol) Site MNCA | 13.39 | 10.82 | 23.73 | 0.006 | 12.58 - 14.62 | 3.88, 17.28 |
| Vitamin E (α-tocopherol) Site NDVA | 15.89 | 9.43 | 68.39 | 0.010 | 15.23 - 16.55 | 3.88, 17.28 |
| Vitamin E (α-tocopherol) Site SKSA | 1.49 | 6.91 | -78.47 | 0.019 | 1.30 - 1.66 | 3.88, 17.28 |
| **Seed Anti-nutrient** | | | | | | |
| Sinapic Acid (% dw) Site MBNW | 1.02 | 0.92 | 10.34 | 0.001 | 0.99 - 1.06 | 0.57, 1.13 |

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**Table VII-1. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| | | | Mean Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (% of Control) | Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
| **Statistical Differences Observed in More than One Individual Site** | | | | | | |
| **Seed Anti-nutrient** | | | | | | |
| Sinapic Acid (% dw) Site MBPL | 0.97 | 0.86 | 12.04 | <0.001 | 0.95 - 0.99 | 0.57, 1.13 |
| Sinapic Acid (% dw) Site MNCA | 1.06 | 0.96 | 10.66 | 0.001 | 1.02 - 1.08 | 0.57, 1.13 |
| Sinapic Acid (% dw) Site NDVA | 1.02 | 0.83 | 23.56 | 0.001 | 1.00 - 1.04 | 0.57, 1.13 |
| Sinapic Acid (% dw) Site SKSA | 0.22 | 0.81 | -73.12 | 0.001 | 0.16 - 0.28 | 0.57, 1.13 |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 16:1 Palmitoleic Site MBNW | 0.21 | 0.23 | -9.71 | 0.015 | 0.20 - 0.21 | 0.17, 0.30 |
| 16:1 Palmitoleic Site MBPL | 0.23 | 0.25 | -10.10 | 0.008 | 0.22 - 0.23 | 0.17, 0.30 |
| 16:1 Palmitoleic Site MNCA | 0.21 | 0.24 | -10.88 | 0.001 | 0.21 - 0.21 | 0.17, 0.30 |
| 16:1 Palmitoleic Site NDVA | 0.20 | 0.22 | -11.05 | 0.036 | 0.20 - 0.20 | 0.17, 0.30 |

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**Table VII-1. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (MON 88302 minus Control): Mean Difference (% of Control) | Mean Difference (MON 88302 minus Control): Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
|---|---|---|---|---|---|---|
| **Statistical Differences Observed in More than One Individual Site** | | | | | | |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 18:3 Linolenic Site MBNW | 9.19 | 8.12 | 13.27 | 0.004 | 8.88 - 9.42 | 6.96, 11.73 |
| 18:3 Linolenic Site MBPL | 9.28 | 7.74 | 19.89 | <0.001 | 9.12 - 9.43 | 6.96, 11.73 |
| 18:3 Linolenic Site NDVA | 8.82 | 7.31 | 20.67 | <0.001 | 8.71 - 8.94 | 6.96, 11.73 |
| 18:3 Linolenic Site SKSA | 10.78 | 8.38 | 28.69 | <0.001 | 10.39 - 11.23 | 6.96, 11.73 |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 20:0 Arachidic Site MBPL | 0.53 | 0.60 | -11.73 | <0.001 | 0.52 - 0.54 | 0.45, 0.80 |
| 20:0 Arachidic Site NDVA | 0.57 | 0.65 | -12.58 | <0.001 | 0.56 - 0.57 | 0.45, 0.80 |
| 20:0 Arachidic Site SKSA | 0.54 | 0.62 | -13.28 | <0.001 | 0.52 - 0.55 | 0.45, 0.80 |

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**Table VII-1. Summary of Differences ($p<0.05$) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (MON 88302 minus Control): Mean Difference (% of Control) | Mean Difference (MON 88302 minus Control): Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
|---|---|---|---|---|---|---|
| **Statistical Differences Observed in More than One Individual Site** | | | | | | |
| **Seed Mineral** | | | | | | |
| Copper (mg/kg dw) Site MBNW | 3.72 | 3.41 | 9.28 | 0.013 | 3.61 - 3.83 | 2.00, 4.43 |
| Copper (mg/kg dw) Site MBPL | 3.47 | 3.97 | -12.50 | 0.016 | 3.35 - 3.56 | 2.00, 4.43 |
| Copper (mg/kg dw) Site MNCA | 4.40 | 4.11 | 6.91 | 0.027 | 4.16 - 4.57 | 2.00, 4.43 |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 22:0 Behenic Site MBPL | 0.27 | 0.30 | -13.00 | <0.001 | 0.26 - 0.27 | 0.19, 0.43 |
| 22:0 Behenic Site NDVA | 0.27 | 0.30 | -9.83 | 0.007 | 0.27 - 0.27 | 0.19, 0.43 |
| **Seed Mineral** | | | | | | |
| Iron (mg/kg dw) Site MBPL | 44.13 | 51.01 | -13.48 | 0.001 | 42.80 - 45.09 | 23.39, 86.23 |
| Iron (mg/kg dw) Site MNCA | 42.57 | 50.64 | -15.93 | 0.007 | 40.56 - 44.18 | 23.39, 86.23 |

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**Table VII-1. Summary of Differences ($p<0.05$) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| | | | Mean Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (% of Control) | Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
| **Statistical Differences Observed in More than One Individual Site** | | | | | | |
| **Seed Mineral** | | | | | | |
| Potassium (g/100g dw) Site MBPL | 0.70 | 0.77 | -8.91 | 0.023 | 0.63 - 0.76 | 0.39, 0.96 |
| Potassium (g/100g dw) Site SKSA | 0.82 | 0.71 | 15.32 | <0.001 | 0.77 - 0.90 | 0.39, 0.96 |
| Zinc (mg/kg dw) Site MBPL | 31.25 | 33.88 | -7.76 | 0.024 | 30.45 - 32.05 | 20.19, 48.23 |
| Zinc (mg/kg dw) Site SKSA | 41.58 | 33.10 | 25.61 | 0.010 | 39.33 - 45.49 | 20.19, 48.23 |
| **Statistical Differences Observed in One Individual Site** | | | | | | |
| **Seed Proximate (% dw)** | | | | | | |
| Carbohydrates Site MNCA | 27.31 | 25.99 | 5.07 | 0.035 | 26.27 - 27.90 | 23.12, 30.77 |
| Moisture (% fw) Site MNCA | 5.52 | 6.69 | -17.46 | <0.001 | 5.37 - 5.61 | 4.33, 6.91 |
| Protein Site SKSA | 23.82 | 22.14 | 7.58 | 0.038 | 23.62 - 24.58 | 17.20, 30.08 |

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**Table VII-1. Summary of Differences ($p<0.05$) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (MON 88302 minus Control) Mean Difference (% of Control) | Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
|---|---|---|---|---|---|---|
| **Statistical Differences Observed in One Individual Site** | | | | | | |
| **Seed Proximate (% dw)** | | | | | | |
| Total Fat Site NDVA | 48.04 | 45.17 | 6.35 | 0.014 | 47.20 - 48.87 | 39.65, 51.24 |
| **Seed Fiber (% dw)** | | | | | | |
| Acid Detergent Fiber Site MBPL | 16.75 | 14.19 | 18.00 | 0.005 | 15.17 - 18.19 | 6.95, 23.92 |
| Neutral Detergent Fiber Site MBPL | 19.45 | 16.87 | 15.31 | 0.017 | 18.35 - 20.02 | 10.07, 25.94 |
| **Seed Amino Acid (% dw)** | | | | | | |
| Tyrosine Site MBPL | 0.72 | 0.71 | 2.46 | 0.028 | 0.72 - 0.73 | 0.57, 0.81 |
| Valine Site MNCA | 1.15 | 1.24 | -7.32 | 0.048 | 1.13 - 1.15 | 0.92, 1.55 |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 16:0 Palmitic Site SKSA | 4.51 | 4.07 | 10.90 | <0.001 | 4.46 - 4.57 | 2.84, 5.26 |
| 20:1 Eicosenoic Site SKSA | 1.24 | 1.13 | 9.55 | 0.005 | 1.22 - 1.26 | 0.83, 1.68 |

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**Table VII-1. Summary of Differences ($p<0.05$) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean[3] | Control[4] Mean | Mean Difference (MON 88302 minus Control) Mean Difference (% of Control) | Significance (p-Value) | MON 88302 Range | Commercial Tolerance Interval[5] |
|---|---|---|---|---|---|---|
| **Statistical Differences Observed in One Individual Site** | | | | | | |
| **Seed Fatty Acid (% Total FA)** | | | | | | |
| 24:0 Lignoceric Site MBPL | 0.16 | 0.19 | -12.24 | 0.029 | 0.16 - 0.17 | 0.033, 0.25 |
| 24:1 Nervonic Site MBPL | 0.13 | 0.16 | -20.37 | 0.031 | 0.12 - 0.13 | 0.041, 0.18 |
| **Seed Anti-nutrient** | | | | | | |
| Alkyl Glucosinolate (µmole/g dw) Site SKSA | 1.61 | 5.82 | -72.32 | 0.005 | 1.19 - 2.17 | 0, 29.02 |
| Indolyl Glucosinolate (µmole/g dw) Site SKSA | 0.86 | 3.30 | -73.88 | 0.001 | 0.49 - 1.31 | 1.37, 6.62 |
| Total Glucosinolate (µmole/g dw) Site SKSA | 2.53 | 9.22 | -72.58 | 0.002 | 1.73 - 3.51 | 0, 32.20 |

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88302 treated with glyphosate.
[3]Mean = least-square mean.
[4]Control refers to the genetically similar, conventional control Ebony.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

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**Table VII-2. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% CI[3] Lower, Upper | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Proximate (% dw)** | | | | | | |
| Ash | 3.96 (0.18) (3.31 - 4.45) | 3.90 (0.18) (3.20 - 5.10) | 0.055 (0.095) (-0.21 - 0.64) | -0.14, 0.25 | 0.565 | 3.32, 4.66 (2.98 - 4.52) |
| Carbohydrates | 25.96 (0.68) (21.83 - 28.81) | 26.13 (0.68) (23.91 - 28.73) | -0.17 (0.54) (-4.18 - 1.94) | -1.42, 1.09 | 0.765 | 23.12, 30.77 (22.53 - 29.96) |
| Moisture (% fw) | 5.35 (0.34) (3.90 - 6.08) | 5.45 (0.34) (4.41 - 6.98) | -0.10 (0.24) (-1.53 - 0.87) | -0.65, 0.45 | 0.688 | 4.33, 6.91 (4.09 - 8.48) |
| Protein | 23.04 (0.70) (19.68 - 25.98) | 23.14 (0.69) (20.29 - 27.02) | -0.10 (0.52) (-2.29 - 2.50) | -1.32, 1.11 | 0.847 | 17.20, 30.08 (18.68 - 28.32) |
| Total Fat | 47.06 (0.83) (43.96 - 49.26) | 46.82 (0.83) (43.65 - 50.24) | 0.24 (0.52) (-2.28 - 4.10) | -1.00, 1.48 | 0.659 | 39.65, 51.24 (40.71 - 50.26) |
| **Fiber (% dw)** | | | | | | |
| Acid Detergent Fiber | 15.32 (1.36) (9.19 - 20.24) | 14.47 (1.36) (8.94 - 18.71) | 0.84 (0.41) (-2.71 - 3.57) | -0.14, 1.83 | 0.082 | 6.95, 23.92 (9.75 - 21.22) |

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**Table VII-2. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% CI[3] Lower, Upper | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fiber (% dw)** | | | | | | |
| Neutral Detergent Fiber | 17.43 (1.38) (9.48 - 21.36) | 16.70 (1.38) (11.56 - 19.58) | 0.74 (0.57) (-2.74 - 4.43) | -0.58, 2.05 | 0.231 | 10.07, 25.94 (10.93 - 22.75) |
| Total Dietary Fiber | 20.90 (0.79) (16.91 - 27.81) | 18.37 (0.78) (14.58 - 23.00) | 2.54 (0.84) (-0.49 - 9.96) | 0.85, 4.23 | 0.004 | 13.97, 24.85 (12.64 - 26.47) |
| **Amino Acid (% dw)** | | | | | | |
| Alanine | 1.02 (0.025) (0.88 - 1.15) | 1.04 (0.025) (0.93 - 1.19) | -0.015 (0.022) (-0.12 - 0.069) | -0.066, 0.035 | 0.502 | 0.77, 1.34 (0.87 - 1.27) |
| Arginine | 1.45 (0.054) (1.23 - 1.72) | 1.51 (0.054) (1.29 - 1.77) | -0.063 (0.032) (-0.27 - 0.15) | -0.13, 0.00082 | 0.052 | 1.10, 1.93 (1.23 - 1.96) |
| Aspartic Acid | 1.65 (0.067) (1.40 - 1.93) | 1.71 (0.067) (1.46 - 1.97) | -0.055 (0.043) (-0.37 - 0.12) | -0.16, 0.045 | 0.238 | 1.33, 2.12 (1.42 - 2.23) |
| Cystine | 0.57 (0.027) (0.48 - 0.73) | 0.58 (0.027) (0.49 - 0.79) | -0.0044 (0.015) (-0.054 - 0.053) | -0.040, 0.031 | 0.781 | 0.38, 0.83 (0.45 - 0.79) |

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**Table VII-2. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% CI[3] Lower, Upper | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Amino Acid (% dw)** | | | | | | |
| Glutamic Acid | 4.06 (0.18) (3.37 - 5.06) | 4.24 (0.17) (3.64 - 5.26) | -0.19 (0.10) (-0.68 - 0.36) | -0.43, 0.049 | 0.103 | 2.73, 5.89 (3.26 - 5.43) |
| Glycine | 1.14 (0.040) (1.02 - 1.32) | 1.19 (0.040) (1.01 - 1.38) | -0.041 (0.025) (-0.18 - 0.044) | -0.10, 0.018 | 0.142 | 0.96, 1.47 (1.01 - 1.50) |
| Histidine | 0.63 (0.023) (0.55 - 0.77) | 0.65 (0.023) (0.57 - 0.78) | -0.015 (0.011) (-0.065 - 0.044) | -0.038, 0.0074 | 0.181 | 0.47, 0.86 (0.54 - 0.80) |
| Isoleucine | 0.93 (0.028) (0.81 - 1.08) | 0.96 (0.028) (0.82 - 1.12) | -0.024 (0.021) (-0.13 - 0.041) | -0.074, 0.026 | 0.299 | 0.70, 1.22 (0.78 - 1.15) |
| Leucine | 1.64 (0.049) (1.40 - 1.90) | 1.68 (0.049) (1.46 - 1.95) | -0.042 (0.039) (-0.25 - 0.086) | -0.13, 0.048 | 0.308 | 1.21, 2.18 (1.36 - 2.07) |
| Lysine | 1.39 (0.041) (1.22 - 1.63) | 1.41 (0.041) (1.25 - 1.65) | -0.019 (0.023) (-0.12 - 0.086) | -0.064, 0.027 | 0.410 | 1.02, 1.90 (1.20 - 1.68) |

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**Table VII-2. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% CI[3] Lower, Upper | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Methionine | 0.46 (0.015) (0.40 - 0.54) | 0.46 (0.015) (0.40 - 0.56) | -0.0018 (0.0089) (-0.038 - 0.034) | -0.022, 0.019 | 0.847 | 0.30, 0.65 (0.36 - 0.57) |
| Phenylalanine | 0.98 (0.029) (0.84 - 1.11) | 1.00 (0.028) (0.87 - 1.15) | -0.024 (0.024) (-0.17 - 0.044) | -0.079, 0.031 | 0.348 | 0.77, 1.26 (0.84 - 1.25) |
| Proline | 1.40 (0.054) (1.20 - 1.71) | 1.42 (0.054) (1.20 - 1.73) | -0.028 (0.027) (-0.16 - 0.17) | -0.093, 0.036 | 0.335 | 0.90, 2.01 (1.12 - 1.78) |
| Serine | 1.02 (0.030) (0.87 - 1.14) | 1.05 (0.030) (0.94 - 1.18) | -0.035 (0.019) (-0.17 - 0.052) | -0.080, 0.0095 | 0.105 | 0.81, 1.32 (0.88 - 1.30) |
| Threonine | 0.98 (0.030) (0.86 - 1.11) | 1.00 (0.030) (0.88 - 1.12) | -0.025 (0.018) (-0.12 - 0.065) | -0.066, 0.016 | 0.192 | 0.82, 1.20 (0.84 - 1.22) |
| Tryptophan | 0.23 (0.010) (0.17 - 0.26) | 0.24 (0.010) (0.19 - 0.31) | -0.013 (0.0093) (-0.063 - 0.036) | -0.032, 0.0059 | 0.172 | 0.13, 0.35 (0.17 - 0.32) |

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**Table VII-2. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% CI[3] Lower, Upper l | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Amino Acid (% dw)** | | | | | | |
| Tyrosine | 0.67 (0.019) (0.59 - 0.75) | 0.69 (0.019) (0.61 - 0.77) | -0.017 (0.013) (-0.11 - 0.028) | -0.048, 0.015 | 0.249 | 0.57, 0.81 (0.60 - 0.84) |
| Valine | 1.20 (0.035) (1.04 - 1.37) | 1.22 (0.035) (1.05 - 1.41) | -0.025 (0.025) (-0.16 - 0.054) | -0.084, 0.034 | 0.352 | 0.92, 1.55 (1.01 - 1.46) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 16:0 Palmitic | 4.23 (0.078) (3.95 - 4.57) | 4.10 (0.077) (3.94 - 4.41) | 0.13 (0.067) (-0.22 - 0.48) | -0.027, 0.28 | 0.094 | 2.84, 5.26 (3.55 - 4.69) |
| 16:1 Palmitoleic | 0.22 (0.0081) (0.20 - 0.26) | 0.24 (0.0081) (0.22 - 0.26) | -0.018 (0.0053) (-0.039 - 0.0074) | -0.030, -0.0059 | 0.008 | 0.17, 0.30 (0.19 - 0.27) |
| 18:0 Stearic | 1.68 (0.044) (1.54 - 1.87) | 1.98 (0.044) (1.78 - 2.19) | -0.30 (0.031) (-0.48 - -0.059) | -0.37, -0.23 | <0.001 | 0.90, 3.05 (1.50 - 2.64) |
| 18:1 Oleic | 62.82 (0.62) (60.51 - 65.20) | 65.79 (0.62) (63.72 - 68.44) | -2.97 (0.31) (-4.30 - -1.52) | -3.69, -2.26 | <0.001 | 56.13, 70.69 (57.86 - 68.53) |

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**Table VII-2. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% CI[3] Lower, Upper | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fatty Acid (% Total FA)** | | | | | | |
| 18:2 Linoleic | 19.26 (0.51) (17.78 - 20.66) | 17.67 (0.51) (15.72 - 19.29) | 1.59 (0.17) (0.40 - 2.42) | 1.20, 1.97 | <0.001 | 12.60, 24.49 (14.12 - 22.57) |
| 18:3 Linolenic | 9.58 (0.27) (8.71 - 11.23) | 7.98 (0.27) (7.19 - 8.99) | 1.60 (0.21) (0.76 - 2.64) | 1.12, 2.07 | <0.001 | 6.96, 11.73 (7.99 - 10.94) |
| 20:0 Arachidic | 0.54 (0.011) (0.50 - 0.57) | 0.60 (0.011) (0.54 - 0.65) | -0.064 (0.0074) (-0.091 - -0.0032) | -0.081, -0.047 | <0.001 | 0.45, 0.80 (0.53 - 0.71) |
| 20:1 Eicosenoic | 1.13 (0.024) (1.06 - 1.26) | 1.09 (0.024) (1.00 - 1.18) | 0.036 (0.017) (-0.042 - 0.14) | -0.0034, 0.076 | 0.068 | 0.83, 1.68 (1.04 - 1.56) |
| 22:0 Behenic | 0.27 (0.0072) (0.24 - 0.29) | 0.28 (0.0072) (0.24 - 0.31) | -0.017 (0.0056) (-0.047 - 0.016) | -0.030, -0.0041 | 0.016 | 0.19, 0.43 (0.27 - 0.38) |
| 24:0 Lignoceric | 0.16 (0.016) (0.049 - 0.23) | 0.16 (0.015) (0.045 - 0.22) | 0.0038 (0.017) (-0.14 - 0.11) | -0.030, 0.038 | 0.823 | 0.033, 0.25 (0.044 - 0.21) |

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**Table VII-2. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% CI[3] Lower, Upper | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 24:1 Nervonic | 0.12 (0.015)<br>(0.046 - 0.20) | 0.11 (0.015)<br>(0.045 - 0.17) | 0.013 (0.014)<br>(-0.072 - 0.081) | -0.020, 0.047 | 0.377 | 0.041, 0.18<br>(0.044 - 0.20) |
| **Mineral** | | | | | | |
| Calcium (g/100g dw) | 0.41 (0.030)<br>(0.30 - 0.51) | 0.40 (0.030)<br>(0.28 - 0.49) | 0.015 (0.012)<br>(-0.068 - 0.081) | -0.0089, 0.039 | 0.210 | 0.16, 0.61<br>(0.25 - 0.53) |
| Copper (mg/kg dw) | 3.78 (0.17)<br>(3.27 - 4.57) | 3.65 (0.17)<br>(2.96 - 4.18) | 0.14 (0.14)<br>(-0.83 - 0.57) | -0.19, 0.46 | 0.361 | 2.00, 4.43<br>(2.52 - 4.93) |
| Iron (mg/kg dw) | 48.73 (4.28)<br>(40.55 - 69.61) | 54.01 (4.24)<br>(41.65 - 77.74) | -5.28 (2.89)<br>(-20.41 - 14.87) | -11.85, 1.30 | 0.102 | 23.39, 86.23<br>(39.16 - 77.92) |
| Magnesium (g/100g dw) | 0.37 (0.014)<br>(0.31 - 0.42) | 0.36 (0.014)<br>(0.31 - 0.42) | 0.0048 (0.0070)<br>(-0.032 - 0.043) | -0.011, 0.021 | 0.508 | 0.32, 0.43<br>(0.30 - 0.45) |
| Manganese (mg/kg dw) | 41.44 (2.02)<br>(35.28 - 51.55) | 40.34 (1.99)<br>(33.12 - 50.97) | 1.10 (1.83)<br>(-8.36 - 12.63) | -2.62, 4.82 | 0.551 | 14.85, 61.05<br>(25.00 - 54.11) |

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**Table VII-2. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% CI[3] Lower, Upper | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Mineral** | | | | | | |
| Phosphorus (g/100g dw) | 0.72 (0.042) (0.56 - 0.87) | 0.72 (0.041) (0.56 - 0.93) | -0.0090 (0.022) (-0.095 - 0.16) | -0.055, 0.037 | 0.692 | 0.38, 1.06 (0.44 - 0.87) |
| Potassium (g/100g dw) | 0.64 (0.053) (0.48 - 0.90) | 0.64 (0.052) (0.53 - 0.81) | 0.0016 (0.025) (-0.097 - 0.14) | -0.056, 0.060 | 0.951 | 0.39, 0.96 (0.50 - 0.92) |
| Zinc (mg/kg dw) | 35.58 (1.78) (29.81 - 45.56) | 33.01 (1.76) (28.46 - 40.66) | 2.57 (1.83) (-4.50 - 11.44) | -1.66, 6.80 | 0.198 | 20.19, 48.23 (22.18 - 47.61) |
| **Vitamin (mg/100g dw)** | | | | | | |
| Vitamin E (α-tocopherol) | 11.06 (2.08) (1.30 - 16.55) | 8.85 (2.08) (3.33 - 11.77) | 2.21 (1.66) (-6.92 - 8.09) | -1.61, 6.03 | 0.218 | 3.88, 17.28 (2.62 - 14.84) |

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control Ebony.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

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**Table VII-3. Statistical Summary of Combined-Site Seed Anti-nutrient Content for MON 88302 vs. the Conventional Control**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% CI[3] Lower, Upper | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Anti-nutrient** | | | | | | |
| Alkyl Glucosinolate (μmole/g dw) | 3.68 (0.43) (1.19 - 5.87) | 5.08 (0.42) (2.45 - 8.28) | -1.40 (0.59) (-6.11 - 1.43) | -2.69, -0.11 | 0.035 | 0, 29.02 (2.32 - 28.33) |
| Indolyl Glucosinolate (μmole/g dw) | 3.50 (0.51) (0.49 - 5.76) | 3.89 (0.50) (1.83 - 5.89) | -0.39 (0.45) (-3.05 - 2.83) | -1.42, 0.64 | 0.408 | 1.37, 6.62 (1.84 - 7.18) |
| Phytic Acid (% dw) | 1.95 (0.18) (1.20 - 2.58) | 2.11 (0.18) (1.46 - 2.77) | -0.16 (0.083) (-0.67 - 0.68) | -0.33, 0.010 | 0.064 | 0.70, 3.52 (1.10 - 2.71) |
| Sinapic Acid (% dw) | 0.86 (0.12) (0.16 - 1.08) | 0.88 (0.12) (0.65 - 0.97) | -0.023 (0.11) (-0.76 - 0.21) | -0.27, 0.22 | 0.837 | 0.57, 1.13 (0.48 - 0.99) |
| Total Glucosinolate (μmole/g dw) | 7.35 (0.87) (1.73 - 11.42) | 9.08 (0.86) (4.38 - 12.72) | -1.73 (1.01) (-9.21 - 3.58) | -4.06, 0.61 | 0.127 | 0, 32.20 (5.52 - 31.98) |

[1]dw = dry weight.
[2]MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control Ebony.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

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**Table VII-4. Literature Ranges for Components in Canola Seed**

| Component[1] | Literature Range[2] |
|---|---|
| **Proximates (% dw)** | |
| Ash | 4.067 – 5.917[a] |
| Carbohydrates | N |
| Moisture (% fw) | 3.177 – 8.045[a]; 7.4 – 10.0[b] |
| Protein | 21.30 – 28.125[a]; 18.7 – 26.0[b]; 17.4 – 23.0[c]; 21.1 – 26.7[d] |
| Total Fat | 35.59 – 44.93[a]; 24.0 – 43.6[b]; 42.0 – 49.5[d] |
| **Fiber (% dw)** | |
| Acid Detergent Fiber (ADF) | 11.934 – 26.799[a];11.6[f]; 12.4[g]; 22.2[h] |
| Neutral Detergent Fiber (NDF) | 18.653 – 34.720[a]; 17.8[f]; 16.49[g]; 31.3[h] |
| Total Dietary Fiber | N |
| **Amino Acids (% dw)** | |
| Alanine | 0.93 – 0.96[b]; 1.15 – 1.38[e] |
| Arginine | 1.13 – 1.21[b]; 2.23 – 2.46[e] |
| Aspartic acid | 1.54 – 1.59[e] |
| Cystine/Cysteine | 0.52 – 0.54[b] |
| Glutamic acid | 4.60 – 4.71[e] |
| Glycine | 1.04 – 1.06[b]; 2.20 – 2.22[e] |
| Histidine | 0.51 – 0.66[b]; 0.80 – 0.82[e] |
| Isoleucine | 0.80 – 0.86[b]; 0.96 – 1.03[e] |
| Leucine | 1.35 – 1.47[b]; 1.83 – 1.99[e] |
| Lysine | 1.03 – 1.19[b]; 1.67 – 1.85[e] |
| Methionine | 0.42 – 0.44[b] |
| Phenylalanine | 0.75 – 0.82[b]; 0.90 – 1.03[e] |
| Proline | 1.19 – 1.33[b]; 3.36 – 3.74[e] |
| Serine | 0.90 – 0.94[b]; 1.44 – 1.55[e] |
| Threonine | 0.87 – 0.94[b]; 1.28 – 1.30[e] |
| Tryptophan | 0.23 – 0.27[b]; |
| Tyrosine | 0.51 – 0.59[b]; 0.81 – 0.92[e] |
| Valine | 1.02 – 1.13[b]; 1.45 – 1.55[e] |
| **Vitamins (mg/kg dw)** | |
| Vitamin E (α-tocopherol) | 71.1 – 108.4[i] |

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**Table VII-4. Literature Ranges for Components in Canola Seed (continued)**

| Component[1] | Literature Range[2] |
|---|---|
| **Minerals** | |
| Calcium (% dw) | 0.29 – 0.48[b]; 0.348 – 0.729[a] |
| Copper (mg/kg dw) | 7[b]; 1.388 – 5.492[a] |
| Iron (mg/kg dw) | ND[b]; 0.0 – 965.6[a] |
| Magnesium (% dw) | 0.29 – 0.31[b]; 0.272 – 0.402[a] |
| Manganese (mg/kg dw) | ND[b]; 33.813 – 64.757[a] |
| Phosphorus (% dw) | 0.48 – 0.85[b]; 0.581 – 0.895[a] |
| Potassium (% dw) | 0.83 – 0.91[b]; 0.681 – 1.016[a] |
| Sodium (% dw) | 0.05[b]; 0.003 – 0.030[a] |
| Zinc (mg/kg dw) | 62[b]; 0 – 126.953[a] |
| **Fatty Acids (% total)** | |
| 16:0 Palmitic | 3.3 – 6.0[b] |
| 16:1 Palmitoleic | 0.1 – 0.6[b] |
| 18:0 Stearic | 1.1 – 2.5[b] |
| 18:1 Oleic | 52.0 – 66.9[b] |
| 18:2 Linoleic | 16.1 – 24.8[b] |
| 18:3 Linolenic | 6.4 – 14.1[b] |
| 20:0 Arachidic | 0.2 – 0.8[b] |
| 20:1 Eicosenoic | 0.1 – 3.4[b] |
| 20:2 Eicosadienoic | 0.0 – 0.1[b] |
| 20:3 Eicosatrienoic | N |
| 20:4 Arachidonic | N |
| 22:0 Behenic | 0.0 – 0.5[b] |
| 22:1 Erucic | 0.0 – 2.0[b] |
| 24:0 Lignoceric | 0.0 – 0.2[b] |
| 24:1 Nervonic | 0.0 – 0.04[b] |
| **Anti-nutrients** | |
| Total Glucosinolates (µmol/g) | 6 – 29[b]; 7.8 – 26.8[c]; 18 – 57[j] |
| Phytic Acid (% dw) | 2.0 – 5.0[b] |
| Sinapine (% dw) | 0.6 – 1.8[b] |

[1]fw = fresh weight; dw = dry weight; dm = dry matter; ND defined as below the level of detection; N defined as not reported.
[2]Literature Range = Values published for low erucic acid rapeseed (canola).
Citations = [a](Dairy One Forage Lab); [b](OECD, 2001); [c](Pritchard et al., 2000); [d](Barthet and Daun, 2005); [e](Wang et al., 1999)6; [f](NRC, 2001); [g](Mustafa et al., 2000); [h](Leupp et al., 2006); [i](Marwede et al., 2004);.[j](Mailer and Pratley, 1990).
Conversions: mg/100g dw × 10 = mg/kg dw; g/100g dw× 10 = mg/g dw.

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## VII.B. Compositional Assessment of MON 88302 Summary and Conclusion

Analyses of nutrient, toxicant, and anti-nutrient levels in MON 88302 and the conventional control were conducted to assess compositional equivalence. The tissue analyzed was seed harvested from plants grown at five field sites in the U.S. and Canada during the 2009 field season. The compositional analysis, conducted in accordance with OECD guidelines, included measurement of nutrient, toxicant, and anti-nutrient components in a genetically similar conventional control variety, Ebony and also commercial canola commercial reference varieties that have a history of safe consumption to establish the natural range of variability. MON 88302, the conventional control, and commercial reference varieties were treated with conventional weed control programs. In addition, MON 88302 plots were treated with glyphosate herbicide at a target rate of 1.6 lb a.e./acre (1800 g a.e./ha).

The significant differences ($\alpha = 0.05$) in nutrient and anti-nutrient components were evaluated using considerations relevant to the safety and nutritional quality of MON 88302 when compared to the conventional control:

1) The relative magnitudes of differences for nutrients that were statistically significant in the combined-site analysis were small (4.52% to 20.01%), when considered relative to the natural variability determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption. The relative magnitude of difference for the anti-nutrient alkyl glucosinolate that was statistically significant in the combined site analysis was small (27.52%) when considered relative to the natural variability determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption.

2) Mean values for these nutrient, toxicant and anti-nutrient components from the combined-site analysis of MON 88302 fell within the 99% tolerance interval established from the commercial references grown concurrently. Therefore, the differences were, within the range of natural variability of those components in commercial canola varieties with a history of safe consumption (Tables VII-2 and VII-3).

3) Assessment of the reproducibility of the combined-site differences at the five individual sites showed similar significant differences ($\alpha = 0.05$) at multiple sites. In all instances the individual site mean values for these components in MON 88302 were within the 99% tolerance interval established from the concurrently grown commercial reference varieties. Therefore, these components were within the range of natural variability in commercial canola varieties with a history of safe consumption.

4) With the exception of alkyl glucosinolates, all of the compositional components identified as significantly different from the conventional control were within the natural variability of these components in commercial canola composition as

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published in the scientific literature. There are no published values for alkyl glucosinolates as a composite category.

This analysis provides a comprehensive comparative assessment of the levels of key nutrients, toxicants, and anti-nutrients in seed of MON 88302 and the conventional control, discussed in the context of natural variability of commercial canola. Results of the comparison indicate that the seed of MON 88302 is compositionally equivalent to that of the conventional canola control. The genetic modification in MON 88302 does not meaningfully impact seed composition and therefore the food and feed safety and nutritional quality of this product is comparable to conventional canola with a history of safe consumption.

Conventional canola processing is described in Section II of this document. The processing of MON 88302 is not expected to be any different from that of conventional canola. As described in this section, detailed compositional analyses of key components of MON 88302 have been performed and have demonstrated that MON 88302 is compositionally equivalent to conventional canola. Additionally, the mode of action of CP4 EPSPS protein, as described in Section VI.A., is well understood, and there is no reason to expect interactions with important nutrients or endogenous toxicants that may be present in canola. Therefore, when MON 88302 and its progeny is used on a commercial scale as a source of food or feed, these products are not expected to be different from the equivalent foods or feeds originating from conventional canola.

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# VIII. USE OF ANTIBIOTIC RESISTANCE MARKER GENES

The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 5, paragraphs 55 through 58 (Codex Alimentarius, 2009).

## VIII.A. Presence of Genes that Encode Resistance to Antibiotics

No genes that encode resistance to an antibiotic marker were inserted into the crop genome during the development of MON 88302. Molecular characterization data presented in Section V. demonstrate the absence of the *aadA* antibiotic resistant marker gene in MON 88302.

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# IX. SUMMARY OF FOOD AND FEED SAFETY ASSESSMENTS

This section provides a concluding discussion of the safety assessment and addresses the relevant factors in Codex Plant Guidelines, Section 5, paragraph 59 (Codex Alimentarius, 2009).

## IX.A. Donor Organism

As described in detail in Section III, the *cp4 epsps* coding sequence is derived from the soil bacterium *Agrobacterium* sp. strain CP4, which is related to microbes commonly present in the soil and in the rhizosphere of plants. *Agrobacterium* species are not commonly known for human or animal pathogenicity, and are not commonly allergenic. Furthermore, according to FAO/WHO, there is no known population of individuals sensitized to bacterial proteins (FAO/WHO, 2001).

## IX.B. Genetic Insert

MON 88302 was developed through *Agrobacterium*-mediated transformation of hypocotyls from canola variety Ebony utilizing plasmid vector PV-BNHT2672. PV-BNHT2672 contains one T-DNA that is delineated by Left and Right Border regions. The T-DNA contains the *cp4 epsps* coding sequence under the control of the *FMV/Tsf1* chimeric promoter, the *Tsf1* leader and intron sequences, and the *E9* 3′ untranslated region. The chloroplast transit peptide CTP2 directs transport of the CP4 EPSPS protein to the chloroplast and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene. After transformation and subsequent rounds of self-pollination, homozygous $R_2$ plants containing only a single T-DNA insertion were identified resulting in production of glyphosate-tolerant canola MON 88302.

Molecular characterization by Southern blot analyses determined that MON 88302 contains one copy of the T-DNA at a single integration locus and all genetic elements are present. These data also demonstrated that MON 88302 does not contain detectable backbone sequences from the plasmid vector. The complete DNA sequence of the insert and adjacent genomic DNA sequences in MON 88302 confirmed the integrity of the inserted *cp4 epsps* expression cassette within the inserted sequences and identified the 5′ and 3′ insert-to-genomic DNA junctions. Southern blot analysis demonstrated that the insert in MON 88302 has been maintained over multiple generations of breeding, thereby confirming the stability of the insert. Further, results from segregation analyses show inheritance and stability of the insert were as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA in MON 88302 at a single chromosomal locus.

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## IX.C. Safety of CP4 EPSPS Protein

As described in detail in Section VI, a multistep approach was used to assess the safety of the CP4 EPSPS protein in MON 88302 in accordance with guidelines established by the Codex Alimentarius Commission and OECD which embody the principles and guidance of the FDA's 1992 policy on foods from new plant varieties. The resulting detailed characterization of the CP4 EPSPS protein confirmed that it is safe for human and animal consumption. These steps include: 1) documentation of the history of safe use of the CP4 EPSPS protein and its homology with proteins that lack adverse effects on human or animal health; 2) characterization of the physicochemical and functional properties of CP4 EPSPS; 3) quantification of CP4 EPSPS expression in plant tissues; 4) examination of the similarity of CP4 EPSPS to known allergens, 5) evaluation of the digestibility of CP4 EPSPS in simulated gastrointestinal fluids; 6) evaluation of the stability of the CP4 EPSPS protein in response to typical food/feed preparation conditions such as heat treatment; 7) examination of the similarity of CP4 EPSPS to known toxins or other biologically active proteins known to have adverse effects on mammals; 8) investigation of potential mammalian toxicity through an animal assay and calculating margins of exposure; and 9) examination of the similarity of putative polypeptides encoded by the insert and flanking sequences to known allergens and toxins, or other biologically active proteins known to have adverse effects on mammals. Additionally, a stepwise approach to assess the potential allergenicity for the newly expressed protein (Codex Alimentarius, 2009) is included. The safety assessment supports the conclusion that dietary exposure to CP4 EPSPS protein derived from MON 88302 poses no meaningful risk to human or animal health.

The CP4 EPSPS protein expressed in MON 88302 is identical to the CP4 EPSPS protein in other Roundup Ready crops, and the *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready corn 2, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton and Roundup Ready alfalfa. Furthermore, the U.S. EPA has established an exemption from the requirement of a tolerance for residues of CP4 EPSPS protein and the genetic material necessary for its production in all plants (U.S. EPA, 1996). Because the MON 88302-produced CP4 EPSPS protein is equivalent to the exempted CP4 EPSPS protein a similar conclusion can be reached, that the MON 88302-produced CP4 EPSPS protein is safe for human and animal consumption. Using the guidance provided by the FDA in its 1992 Policy Statement regarding the evaluation of New Plant Varieties, a conclusion of "no concern" was reached for the donor organism and the CP4 EPSPS protein. The food and feed products containing MON 88302 or derived from MON 88302 and its progeny are as safe as canola currently on the market for human and animal consumption.

## IX.D. Compositional Characteristics of MON 88302

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by the FDA. The CP4 EPSPS protein expressed in MON 88302 is identical to the CP4 EPSPS protein in other Roundup Ready crops and the mode of action of the

CP4 EPSPS protein is well understood. Previous Roundup Ready crops reviewed by the FDA have had no biologically relevant compositional changes identified, and there is no reason to expect expression of the CP4 EPSPS protein in MON 88302 would affect nutritionally important nutrients and anti-nutrients present in seed from this new product.

Detailed compositional analyses were conducted in accordance with OECD guidelines to determine whether levels of key nutrients, toxicants and anti-nutrients in seed derived from MON 88302 were equivalent to levels present in the genetically similar conventional control variety Ebony, which has a history of safe consumption, and several commercial reference varieties (Section VII.A.). The commercial reference varieties were used to establish a range of natural variability for each analyte in commercial canola varieties that have a history of safe consumption. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and total fat), fibers (acid detergent fiber [ADF], neutral detergent fiber [NDF], and total dietary fiber [TDF]), amino acids (18 components), fatty acids (FA; C8-C24,), vitamin E ($\alpha$-tocopherol), and minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc) in seed. The toxicants assessed in seed included erucic acid and glucosinolates (alkyl glucosinolates [including 3-butenyl, 4-pentenyl, 2-hydroxy-3-butenyl, and 2-hydroxy-4-pentenyl glucosinolates], indolyl glucosinolates [including 3-indolylmethyl and 4-hyrdoxy-3-indolymethyl], and total glucosinolates). The anti-nutrients assessed in seed included phytic acid and sinapine (as sinapic acid).

Combined-site analyses were conducted to determine statistically significant differences ($\alpha = 0.05$) between MON 88302 and the conventional control. Statistical results from the combined-site data were evaluated using considerations relevant to safety and/or nutritional value. Considerations used to assess the relevance of each combined-site statistically significant difference included: 1) the relative magnitude of the difference in the mean values of nutrient, toxicant and anti-nutrient components of MON 88302 and the conventional control, 2) whether the MON 88302 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of the commercial reference varieties grown concurrently in the same trial, 3) evaluation of the reproducibility of the statistically significant ($\alpha = 0.05$) combined-site component differences at individual sites, and 4) an assessment of the differences within the context of natural variability of commercial canola composition published in the scientific literature.

Assessment of the analytical results confirmed that the differences observed in the combined-site analysis were not meaningful to food and feed safety or the nutritional quality of MON 88302 compared to conventional canola. In addition, the levels of assessed components in MON 88302 were compositionally equivalent to the conventional control and within the range of variability of commercial reference varieties grown concurrently. These results support the overall conclusion that MON 88302 seed is compositionally equivalent to conventional canola in accordance with OECD guidelines.

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## IX.E. Summary of Food and Feed Safety Assessment of MON 88302

These data, along with the history of safe use of canola as a common source of human food and animal feed, collectively support a conclusion of “no concerns” for every criterion specified in the flowcharts outlined in the FDA’s Food Policy document (FDA, 1992) and shown in Figure IX-1. MON 88302 is not materially different in composition, safety or nutrition from conventional canola other than the introduction of the glyphosate-tolerance trait. Sales and consumption of canola seed and processed products derived from MON 88302 and its progeny would be fully consistent with the FDA’s Food Policy, the Federal Food, Drug and Cosmetic Act, and current practices for the development and introduction of new canola varieties and biotechnology-derived traits.

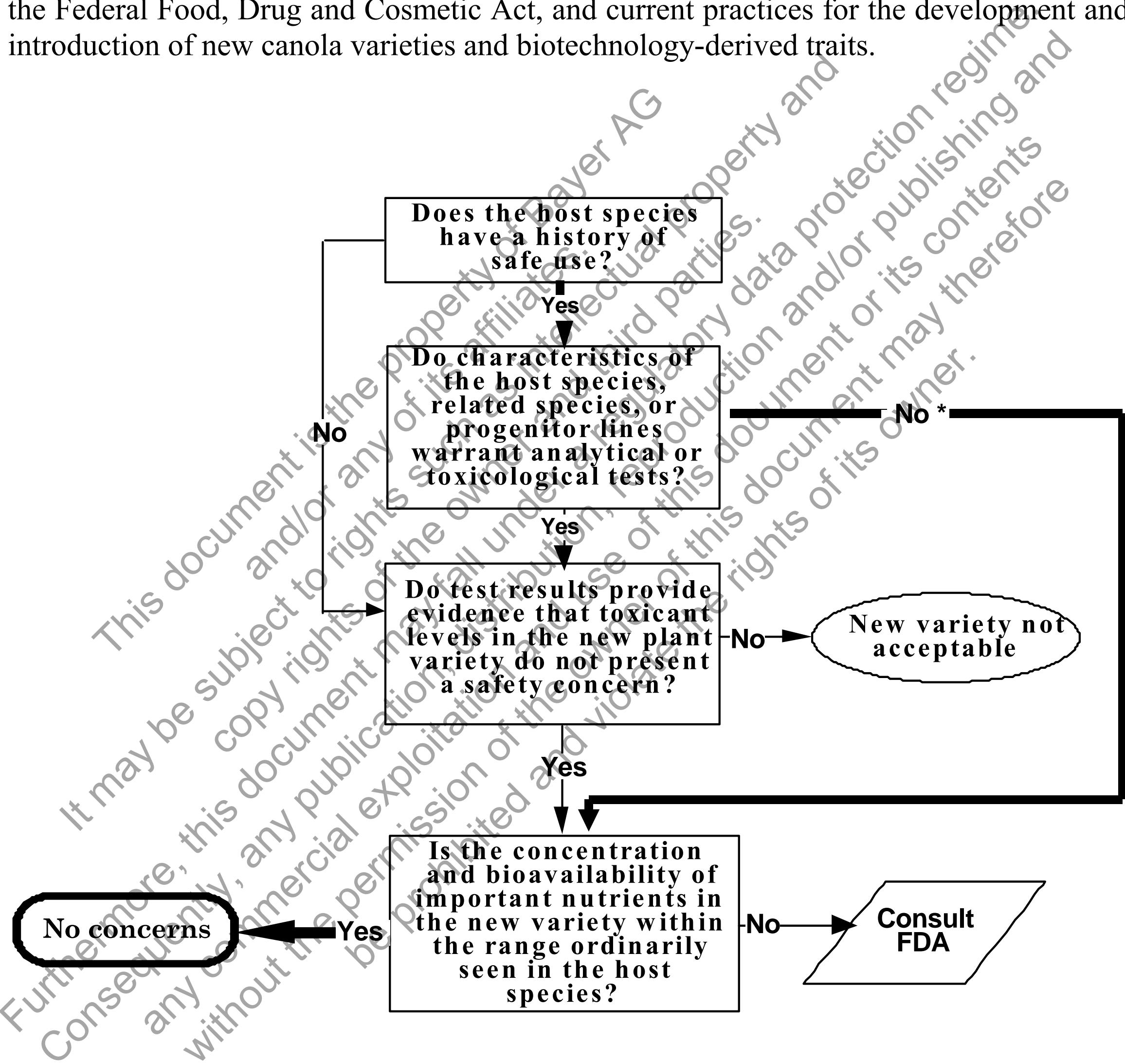



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**Figure IX-1. Safety Assessment of New Varieties: The Host Plant**

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# APPENDICES

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## Appendix A: Materials and Methods Used for Molecular Analyses of MON 88302

### A.1. Materials

The genomic DNA used in molecular analyses was isolated from leaf tissue of the $R_3$ generation of MON 88302 and the conventional control (Ebony). The leaf tissue was harvested from a greenhouse production in 2009. For generational stability analysis, genomic DNA was extracted from leaf tissue of the $R_2$, $R_3$, $R_4$, $R_{5a}$ and $R_{5b}$ generations of MON 88302. The leaf tissue was harvested from production plan PPN-09-523. The reference substance, PV-BNHT2672 (Figure IV-1), was used as a positive hybridization control in Southern blot analyses. Probe templates generated from PV-BNHT2672 were used as additional positive hybridization controls. As additional reference standards, the 1 Kb DNA Extension Ladder and λ DNA/*Hind* III Fragments from Invitrogen (Carlsbad, CA) were used for size estimations on agarose gels and subsequential Southern blots. The 1 Kb DNA Ladder from Invitrogen was used for size estimations on agarose gels for PCR analyses.

### A.2. Characterization of the Materials

The identity of the source materials was verified by methods used in molecular characterization to confirm the presence or absence of MON 88302. The stability of the genomic DNA was confirmed by observation of interpretable signals from digested DNA samples on ethidium bromide stained agarose gels and/or specific PCR products, and the samples did not appear visibly degraded on the ethidium bromide stained gels.

### A.3. DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA was isolated from MON 88302 leaf tissue using a modified sarkosyl method. The leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and a pestle. Approximately 4 ml of ground leaf tissue was transferred to each 50 ml conical tube. Twenty milliliter of the lysis buffer (50 mM Tris HCl, 2% w/v PVP, 20 mM EDTA, 500 mM NaCl, 1.2% w/v SDS, 0.5% w/v sarkosyl, and 0.4% w/v sodium bisulfate) and 300 µg of RNase A were added to each tube. After suspending the powder in the buffer, the samples were incubated at 60-70 °C for 60-90 min with intermittent mixing. Following the completion of the incubation, the samples were allowed to come to room temperature and 20 ml of phenol/chloroform/isoamyl alcohol (PCI) [25:24:1(v/v)] were added to each sample. The samples were then mixed by inversion with hand for 2-3 minutes followed by centrifugation at 2,000 x *g* for 20-25 min at 2-8 °C to separate the phases. The upper aqueous phase was transferred to a clean tube and the PCI extraction and centrifugation process was repeated at least once followed by a chloroform extraction. The aqueous phase was transferred to a clean tube and the amount of aqueous phase was recorded. The DNA was precipitated with an equal amount of 100% ethanol and spooled into a tube with 10-12 ml of 70% ethanol to wash the DNA. The DNA was pelleted by centrifugation at 4,000 x *g* for 5 min at 2-8 °C and the 70% ethanol was discarded. After being air-dried, the DNA pellet was suspended in appropriate amount of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH8.0) and stored in a 4 ºC refrigerator or a -20 °C freezer.

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## A.4. Quantification of Genomic DNA

Genomic DNA was quantified using a DyNA Quant 200 Fluorometer (Hoefer, Inc., Holliston, MA). Molecular Size Marker IX (Roche, Indianapolis, IN) was used as the calibration standard.

## A.5. Restriction Enzyme Digestion of Genomic DNA

Approximately ten micrograms (µg) of genomic DNA extracted from MON 88302 and conventional control were digested with restriction enzyme *Ase* I (New England Biolabs, Inc. Ipswich, MA) and a combination of restriction enzymes *Sal* I and *Sca* I (New England Biolabs, Inc.). All digests were conducted in 1X NEBuffer 3 (New England Biolabs, Inc.) at 37°C in a total volume of ~500 microliters (µl) with ~50 units of each restriction enzyme. Digests conducted with the combination of restriction enzymes *Sal* I and *Sca* I also included 1X BSA (New England Biolabs, Inc.) in the reaction. For the purpose of running positive hybridization controls, ~10 µg of genomic DNA extracted from the conventional control was digested with the restriction enzyme *Ase* I and the appropriate positive hybridization control(s) were added to these digests prior to loading the agarose gel.

## A.6. Agarose Gel Electrophoresis

Digested DNA was resolved on ~0.8% (w/v) agarose gels. For T-DNA insert/copy number and plasmid vector backbone analyses, individual digests containing ~10 µg each of MON 88302 and conventional control genomic DNA were loaded on the same gel in a long run/short run format. The long run allows for greater resolution of large molecular weight DNA, whereas the short run allows for retaining the small molecular weight DNA on the gel. The positive hybridization controls were only run in the short run format. For the insert stability analysis, individual digests of ~10 µg each of genomic DNA extracted from five leaf samples from multiple generations of MON 88302 and the conventional control along with the positive hybridization controls were loaded on the agarose gel in a single run format.

## A.7. DNA Probe Preparation for Southern Blot Analyses

Probe templates were prepared by PCR amplification using the PV-BNHT2672 DNA as template. The PCR products were separated on an agarose gel by electrophoresis and purified from the gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. The probe templates were designed based on the nucleotide composition (%GC) of the sequence in order to optimize the detection of DNA sequences during hybridization. When possible, probes possessing similar melting temperature (Tm) were combined in the same Southern blot hybridization. Approximately 25 ng of each probe template were radiolabeled with either [α-$^{32}$P] deoxycytidine triphosphate (dCTP) (6000 Ci/mmol) or [α-$^{32}$P] deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using RadPrime DNA Labeling System (Invitrogen, Carlsbad, CA) according to manufacturer's instruction.

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## A.8. Southern Blot Analyses of Genomic DNA

Genomic DNA isolated from MON 88302 and the conventional control was digested and evaluated using Southern blot analyses (Southern, 1975). The PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I was added to conventional control genomic DNA digested with *Ase* I to serve as positive hybridization control on each Southern blot. When multiple probes were hybridized simultaneously to one Southern blot, the probe templates were spiked in the digested conventional control genomic DNA to serve as additional positive hybridization controls on the Southern blot. The DNA was then separated by agarose gel electrophoresis and transferred onto a nylon membrane. Southern blots were hybridized and washed at 55 °C, 60 °C, or 65 °C, depending on the calculated melting temperature (Tm) of the probes that were used. Table A-1 lists the radiolabeling conditions and hybridization temperatures of the probes used in this study. Multiple exposures of each blot were then generated using Kodak Biomax MS film (Eastman Kodak, Rochester, NY) in conjunction with one Kodak Biomax MS intensifying screen in a -80 °C freezer.

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## Appendix B: Materials and Methods for Characterization of CP4 EPSPS Protein Produced in MON 88302

### B.1. Materials

The MON 88302-produced CP4 EPSPS protein (lot 11266369) was purified from seed of MON 88302 (lot 11225246). The MON 88302-produced CP4 EPSPS protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM dithiothreitol (DTT), 1 mM benzamidine-HCl, and 25% glycerol.

The *E. coli*-produced CP4 EPSPS protein (lot 10000739) was used as the reference substance. The CP4 EPSPS protein reference substance was generated from cell paste produced by large-scale fermentation of *E. coli* containing the pMON21104 expression plasmid. The coding sequence for *cp4 epsps* contained on the expression plasmid (pMON21104) was confirmed prior to and after fermentation. The *E. coli*-produced CP4 EPSPS protein was previously characterized.

### B.2. Description of Assay Controls

Protein MW standards (Precision Plus Protein Standards Dual color; Bio-Rad, Hercules, CA) were used to calibrate some SDS-PAGE gels and verify protein transfer to polyvinylidene difluoride (PVDF) and nitrocellulose membranes. Broad Range SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA) were used to generate a standard curve for the apparent MW estimation. The *E. coli*-produced CP4 EPSPS reference standard was used to construct a standard curve for the estimation of total protein concentration using a Bio-Rad protein assay. A phenylthiohydantoin (PTH) amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to calibrate the instrument for each analysis. A peptide mixture (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass and a bovine serum albumin (BSA) standard (NIST, Gaithersburg, MD) was used to calibrate the MALDI-TOF mass spectrometer for intact mass analysis. Transferrin (Sigma-Aldrich, St. Louis, MO) was used as positive control for glycosylation analysis.

### B.3. Protein Purification

The plant-produced CP4 EPSPS protein was purified from seed of MON 88302. The purification procedure was not performed under a GLP plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. The CP4 EPSPS protein was purified at ~4 °C from an extract of ground seed using a combination of ammonium sulfate fractionation, hydrophobic interaction chromatography, anion exchange chromatography, and cellulose phosphate affinity chromatography. The purification procedure is briefly described below.

Approximately 500 g of seed of MON 88302 was frozen with liquid nitrogen in a mortar and ground with a pestle. The partially crushed seed was further ground using a Magic Bullet grinder. The ground seed was then defatted by extraction with heated hexane (~50 °C) followed by vacuum filtration. This was repeated three times at a ground seed

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(g) to hexane volume (ml) ratio of approximately 1:5. The defatted ground seed was allowed to dry overnight at room temperature in a fume hood. The following day the defatted ground seed was mixed with extraction buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM benzamidine-HCl, 4 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1% polyvinylpolypyrrolidone, and 10% glycerol) for ~1.5 h at a sample weight (g) to buffer volume (ml) ratio of approximately 1:10. The slurry was centrifuged at 15,182 x *g* for 1 h at ~ 4 ºC. The supernatant (~3.5 liters) was collected and brought to 45% ammonium sulfate saturation by slow addition of 903 g of ammonium sulfate in a cold room (~4 ºC). The solution was stirred for ~1 h at ~4 ºC and then centrifuged at 15,182 x *g* for 1 h. The supernatant (~3.8 liters) was again collected and 592 g of ammonium sulfate was added to bring the solution to 70% ammonium sulfate saturation. The solution was stirred for ~1 h in a cold room and the pellet was collected by centrifugation at 15,182 x *g* for 1 h. The pellet was re-suspended in 1 liter of PS(A) buffer [50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% (v/v) glycerol,1.5 M ammonium sulfate]. The sample was loaded onto a 460 ml column (5 cm x 23 cm) of Phenyl Sepharose Fast Flow (GE Healthcare, Piscataway, NJ) equilibrated with PS(A) buffer. Proteins were eluted with a linear salt gradient that decreased from 1.5 M to 0 M ammonium sulfate over a volume of 2.3 liters. Fractions containing the CP4 EPSPS protein, identified based on immunoblot analysis and SDS-PAGE analysis, were pooled to a final volume of ~440 ml. The pooled sample was desalted by dialysis against 20 liters of QS(A) buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM benzamidine-HCl, 4 mM DTT) at ~4 °C using a dialysis tubing [Spectrum Laboratories, Inc., Rancho Dominguez, CA; Molecular Weight Cutoff (MWCO): 3.5 kDa] for a total of 16 h.

The desalted sample (600 ml) was loaded onto a 180 ml column (5 cm x 9.2 cm) of Q Sepharose Fast Flow anion exchange resin (GE Healthcare, Piscataway, NJ) equilibrated with QS(A) buffer. The bound CP4 EPSPS was eluted with a linear salt gradient that increased from 0 M to 0.4 M KCl in QS(A) buffer over 2.1 liters. Fractions containing CP4 EPSPS, identified by immunoblot analysis, were pooled to a final volume of ~ 280 ml. The pooled sample was dialyzed against 20 liters CP(A) buffer (10 mM sodium citrate, pH 5.0, 1 mM benzamidine-HCl, 2 mM DTT) for a total of 18 h at ~4 °C using a dialysis tubing (Spectrum Laboratories, Inc. Rancho Dominguez, CA; MWCO: 3.5 kDa).

Of the 350 ml recovered after dialysis, approximately 50 ml of the dialyzed sample was loaded onto a 5 ml column (1.6 x 2.5 cm) of cellulose phosphate P11 cation exchange (Whatman, Kent, UK) pre-equilibrated with CP(A) buffer. After an initial wash with 40 ml of CP(A) buffer, the column was washed with 50 ml of CP(B) buffer [CP(A) buffer with pH adjusted to 5.2 and supplemented with 0.5 mM phosphoenolpyruvate (PEP)]. The column was further washed with CP(C) buffer [CP(A) buffer with pH adjusted to 5.4 and supplemented with 0.5 mM PEP]. The bound CP4 EPSPS protein was eluted with of CP(D) buffer [CP(A) buffer with pH adjusted to 5.7 and supplemented with 0.5 mM PEP and 0.5 mM shikimate-3-phosphate (S3P)] over 90 ml. Fractions containing CP4 EPSPS protein, based on SDS-PAGE analysis and confirmed by immunoblot analysis, were pooled (~22 ml), supplemented with 10% glycerol, labeled Pool 1, and stored at -20 °C. Approximately 200 ml of the remaining dialyzed sample was then loaded onto a freshly prepared 20 ml column (2.6 x 3.7 cm) of cellulose

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phosphate P11 cation exchange (Whatman, Kent, UK) pre-equilibrated with freshly prepared CP(A) buffer. After an initial wash with 200 ml of CP(A) buffer, the column was washed with 160 ml of freshly prepared CP(B) buffer. The column was further washed with freshly prepared CP(C) buffer. The bound CP4 EPSPS protein was eluted with freshly prepared CP(D) buffer. Fractions containing CP4 EPSPS protein, based on SDS-PAGE analysis and confirmed by immunoblot, were pooled (Pool 2). Pool 1 and Pool 2 were combined (~82 ml) and divided between four iCon™ concentrators (MWCO: 20 kDa; size: 20 ml; Pierce, Rockford, IL) and concentrated by centrifugation at 4,000 x *g* for 30 min at ~4 °C. Buffer exchange was carried out in the same concentrators by the addition of ~19 ml an initial buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl followed by centrifugation at 4,000 x *g* for 30 min at ~4 °C repeated four times. After the fourth buffer exchange the remaining sample (~10 ml) was transferred to a new iCon concentrator (MWCO: 20 kDa; size: 20 ml; Pierce, Rockford, IL), supplemented with equal volume of the buffer containing 50 mM Tris-HCl pH 7.5, 50 mm KCl, 2 mm DTT, 50% glycerol and 1 mm benzamidine-HCl, and the sample was concentrated to ~2.4 ml. The final buffer composition of the sample was: 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl and 25% glycerol. This CP4 EPSPS protein purified from the seed of MON 88302 was aliquoted and stored in a -80 °C freezer.

### B.4. Methods for Characterization

#### B.4.1. N-Terminal Sequencing

N-terminal sequencing, carried out by automated Edman degradation chemistry, was used to confirm the identity of the MON 88302-produced CP4 EPSPS.

MON 88302-produced CP4 EPSPS was separated by SDS-PAGE and transferred to PVDF membrane. The blot was stained using Coomassie Blue R-250. The major band at ~44 kDa containing the test protein was excised from the blot and was used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapiller et al., 1983). An Applied Biosystems 494 Procise Sequencing System with a 140C Microgradient pump and a 785 Programmable Absorbance Detector was controlled with Procise® Control (version 1.1a) software. Chromatographic data were collected using Atlas 2003 software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A control protein, β-lactoglobulin, (Applied Biosystems, Foster City, CA) was analyzed before and after the sequence analysis of the CP4 EPSPS protein to verify that the sequencer met performance criteria for repetitive yield and sequence identity. Identity was established if ≥ 8 amino acids, consistent with the predicted sequence of the N-terminus of the MON 88302-produced CP4 EPSPS, were observed during analysis.

#### B.4.2. MALDI-TOF Tryptic Mass Map Analysis

MALDI-TOF tryptic mass fingerprint analysis was used to confirm the identity of the MON 88302-produced CP4 EPSPS protein. MON 88302-produced CP4 EPSPS protein was subjected to SDS-PAGE and the gel was stained using Brilliant Blue G Colloidal

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stain. Each ~44 kDa band was excised and transferred to a microcentrifuge tube. The gel bands were washed in 100 mM ammonium bicarbonate and then, to reduce the protein in each, gel bands were incubated in 100 µl of 10 mM DTT at ~37°C for 2 h. The protein was then alkylated in the dark for 25 min with 100 µl of 20 mM iodoacetic acid and washed with 200 µl of 25 mM ammonium bicarbonate for 3 x 20 min washes. Gel bands were dried with a Speed-Vac® concentrator (Thermo Fisher Scientific, Waltham, MA) and then rehydrated with 20 µl of trypsin solution (20 µg/ml). After 1 h, excess liquid was removed and the gel was incubated at ~37 °C for 16 h in 40 µl of 10% acetonitrile in 25 mM ammonium bicarbonate. Gel bands were sonicated for 5 min to further elute proteolytic fragments. The resulting extracts were transferred to new microcentrifuge tubes labeled Extract 1 and dried using Speed-Vac concentrator. The gel bands were re-extracted twice with 30 µl of a 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% β-octyl-glucopyranoside solution and sonicated for 5 min. Both 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% β-octyl-glucopyranoside extracts were pooled into a new tube labeled Extract 2 and dried with a Speed-Vac concentrator. A solution of 0.1% trifluoroacetic acid (TFA) was added to all Extract 1 and 2 tubes and they were dried as before. To acidify the extracts, a solution of 50% acetonitrile, 0.1% TFA was added to each tube and all were sonicated for 5 min. Each extract (0.3 µl) was spotted to three wells on an analysis plate. For each extract 0.75 µl of 2, 5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α Cyano), or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) (Waters Corp., Milford, MA) was added to one of the spots. The samples in DHB matrix were analyzed in the 300 to 7000 Da range. Samples in α-Cyano and Sinapinic acid were analyzed in the 500 to 5000 and 500 to 7000 Da range, respectively. Protonated peptide masses were monoisotopically resolved in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). CalMix 2 was used as the external calibrant (Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) for the analysis. GPMAW32 software (Lighthouse Data, Odense M, Denmark) was used to generate a theoretical trypsin digest of the CP4 EPSPS protein sequence. Those experimental masses within 1 Da of a theoretical mass were matched. All matching masses were tallied and a coverage map was generated for the mass fingerprint. The tryptic mass fingerprint coverage was considered acceptable if ≥ 40% of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments.

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### B.4.3. Western Blot Analysis-Immunoreactivity

Western blot analysis was performed to confirm the identity of the CP4 EPSPS protein purified from seed of MON 88302 and to compare the immunoreactivity of the MON 88302- and *E. coli*-produced proteins.

The MON 88302- and *E. coli*-produced CP4 EPSPS proteins were analyzed concurrently on the same gel using three loadings of 1, 2 and 3 ng. Loadings of the three concentrations were made in duplicate on the gel. Aliquots of each protein were diluted in water and 5X Laemmli buffer (LB) containing 312 mM Tris-HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) SDS, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8), heated at ~99 °C for 3 min, and applied to a 15-well pre-cast Tris-

glycine 4-20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). Pre-stained molecular weight markers (Precision Plus Protein Standards Dual color; Bio-Rad, Hercules, CA) were loaded in parallel to verify electrotransfer of the proteins to the membrane and to estimate the size of the immunoreactive bands observed. Electrophoresis was performed at a constant voltage of 130 V for 90 min. Electrotransfer to a 0.45 µm nitrocellulose membrane (Invitrogen, Carlsbad, CA) was performed for 90 min at a constant voltage of 30 V. After electrotransfer, the membrane was blocked for 1 h with 5% (w/v) non-fat dried milk (NFDM) in 1X phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST). The membrane was then probed with a 1:1000 dilution of goat anti-CP4 EPSPS antibody (lot 10000787) in 5% NFDM in PBST overnight at 4 °C. Excess antibody was removed using three 10 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Thermo, Rockford, IL) at a dilution of 1:10,000 in 5% NFDM in PBST for 1 h at room temperature. Excess HRP-conjugate was removed using three 10 min washes with PBST. All washes were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (GE, Healthcare, Piscataway, NJ) with exposure (1 and 3 min) to Amersham Hyperfilm ECL (GE, Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

Quantification of the bands on the blot was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA) using the lane finding and contour tools. The signal intensities of the immunoreactive bands observed for the MON 88302- and *E. coli*-produced proteins migrating at the expected position on the blot film were quantified as "contour quantity" values. The raw data was exported to a Microsoft Excel (2007) file for the pair wise comparison of the average of the load replicates. An average difference was calculated for each comparison to assess the immunoreactivity equivalence. The immunoreactivity of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were considered equivalent if the signal intensity of the CP4 EPSPS bands were within 35% of one another.

### B.4.4. Molecular Weight and Purity Estimation SDS-PAGE

An aliquot of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were mixed with 5X LB and diluted with $H_2O$ to a final total protein concentration of 0.2 µg/µl. Molecular Weight Standards, Bio-Rad broad range (Hercules, CA) were diluted to a final total protein concentration of 0.9 µg/µl. The MON 88302 produced CP4 EPSPS was analyzed in duplicate at 1, 2, and 3 µg protein per lane. The *E. coli*-produced CP4 EPSPS reference standard was analyzed at 1 µg total protein in a single lane. The samples were loaded onto a 10-well pre-cast Tris glycine 4-20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA) and electrophoresis was performed at a constant voltage of 130 V for 95 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) acetic acid for ~30 min, stained for ~16 h with Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). Gels were destained for 30 to 45 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and for ~7 h with 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

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The apparent MW of each observed band was estimated from a standard curve generated by the Quantity One software which was based on the MWs of the markers and their migration distance on the gel. To determine purity, all visible bands within each lane were quantified using Quantity One software. The purity of the MON 88302-produced CP4 EPSPS protein was reported as the percent of the total of all quantified bands in a lane. Apparent MW and purity were reported as an average of all six lanes containing the MON 88302-produced CP4 EPSPS

### B.4.5. Glycosylation Analysis

Glycosylation analysis was used to determine whether the MON 88302-produced CP4 EPSPS was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 88302-produced CP4 EPSPS protein, the *E. coli*-produced CP4 EPSPS (negative control) and the positive control, transferrin (Sigma-Aldrich, St Louis, MO), were each diluted with water and mixed with 1X LB. These samples were heated at ~95 °C for 3 min. The MON 88302- and the *E. coli*- produced CP4 EPSPS proteins were loaded at approximately 100 and 200 ng per lane and transferrin was loaded at approximately 50, 100, 150 and 200 ng on a Tris-glycine 10-well 4-20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA). Precision Plus Protein Dual color Standards (Bio-Rad, Hercules, CA) were also loaded to verify electrotransfer of the proteins to the membrane and as markers for molecular weight. Electrophoresis was performed at a constant voltage of 155 V for 75 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 35 min at a constant voltage of 100 V.

Carbohydrate detection was performed directly on the PVDF membrane at room temperature using the Amersham ECL glycoprotein Detection Module (GE, Healthcare, Piscataway, NJ). With this module, carbohydrate moieties of proteins are oxidized with sodium metaperiodate and are then biotinylated with biotin-X-hydrazide. The biotinylated proteins can be detected on the blot by addition of streptavidin conjugated to HRP for luminol-based detection using ECL reagents (GE, Healthcare, Piscataway, NJ) and with subsequent exposure (1, 2 and 3 min) to Amersham Hyperfilm (GE, Healthcare). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

A second identical blot run in parallel to that used for the glycosylation analysis was stained to visualize the proteins present on the membrane. Proteins were stained for 30 sec to 2 min using Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, Hercules, CA) and then destained with 1X Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad) for 5 min. After washing with water, the blot was dried and scanned using Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0).

### B.4.6. Functional Activity

Prior to functional activity analysis, both MON 88302- and *E. coli*-produced proteins were diluted to a purity corrected concentration of ~50 µg/ml with 50 mM HEPES,

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pH 7.0 buffer. Assays for both proteins were conducted in triplicate. The reactions were performed in 50 mM HEPES, pH 7.0, 0.1 mM ammonium molybdate, 1 mM PEP and 5 mM potassium fluoride with or without 2 mM S3P for 2 min at ~25 °C. The reactions were initiated by the addition of PEP. After 2 min, the reactions were quenched with phosphate assay reagent (0.033% malachite green, 1.1% ammonium molybdate) and then fixed with 33% (w/v) sodium citrate. A standard curve was prepared using 0 to 10 nmoles of inorganic phosphate in water treated with the phosphate assay reagent and 33% (w/v) sodium citrate. The absorbance of each reaction and each standard was measured in duplicate at 660 nm using a PowerWave Xi™ (Bio-Tek, Richmond, VA) microplate reader. The amount of inorganic phosphate released from PEP in each reaction was determined using the standard curve. For CP4 EPSPS, the specific activity was defined in unit per mg of protein (U/mg), where a unit (U) is defined as 1 µmole of inorganic phosphate released from PEP per min at 25 °C. Calculations of the specific activities were performed using Microsoft Excel (2007).

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## Appendix C: Materials and Methods Used for the Analysis of the Levels of CP4 EPSPS Protein in MON 88302

### C.1. Materials

Seed, forage, over-season leaf (OSL-1-4), and root (Root-1-2) tissue samples from MON 88302 were harvested from three field sites in the U.S. and three field sites in Canada during the 2009 growing season from starting seed lot 11225246. An *E. coli*-produced CP4 EPSPS protein (lot 10000739) was used as the analytical reference standard.

### C.2. Characterization of the Materials

The identity of MON 88302 was confirmed by verifying the chain of custody documentation prior to analysis. To further confirm the identities of MON 88302 event-specific polymerase chain reaction (PCR) analyses were conducted on the harvested seed from each site. Any seed sample and its associated tissues, for which three or more pools out of four tested unexpectedly during PCR verification, were not analyzed in this study.

### C.3. Field Design and Tissue Collection

Field trials were initiated during the 2009 planting season to generate MON 88302 samples at various canola growing locations in the U.S. and Canada. The forage, seed, OSL-1-4, and Root-1-2 tissue samples from the following field sites were analyzed: Power County, Idaho, U.S. (IDAF), Wilkin County, Minnesota, U.S (MNCA), McHenry County, North Dakota, U.S. (NDVA), Portage la Prairie, Manitoba, Canada (MBPL), Newton, Manitoba, Canada (MBNW), and Saskatoon, Saskatchewan, Canada (SKSA). These field sites were representative of canola producing regions suitable for commercial production. At each site, four replicated plots of plants containing MON 88302 were planted using a randomized complete block field design. OSL-1-4, forage, seed, and Root-1-2 samples were collected from each replicated plot at all field sites. See Table VI-5 for a detailed description of when the samples were collected.

From the IDAF site, seed and Root-2 samples were excluded from the study due to inclement weather during collection which impacted sample quality and quantity.

### C.4. Tissue Processing and Protein Extraction

Tissue samples were shipped to Monsanto, St. Louis. The following tissues were not received by Sample Management: all OSL-2 tissue samples from sites MBPL, MBNW, and MNCA, and one Root-2 sample from site NDVA. The following tissues were received but not processed by sample management due to compromised sample integrity: all OSL-1 samples from site MBNW, one Root-1 sample from site SKSA, all Root-2 samples from site SKSA, and one Root-2 sample from site NDVA. The processed tissue samples were stored in a -80 °C freezer.

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CP4 EPSPS protein was extracted from the tissue samples as described in Table C-1. CP4 EPSPS protein was extracted from all tissues samples using a Harbil Mixer with the appropriate amount of Tris-borate buffer with L-ascorbic acid (1× TBA) [0.1 M Tris, 0.1 M $Na_2B_4O_7 \cdot 10H_2O$, 0.005 M • $6H_2O$ $MgCl_2$, 0.05% (v/v) Tween-20 at pH 7.8, 0.2% (w/v) L-ascorbic acid]. Insoluble material was removed from all tissue extracts using a serum filter (Fisher Scientific, Pittsburgh, PA). The extracts were aliquotted and stored frozen in a -80 °C freezer until ELISA analysis.

**Table C-1. Protein Extraction Methods for Tissue Samples**

| Sample Type | Tissue-to-Buffer Ratio | Extraction Buffer |
|---|---|---|
| Leaf[2] | 1:100 | 1× TBA |
| Root[3] | 1:100 | 1× TBA |
| Forage | 1:100 | 1× TBA |
| Seed | 1:100 | 1× TBA |

[1]Over- season leaf (OSL-1, OSL-2, OSL-3, and OSL-4).
[2]Root (Root-1 and Root-2).

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## C.5. CP4 EPSPS Antibodies

Mouse monoclonal antibody clone 39B6.1 (IgG2a isotype, kappa light chain; lot 10002190) specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Protein-A Sepharose affinity chromatography and was used as the capture antibody in the CP4 EPSPS ELISA. The concentration of the purified IgG was determined to be 2.3 mg/ml by spectrophotometric methods. Production of the 39B6.1 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 20 mM sodium phosphate, 150 mM NaCl, and 15 ppm Proclin 300 (Sigma-Aldrich, St. Louis, MO).

The detection reagent was goat anti-CP4 EPSPS antibody, otherwise known as anti-protein 4 (Sigma-Aldrich, catalog number P-5867) conjugated to horseradish peroxidase (HRP).

## C.6. CP4 EPSPS ELISA Method

Mouse anti-CP4 EPSPS antibodies were diluted in coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) to a final concentration of 2.0 µg/ml, and immobilized onto 96-well microtiter plates followed by incubation in a 4 °C refrigerator for >8 hours. Prior to each step in the assay, plates were washed with 1× PBST. CP4 EPSPS protein standard or sample extract was added at 100 µl per well and incubated for 1 hour at 37 °C. The captured CP4 EPSPS protein was detected by the addition of 100 µl per well of anti-CP4 EPSPS HRP conjugate. Plates were developed by adding 100 µl per well of 3,3',5,5' tetramethyl-benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M $H_3PO_4$. Quantification of the CP4 EPSPS protein was accomplished by interpolation from a CP4 EPSPS protein standard curve that ranged from 0.456-14.6 ng/ml.

## C.7. Moisture Analysis

Tissue moisture content was determined using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO). A homogeneous tissue-specific site pool (TSSP) was prepared consisting of samples of a given tissue type grown at a given site. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$\text{DWCF} = 1 - \left(\frac{\text{Mean\% TSSP Moisture}}{100}\right)$$

The DWCF was used to convert protein levels assessed on a µg/g fresh weight (fw) basis into levels reported on a µg/g dry weight (dw) basis using the following calculation:

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$$\text{Protein Level in Dry Weight} = \left(\frac{\text{Protein Level Fresh Weight}}{\text{DWCF}}\right)$$

The protein levels (ng/ml) that were reported to be less than or equal to the limit of detection (LOD) or less than the limit of quantitation (LOQ) on a fresh weight basis were not reported on a dry weight basis.

### C.8. Data Analyses

All CP4 EPSPS ELISA plates were analyzed on a SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. All protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620-655 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GxP version 5.0.1. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was converted to a µg/g fw basis for data that were greater than or equal to the LOQ. This conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The protein values expressed as µg/g fw were also converted to µg/g dw by applying the DWCF. Microsoft Excel 2007 (Version (12.0.6535.5002) SP2 MSO (12.0.6535.5002) Microsoft, Redmond, WA) was used to calculate the CP4 EPSPS protein level in canola tissues. The sample means, standard deviations, and ranges were also calculated by Microsoft Excel 2007.

Any MON 88302 sample extracts that resulted in unexpectedly negative results by ELISA analysis were re-extracted twice for the protein of interest and re-analyzed by ELISA to confirm the results. Samples with confirmed unexpected results were omitted from all calculations.

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## Appendix D: Western Blot Analysis of CP4 EPSPS Protein in MON 88302 Leaf across Multiple Generations

### D.1. Materials

Leaf tissues of MON 88302 were collected from plants of multiple breeding generations grown in a U.S. greenhouse (St. Louis, MO).

### D.2. MON 88302 Materials

A summary of the MON 88302 leaf samples and the starting seed lot numbers are listed in the table below.

| Generation | Lot Number | Starting Seed Lot Number |
|---|---|---|
| $R_2$ | 11265249 | 11236102 |
| $R_3$ | 11264677 | 11236103 |
| $R_4$ | 11264679 | 11225246 |
| $R_{5a}$ | 11264682 | 11263713 |
| $R_{5b}$ | 11265250 | 11261829 |

### D.3. Control Material

The negative control substance was a conventional canola variety (Ebony) in a similar genetic background to MON 88302. The conventional control does not contain the CP4 EPSPS protein. Leaf tissue of the conventional control was evaluated.

| Description | Lot Number | Starting Seed Lot Number |
|---|---|---|
| Conventional Control | 11264672 | 11225244 |

### D.4. Characterization of MON 88302 and Control Materials

The identities of MON 88302 and the conventional control were confirmed by verifying the chain of custody documentation. The identities of the MON 88302 generations $R_4$, $R_5$a and the conventional control were further confirmed by event-specific polymerase chain reaction (PCR) analyses of the starting seeds. The identities of the MON 88302 generations $R_2$, $R_3$ and $R_{5b}$ were further confirmed by endpoint TaqMan PCR analysis used on the starting seed DNA by testing leaf punches taken from each individual plant. This analysis was performed by the Monsanto Company Seed Quality Technologies Group (SQT) and copies of the results were archived with this study file.

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### D.5. Reference Material

The *E. coli*-produced CP4 EPSPS (lot 10000739) protein was used as the analytical reference standard for the western blot analysis.

### D.6. Methods

Leaf tissue samples from multiple breeding generations of MON 88302 were analyzed by western blot to demonstrate the presence of the CP4 EPSPS protein in the $R_2$, $R_3$, $R_4$, $R_{5a}$, and $R_{5b}$ generations. Leaf tissue from the conventional control substance was also analyzed by western blot to confirm the expected absence of the CP4 EPSPS protein. The presence or absence of the CP4 EPSPS protein was determined using a goat anti-CP4 EPSPS polyclonal antibody (Lot 047K6082) and the *E. coli*-produced protein standard (lot 10000739) was used as a reference for molecular weight comparison.

### D.7. MON 88302 Tissue Processing

All samples were processed by the Monsanto Sample Management Team in St. Louis, Missouri. The processed tissue samples were stored in a -80 °C freezer until transferred on dry ice to the analytical facility.

### D.8. Extraction

The CP4 EPSPS protein was extracted from processed leaf samples in 1× Tris-Borate with L-Ascorbic Acid (TBA). All processed tissues were kept on dry ice during extract preparation. The CP4 EPSPS protein was extracted from each tissue by adding the appropriate volume of extraction buffer, and shaking in a Harbil mixer. Insoluble material was removed from the extracts by using a serum filter. The extracts were aliquoted and stored in a -80 °C freezer until analysis.

### D.9. SDS-PAGE

- Prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, MON 88302 and conventional control extracts were diluted 1:2.5 (v/v) in dilution buffer; 1 × Phosphate-Buffered Saline with 0.05% (v/v) Tween-20 (PBST), then 1:2 (v/v) using 2 × Laemmli Buffer (Bio-Rad, Hercules, CA). Two nanograms of CP4 EPSPS protein reference standard prepared in 1× PBST was loaded onto the gel along with the MON 88302 and conventional control extracts. Sample extracts were loaded on a Novex Tris-glycine 4-20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA), along with the Precision Plus molecular weight marker (Bio-Rad) to demonstrate the transfer of protein to membrane and the MagicMark molecular weight marker (Invitrogen) to show the molecular weights of the protein. Electrophoresis was conducted at 125 V for approximately 90 min in 1× Novex Tris-Glycine SDS running buffer (Invitrogen) until the dye front reached the end of the gel.

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**D.10. Western Blot Analysis (Immunoblotting)**

Proteins separated by SDS-PAGE were electrophoretically transferred to a 0.45 µm Invitrolon Polyvinylidene Fluoride (PVDF) membrane (Invitrogen) using 1× Novex Tris-glycine transfer buffer (Invitrogen) containing 20% methanol. After transfer, non-specific sites were blocked using 5% (w/v) non-fat dry milk (NFDM, Schnucks) in 1× PBST. The membrane was probed for the presence of the CP4 EPSPS protein using a 1:4000 dilution of purified goat anti-CP4 EPSPS antibody (Lot 047K6082) in 1× PBST with 2% (w/v) NFDM. The membrane was washed three times for 10 min each time in 1× PBST to remove unbound antibody. Bound antibody was then probed with a 1:1250 dilution of anti-goat IgG antibody conjugated to horseradish peroxidase (HRP; Thermo Scientific, Rockford, IL) in 1× PBST with 2% (w/v) NFDM. The membrane was washed four times for 10 min each time in 1× PBST to remove unbound antibody. The SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL) was added to the membrane according to the manufacturers' instructions. The membrane was exposed using the Kodak Gel Logic 2200 imaging system.

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## Appendix E: Bioinformatics Evaluation of MON 88302

### E.1. Bioinformatics Evaluation of the CP4 EPSPS Protein in MON 88302

#### E.1.1. Sequence Database Preparation

The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from FARRP (2010) and was used as provided. The AD_2010 database contains 1,471 sequences.

GenBank protein database, release 175.0 was downloaded from National Center for Biotechnology Information (NCBI) and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2010 database and contains 17,815,538 sequences.

The toxin database is a subset of sequences derived from the PRT_2010 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins It is referred to herein as the TOX_2010 database and contains 8,448 sequences.

#### E.1.2. Sequence Database Searches

FASTA analyses using the AD_2010, PRT_2010 and TOX_2010 databases were performed on a virtual machine loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The structural similarity of the translated protein sequences to sequences in each database (AD_2010, TOX_2010 and PRT_2010) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (*k-tuple*), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E*-score of $1 \times 10^{-5}$ or smaller to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

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If two proteins share sufficient linear sequence similarity and identity, they will also share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of the MON 88302 CP4 EPSPS protein sequence to known allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2010). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Goodman et al., 2002; Hileman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

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### E.1.3. Significance of the Alignment

An *E*-score of $1\times10^{-5}$ was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than $1\times10^{-5}$ was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2010 database were inspected to determine whether they exceeded the Codex threshold of 35% or greater identity in 80 or greater amino acids amino acids.

## E.2. Bioinformatics Evaluation of the Transfer DNA Insert in MON 88302

### E.2.1. Methods

#### E.2.1.1. Database Assembly

The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from FARRP (2010) and was used as provided. The AD_2010 database contains 1,471 sequences.

GenBank protein database, release 175.0 was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2010 database and contains 17,815,538 sequences.

The toxin database is a subset of sequences derived from the PRT_2010 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. It is referred to herein as the TOX_2010 database and contains 8,448 sequences.

#### E.2.1.2. Translation of Query Sequences

The DNA insert sequence was translated beginning with nucleotide 1, 2 or 3 through the final nucleotide to yield frames 1, 2 or 3, respectively. Likewise, the reverse complement (anti-sense) strand of the above described sequence was translated beginning with nucleotide 1, 2 or 3 through the final nucleotide to yields frame 4, 5, or 6, respectively. All sequences were translated using standard genetic code with DNAStar, version 8.0.2 (13), 412. The resultant amino acid sequences were used to search the AD_2010, PRT_2010 and TOX_2010 databases.

#### E.2.1.3. Sequence Database Searches

FASTA analyses using the AD_2010, PRT_2010 and TOX_2010 databases were performed on a virtual machine loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The structural similarity of the translated protein sequences to sequences in each database (AD_2010, TOX_2010 and PRT_2010) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

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FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (*k-tuple*), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E*-score of $1\times10^{-5}$ or smaller to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of each putative polypeptide to allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2010). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Hileman et al., 2002;

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Goodman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, (Silvanovich et al., 2006) demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

#### E.2.1.4. Significance of the Alignment

An *E*-score of $1 \times 10^{-5}$ was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than $1 \times 10^{-5}$ was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2010 database were inspected to determine whether they exceeded the Codex threshold of 35% or greater identity in 80 or greater amino acids.

### E.2.2. Bioinformatics Evaluation of DNA Sequences Flanking the 5′ and 3′ Junctions of Inserted DNA in MON 88302: Assessment of Putative Polypeptides

#### E.2.2.1. Sequence Database Preparation

The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from FARRP (2010) and was used as provided. The AD_2010 database contains 1,471 sequences.

GenBank protein database, release 175.0 was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2010 database and contains 17,815,538 sequences.

The toxin database is a subset of sequences derived from the PRT_2010 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. It is referred to herein as the TOX_2010 database and contains 8,448 sequences.

#### E.2.2.2. Translation of Putative Polypeptides

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DNA sequence spanning the 5′ and 3′ junctions of the MON 88302 insertion site was analyzed for translational stop codons (TGA, TAG, TAA). All six reading frames originating or terminating within the MON 88302 T-DNA insert and originating or terminating within the intervening sequence were translated using the standard genetic code from stop codon to stop codon using DNAStar, version 8.0.2 (13), 412. A total of twenty sequences of eight amino acids or greater that spanned the junction(s) were analyzed.

### E.2.2.3. Sequence Database Searches

FASTA analyses using the AD_2010, TOX_2010 and PRT_2010 databases were performed on a virtual machine loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The DNA sequence was translated to the amino acid sequence with DNAStar, version 8.0.2 (13), 412 or SeqBuilder 8.0.2 (13). The structural similarity of the translated protein sequences to sequences in each database (AD_2010, TOX_2010, and PRT_2010) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (*k-tuple*), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E*-score of $1\times10^{-5}$ or smaller to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-

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reactivity caused by proteins is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of each putative polypeptide to known allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2010). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Hileman et al., 2002; Goodman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

#### E.2.2.4. Significance of the Alignment

An *E*-score of $1\times10^{-5}$ was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than $1\times10^{-5}$ was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2010 database were inspected to determine whether they exceeded the Codex threshold of 35% or greater identity in 80 or greater amino acids amino acids.

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## Appendix F: Methods Used in Assessing Stability of Proteins in Simulated Digestive Fluid

### F.1. Materials

Purified *E. coli*-produced CP4 EPSPS protein (Lot 5192245) was used as the test substance. The *E. coli*-produced CP4 EPSPS protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT and 50% glycerol. The *E. coli*-produced CP4 EPSPS protein has a purity of 90% and a concentration of 3.96 mg/ml. The protein was diluted to 1.68 mg/ml with PBS before use.

Simulated gastric fluid (SGF) contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH of 1.2. The SGF was prepared using a highly purified form of pepsin (catalog number P 6887, Sigma Company, St. Louis, MO).

### F.2. Digestion of CP4 EPSPS in Simulated Gastric Fluid (SGF) Method

Digestions were initiated by addition of *E. coli*-produced CP4 EPSPS to tubes containing simulated gastric fluid (SGF), where 10 units of pepsin activity were used per 1 µg of total protein. Digestions were incubated at 37 ± 2 °C in separate tubes for various durations, and the reactions were quenched by addition of a sodium carbonate solution to each tube. The zero incubation time point (T = 0) was quenched by addition of sodium carbonate solution to SGF prior to addition of the *E. coli*-produced CP4 EPSPS. The SGF was assayed before conducting the timed incubations to demonstrate that pepsin was active. Experimental controls were prepared to demonstrate the stability of *E. coli*-produced CP4 EPSPS in the system without pepsin. These controls were incubated for 0 and 60 min and were designated with the letter "P" (P0 and P9, respectively). Additionally, experimental controls to characterize the system without *E. coli*-produced CP4 EPSPS were also included. These experimental controls were prepared by substituting buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT and 25% glycerol) for *E. coli*-produced CP4 EPSPS. These controls were incubated for 0 and 60 min and were designated with the letter "N" (N0 and N9).

All quenched specimens were stored in a –20 ºC freezer until analyzed. The digestibility of *E. coli*-produced CP4 EPSPS in SGF was assessed using SDS-PAGE gel followed by Brilliant Blue G Colloidal dye (Sigma P/N B-2025) staining and immunoblotting. Limits of detection (LOD) were determined for the gel staining and immunoblot methods.

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## Appendix G: Heat Stability of CP4 EPSPS Protein

The previously characterized *E. coli*-produced CP4 EPSPS protein (lot 10000739) was used as both the test substance and reference protein. As reference protein, CP4 EPSPS protein was maintained at -80 °C until the heat treatment samples were ready for analysis. The reference protein was evaluated along with the heat treatment samples in the functional assay and the SDS-PAGE analysis.

### G.1. Heat Treatment

The CP4 EPSPS protein was thawed on wet ice and diluted in 50 mM Tris HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine HCl to a final total protein concentration of 1 mg/ml. Aliquots of 200 µl of the diluted CP4 EPSPS protein were transferred to six tubes. The six aliquots in tubes were maintained on wet ice until the heat treatments were initiated. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95 °C, each ± 2 °C) and incubated for 30 ± 1 min. The sixth tube, a control treatment, was maintained on wet ice throughout the heat treatment incubation period. All temperature-treated samples were returned immediately to wet ice following the incubation period.

Following the heat treatments, 20 µl of each temperature treated sample (including the control treatment) was transferred to a clean tube and mixed with 5 µl of 5X LB (0.312 M Tris HCl, pH 6.8, 10% SDS, 50% glycerol, 3.6 M 2-mercaptoethanol, 0.025% Bromophenol Blue) in preparation for SDS-PAGE analysis. The 20 µl samples were heated at 95 ± 5 °C for 3-5 min, quick frozen by placement on dry ice, and stored at -80 °C until analysis. The remainder of each temperature-treated sample (approximately 180 µl each) was maintained on wet ice and used for functional activity assessment.

### G.2. Functional Activity Assay

The CP4 EPSPS functional activity of the heat treated samples, the control treatment sample, and the reference protein were determined using the functional activity assay described in Appendix B section B.4.6. All samples were diluted to total protein concentration of 0.05 mg/ml in 50 mM HEPES, pH 7.0 prior to analysis. Two replicates of each diluted protein sample were used for the analysis.

### G.3. SDS-PAGE

The samples prepared above (Section G.1) for SDS-PAGE analysis, were thawed, heated at 95 ± 5 °C for 3-5 min, and loaded on one 4-20% polyacrylamide gradient gel at 0.8 mg total protein/ml. The reference protein was loaded on the same gel at 0.8 mg/ml and at 0.08 mg/ml. Following electrophoresis, gels were stained with Brilliant Blue G Colloidal (Sigma, St. Louis, MO).

After staining, the stability of CP4 EPSPS at each heat treatment was evaluated qualitatively. The intensity of the major protein band at 43.8 kDa in the heat treatment lanes was compared visually to the same band in the lanes with the control treatment, 100% reference protein equivalent, and 10% reference protein equivalent.

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# Appendix H: Materials, Methods, and Individual Site Results for Compositional Analysis of MON 88302 Canola Seed

## H.1. Materials

Seed from MON 88302 (Seed Lot Number 11225246) and the conventional control (Seed Lot Number 11225244) was evaluated. The conventional control has background genetics similar to that of MON 88302 but does not contain the *cp4 epsps* expression cassette. The commercial reference varieties were seven conventional canola varieties (Table H-1).

**Table H-1. Commercial Reference Varieties**

| Material Name | Seed Lot Number | Field Sites[1] |
|---|---|---|
| Q2 | 10001931 | MBPL, MBNW, SKSA, NDVA, MNCA |
| Hyola 401 | 10001850 | NDVA, MBPL, SKSA |
| SP Armada | 10001932 | MBPL, SKSA, NDVA |
| Croplan 601 | 10001849 | MBPL, SKSA, NDVA |
| SValof Sponsor | 10002116 | MNCA, MBNW |
| SValof Senator | 10002115 | MNCA, MBNW |
| DSV Ability | 10002117 | MNCA, MBNW |

[1]Field sites described in Section VII.A.

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## H.2. Characterization of the Materials

The identities of MON 88302, the conventional control, and commercial reference varieties were confirmed by verifying the chain of custody documentation prior to analysis. To further confirm the identities of MON 88302, the conventional control, and commercial reference varieties, event-specific polymerase chain reaction (PCR) analyses were conducted on the harvested seed from each site to confirm the presence or absence of the *cp4 epsps* expression cassette.

## H.3. Field Production of the Samples

Seed of the MON 88302, the conventional control and commercial reference varieties was collected from replicated plots at each of two U.S. sites [Wilkin County, MN (MNCA); and McHenry County, North Dakota (NDVA)] and three Canadian sites [Portage la Prairie, Manitoba (MBPL); Newton, Manitoba (MBNW); and Saskatoon, Saskatchewan (SKSA)**]**. Seeds were planted in a randomized complete block design with four replicates per site. The MON 88302 plots were treated with glyphosate applications between the 5-6 leaf stage, at a target rate of 1800 g a.e./ha. All samples at the field sites were grown under normal agronomic field conditions for their respective geographic regions. Seed samples were harvested from all plots and shipped at ambient temperature from the field sites to Monsanto Company (St. Louis, MO). Sub-samples were ground to a powder, stored in a freezer set to maintain -20 °C located at Monsanto Company (St. Louis, MO), and then shipped on dry ice to Covance Laboratories Inc. (Madison, WI) for analysis.

## H.4. Summary of Analytical Methods

Ground grain samples were analyzed by Covance Laboratories Inc. Upon receipt, the samples were stored in a freezer set to maintain -20 °C until their use. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), amino acids, fatty acids (C8-C24), vitamin E (α-tocopherol), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc) in the grain. The anti-nutrients assessed in grain included erucic acid, glucosinolates (alkyl glucosinolates, indolyl glucosinolates, and total glucosinolates), phytic acid and sinapic acid.

### H.4.1. Acid Detergent Fiber

The ANKOM2000 Fiber analyzer automated the process of removal of proteins, carbohydrates, and ash. Fats and pigments were removed with an acetone wash prior to analysis. The fibrous residue that is primarily cellulose, lignin, and insoluble protein complexes remained in the Ankom filter bag, and were determined gravimetrically. (Komarek et al., 1994; USDA, 1970). The results are reported on fresh weight basis. The limit of quantitation was 0.100%.

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### H.4.2. Amino Acid Composition

The following 18 amino acids were analyzed:

| | |
|---|---|
| Total threonine | Total aspartic acid (including asparagine) |
| Total serine | Total tyrosine |
| Total phenylalanine | Total glutamic acid (including glutamine) |
| Total proline | Total histidine |
| Total glycine | Total lysine |
| Total alanine | Total arginine |
| Total valine | Total tryptophan |
| Total isoleucine | Total methionine |
| Total leucine | Total cystine (including cysteine) |

The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct acid hydrolysis with hydrochloric acid. Once hydrolyzed, the individual amino acids were then quantified using an automated amino acid analyzer (AOAC, 2005a). The limit of quantitation was 0.100 %.

***Reference Standards:***

- Thermo Scientific, K18 amino acid standard, H,, 2.5 ± 0.1 µmol/mL per constituent (except cystine 1.25 ± 0.1 µmol/mL), Lot Number KG137091
- Sigma, L-Tryptophan, 100%, Lot Number 097K0119
- Sigma/BioChemika, L-Cysteic Acid Monohydrate, 99.5% (used as 100%), Lot Number 1305674
- Sigma, L-Methionine Sulfone, 100%, Lot Number 047K1321
- Sigma, L-Norvaline, 100%, 087K1954

### H.4.3. Ash

The sample was placed in an electric furnace at 550 °C and ignited. The nonvolatile matter remaining was quantified gravimetrically and calculated to determine percent ash (AOAC, 2005b). The limit of quantitation was 0.100%.

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#### H.4.4. Carbohydrates

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation:

$$\% \text{ carbohydrates} = 100\% - (\% \text{ protein} + \% \text{ fat} + \% \text{ moisture} + \% \text{ ash})$$

The results are reported on fresh weight basis (USDA, 1973). The limit of quantitation was 0.100%.

#### H.4.5. Fat by Soxhlet Extraction

The sample was weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was then evaporated, dried, and weighed (AOAC, 2005c). The results are reported on fresh weight basis. The limit of quantitation was 0.100%.

#### H.4.6. Fatty Acids as Triglycerides

The lipid was extracted, saponified with 0.5 N methanolic sodium hydroxide, and methylated with 14% boron trifluoride in methanol. The resulting methyl esters of the fatty acids were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation (AOAC, 2005d; AOCS, 1997; 2007). The results are reported on fresh weight basis. The limit of quantitation was 0.0400%.

***Reference Standards:***

- Nu Chek Prep GLC Reference Standard Hazleton No. 1, *, Lot Number MA30-U
- Nu Chek Prep GLC Reference Standard Hazleton No. 2, *, Lot Number AU24-T
- Nu Chek Prep GLC Reference Standard Hazleton No. 3, *, Lot Number JY17-T
- Nu Chek Prep GLC Reference Standard Hazleton No. 4, *, Lot Number MA30-U
- Nu Chek Prep Methyl Gamma Linolenate, used as 100%, Lot Number U-63M-08-T
- Nu Chek Prep Methyl Tridecanoate, used as 100%, Lot Number N-13M-MA25-T
- Nu Chek Prep Methyl Erucate, used as 100%, Lot Numbers U-79M-JA28-T
- Nu Chek Prep Methyl Lignocerate, used as 100%, Lot Number N-24M-S8-T
- Nu Chek Prep Methyl Docosapentaenoate, used as 100%, Lot Number U-101M-D4-T
- Nu Chek Prep Methyl Docosahexaenoate, used as 100%, Lot Number U-84M-JA15-U
- Nu Chek Prep Methyl Eicosapentaenoate, used as 100%, Lot Number U-99M-S22-T
- Nu Chek Prep Methyl Nervonate, used as 100%, Lot Number U-88M-MA31-U

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- Cayman Chemicals Steariodonic Acid Methyl Ester, 100%, Lot Number 0407775

*Overall purity of the sum of the mixture of components was used as 100%

### H.4.7. Glucosinolates

Glucosinolates were extracted using 70% methanol at 75 °C. They were then purified and enzymatically desulfatated on ion-exchange resin. Determination was by reversed-phase high performance liquid chromatography with gradient elution and ultraviolet detection using an internal standard. Quantification was performed based on the relative responses to the internal standards. Peak identification was made based on retention times determined by comparing the chromatograms of internal standard(s) and three BCR certified rapeseed controls (ISO, 1992). The results are reported on fresh weight basis. The limit of quantitation was 0.00300 µmole/g.

***Reference Standard:***

- Chromadex, Glucotropaeolin Potassium Salt, 98.7%, Lot Number 07300-304

### H.4.8. ICP Emission Spectrometry

The sample was dried, precharred, and ashed overnight in a muffle furnace set to maintain 500 °C. The ashed sample was re-ashed with nitric acid, treated with hydrochloric acid, taken to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured on the inductively coupled plasma spectrometer, with the emission of the standard solutions (AOAC, 2005e). The results are reported on fresh weight basis.

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***Reference Standards:***

Inorganic Ventures Reference Standards and Limits of Quantitation:

| Mineral | Lot Numbers | Concentration (µg/mL) | Limit of Quantitation (ppm) |
|---|---|---|---|
| Calcium | D2-MEB322092MCA, D2-MEB322094 | 200, 1000 | 20.0 |
| Copper | D2-MEB322092MCA, D2-MEB322093MCA | 2.00, 10.0 | 0.500 |
| Iron | D2-MEB322092MCA, D2-MEB322095 | 10.0, 50.0 | 2.00 |
| Magnesium | D2-MEB322092MCA, D2-MEB322093MCA | 50.0, 250 | 20.0 |
| Manganese | D2-MEB322092MCA, D2-MEB322093MCA | 2.00, 10.0 | 0.300 |
| Phosphorus | D2-MEB322092MCA, D2MEB322094 | 200, 1000 | 20.0 |
| Potassium | D2-MEB322092MCA, D2-MEB322094 | 200, 1000 | 100 |
| Sodium | D2-MEB322092MCA, D2-MEB322094 | 200, 1000 | 100 |
| Zinc | D2-MEB322092MCA, D2-MEB322093MCA | 10.0, 50.0 | 0.400 |

### H.4.9. Moisture

The sample was dried in a vacuum oven at approximately 100 °C to a constant weight. The moisture weight loss was determined and converted to percent moisture (AOAC, 2005f). The results are reported on fresh weight basis. The limit of quantitation was 0.100%.

### H.4.10. Neutral Detergent Fiber, Enzyme Method

The ANKOM2000 Fiber Analyzer automated the process of the removal of proteins, carbohydrates, and ash. The fats and pigments were removed with an acetone wash prior to analysis. Hemicellulose, cellulose, lignin and insoluble protein fraction was left in the filter bag and determined gravimetrically (AACC, 1998; Komarek et al., 1994; USDA, 1970). The results are reported on fresh weight basis. The limit of quantitation was 0.100%.

### H.4.11. Phytic Acid

The sample was extracted using 0.5 M HCl with ultrasonication. Purification and concentration were accomplished on a silica-based anion-exchange column. The sample was analyzed on a polymer high-performance liquid chromatography column PRP-1, 5 µm (150 x 4.1mm) with a refractive index detector (Lehrfeld, 1989; Lehrfeld, 1994). The results are reported on fresh weight basis. The limit of quantitation was 0.100%.

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***Reference Standard:***

Sigma-Aldrich, Phytic Acid Sodium Salt Hydrate, 96%, Lot Number 089K0159**H.4.12. Protein**

The protein and other organic nitrogen in the sample were converted to ammonia by digesting the sample with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25 (AOAC, 2005g; AOCS, 1998). The results are reported on fresh weight basis. The limit of quantitation was 0.100%.

**H.4.13. Sinapic Acid**

The ground sample was extracted with methanol followed by alkaline hydrolysis and buffering prior to injection on an analytical high-performance liquid chromatography (HPLC) system for quantification of sinapic acid by ultra violet (UV) detection (Hagerman and Nicholson, 1982). The results are reported on fresh weight basis. The limit of quantitation was 200 ppm.

***Reference Standard:***

Sigma, Sinapic Acid, 99.3%, Lot No. 079K1171.

**H.4.14. Total Dietary Fiber**

Duplicate samples were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber. The sample was filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated using protein and ash values (AOAC, 2005h). The results were reported on fresh weight basis. The limit of quantitation was 1.00%.

**H.4.15. Vitamin E**

The sample was saponified to break down any fat and release vitamin E. The saponified mixture was extracted with ethyl ether and then quantified by high-performance liquid chromatography using a silica column (Cort et al., 1983; McMurray et al., 1980; Speek et al., 1985). The results are reported on fresh weight basis. The limit of quantitation was 0.500 mg/100g.

***Reference Standard:***

USP, α-Tocopherol, 98.9%, Lot Number N0F068

**H.5. Data Processing and Statistical Analysis**

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After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Monsanto Company for review. Data were then transferred to Certus International (Chesterfield, MO) where they were converted into the appropriate units and statistically analyzed. The formulas that were used for re-expression of composition data for statistical analysis are listed in Table H-2.

**Table H-2. Re-expression Formulas for Statistical Analysis of Composition Data**

| Component | From (X) | To | Formula[1] |
|---|---|---|---|
| Proximates (excluding Moisture), Fiber, Phytic Acid | % fw | % dw | X/d |
| Alkyl Glucosinolate, Indolyl Glucosinolate, Total Glucosinolate | μmole/g fw | μmole/g dw | X/d |
| Sinapic Acid | ppm fw | % dw | $X/(10^4 d)$ |
| Calcium, Magnesium, Phosphorus, Potassium, Sodium | ppm fw | g/100g dw | $X/(10^4 d)$ |
| Copper, Iron, Manganese, Zinc | ppm fw | mg/kg dw | X/d |
| Vitamin E | mg/100g fw | mg/100g dw | X/d |
| Amino Acids (AA) | mg/g fw | % dw | X/(10d) |
| Fatty Acids (FA) | % fw | % Total FA | $(100)X_j/\Sigma X$, for each $FA_j$ where $\Sigma X$ is over all the FA |

[1]'X' is the individual sample value; 'd' is the fraction of the sample that is dry matter.

In order to complete a statistical analysis for a compositional component in this study, at least 50% of the values for a component had to be greater than the assay limit of quantitation (LOQ). Components with more than 50% of observations below the assay LOQ were excluded from summaries and analysis. The following 19 components with more than 50% of the observations below the assay LOQ were excluded: 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma-linolenic acid, 18:4 octadecatetraenoic acid; 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, 20:5 eicosapentaenoic acid, 22:1 erucic acid, 22:5 docosapentaenoic acid, 22:6 docosahexaenoic acid, and sodium.

If less than 50% of the observations for a component were below the LOQ, individual analyses that were below the LOQ were assigned a value equal to one-half the LOQ. In this study 24 values for 24:0 lignoceric acid and 34 values for 24:1 nervonic acid were assigned a value of 0.02% total fw.

The data were assessed for potential outliers using a studentized PRESS residuals calculation. A PRESS residual is the difference between any value and its value predicted from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between $\pm 3$. Extreme data points that are also outside of the $\pm 6$ studentized PRESS residual range are considered for exclusion, as outliers, from the final analyses. One 18:3 linolenic value from MON 88302 at the MNCA site, one alkyl glucosinolate value and one total glucosinolate

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value from one commercial reference at the MBPL site were identified as outliers, but the values were either similar to other nearby data points or were not the extreme highest or lowest value, and were not removed from statistical analysis. One carbohydrate value and one total fat value from one commercial reference at the MBNW site were extreme data points that were outside the ± 6 studentized PRESS residual range and were removed from the statistical analysis.

All canola components were statistically analyzed using a mixed model analysis of variance. The five replicated field sites were analyzed individually and as a combined data set. Individual replicated site analyses used model (1).

(1) $Y_{ij} = U + T_i + B_j + e_{ij}$,

where $Y_{ij}$ = unique individual observation, $U$ = overall mean, $T_i$ = substance effect, $B_j$ = random block effect, and $e_{ij}$ = residual error.

Combined-site analyses used model (2).

(2) $Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk}$,

where $Y_{ijk}$ = unique individual observation, $U$ = overall mean, $T_i$ = substance effect, $L_j$ = random site effect, $B(L)_{jk}$ = random block within site effect, $LT_{ij}$ = random site by substance interaction effect, and $e_{ijk}$ = residual error.

For each compositional component, a range of observed values and a 99% tolerance interval were calculated. A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured. The calculated tolerance intervals are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of conventional canola. Each tolerance interval estimate was based upon the average observation for each unique reference material. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

SAS® (Version 9.2) software was used to generate all summary statistics and perform all analyses.

Report tables present p-values from SAS as either <0.001 or the actual value truncated to three decimal places.

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**Table H-3. Statistical Summary of Site MBNW Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Proximate (% dw)** | | | | | | |
| Ash | 3.98 (0.087)<br>(3.72 - 4.10) | 3.84 (0.10)<br>(3.66 - 4.03) | 0.14 (0.13)<br>(0.076 - 0.41) | -0.28, 0.56 | 0.367 | 3.32, 4.66<br>(2.98 - 4.52) |
| Carbohydrates | 27.18 (0.29)<br>(26.75 - 28.02) | 26.02 (0.33)<br>(25.81 - 26.35) | 1.16 (0.40)<br>(1.08 - 1.67) | -0.13, 2.45 | 0.063 | 23.12, 30.77<br>(22.53 - 29.96) |
| Moisture (% fw) | 5.26 (0.16)<br>(4.99 - 5.56) | 4.90 (0.18)<br>(4.69 - 5.13) | 0.36 (0.24)<br>(0.12 - 0.87) | -0.39, 1.12 | 0.225 | 4.33, 6.91<br>(4.09 - 8.48) |
| Protein | 21.00 (0.62)<br>(19.68 - 22.64) | 20.78 (0.71)<br>(20.29 - 21.61) | 0.22 (0.94)<br>(-0.86 - 0.49) | -2.78, 3.22 | 0.830 | 17.20, 30.08<br>(18.68 - 28.32) |
| Total Fat | 47.84 (0.47)<br>(46.87 - 49.26) | 49.35 (0.54)<br>(48.89 - 49.93) | -1.51 (0.72)<br>(-2.28 - -0.61) | -3.79, 0.77 | 0.125 | 39.65, 51.24<br>(40.71 - 50.26) |
| **Fiber (% dw)** | | | | | | |
| Acid Detergent Fiber | 16.26 (0.63)<br>(15.05 - 17.66) | 14.93 (0.71)<br>(13.64 - 16.34) | 1.32 (0.81)<br>(0.019 - 3.09) | -1.25, 3.90 | 0.199 | 6.95, 23.92<br>(9.75 - 21.22) |

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**Table H-3. Statistical Summary of Site MBNW Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fiber (% dw)** | | | | | | |
| Neutral Detergent Fiber | 19.08 (0.75) (17.16 - 21.36) | 17.16 (0.87) (16.68 - 17.45) | 1.92 (1.07) (-0.29 - 3.97) | -1.50, 5.33 | 0.171 | 10.07, 25.94 (10.93 - 22.75) |
| Total Dietary Fiber | 22.93 (1.65) (19.17 - 27.81) | 19.30 (1.87) (15.21 - 22.29) | 3.63 (2.10) (-0.29 - 7.36) | -3.05, 10.32 | 0.181 | 13.97, 24.85 (12.64 - 26.47) |
| **Amino Acid (% dw)** | | | | | | |
| Alanine | 0.95 (0.026) (0.88 - 1.02) | 0.95 (0.030) (0.93 - 0.98) | 0.0018 (0.040) (-0.047 - 0.011) | -0.13, 0.13 | 0.967 | 0.77, 1.34 (0.87 - 1.27) |
| Arginine | 1.35 (0.042) (1.23 - 1.44) | 1.37 (0.049) (1.36 - 1.38) | -0.019 (0.065) (-0.12 - 0.025) | -0.23, 0.19 | 0.784 | 1.10, 1.93 (1.23 - 1.96) |
| Aspartic Acid | 1.60 (0.057) (1.44 - 1.72) | 1.58 (0.066) (1.55 - 1.64) | 0.018 (0.087) (-0.10 - 0.090) | -0.26, 0.30 | 0.846 | 1.33, 2.12 (1.42 - 2.23) |
| Cystine | 0.52 (0.020) (0.48 - 0.59) | 0.51 (0.023) (0.50 - 0.54) | 0.0048 (0.031) (-0.043 - 0.0090) | -0.093, 0.10 | 0.886 | 0.38, 0.83 (0.45 - 0.79) |

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**Table H-3. Statistical Summary of Site MBNW Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Glutamic Acid | 3.68 (0.12) (3.37 - 4.02) | 3.71 (0.14) (3.64 - 3.84) | -0.030 (0.19) (-0.27 - 0.043) | -0.64, 0.58 | 0.886 | 2.73, 5.89 (3.26 - 5.43) |
| Glycine | 1.09 (0.028) (1.02 - 1.16) | 1.09 (0.032) (1.06 - 1.12) | 0.0020 (0.043) (-0.046 - 0.014) | -0.13, 0.14 | 0.965 | 0.96, 1.47 (1.01 - 1.50) |
| Histidine | 0.59 (0.017) (0.55 - 0.64) | 0.58 (0.019) (0.57 - 0.60) | 0.0052 (0.026) (-0.023 - 0.0092) | -0.076, 0.087 | 0.851 | 0.47, 0.86 (0.54 - 0.80) |
| Isoleucine | 0.87 (0.028) (0.81 - 0.94) | 0.86 (0.032) (0.82 - 0.90) | 0.010 (0.042) (-0.029 - 0.0069) | -0.12, 0.14 | 0.820 | 0.70, 1.22 (0.78 - 1.15) |
| Leucine | 1.51 (0.044) (1.40 - 1.62) | 1.51 (0.051) (1.48 - 1.56) | 0.00056 (0.067) (-0.082 - 0.026) | -0.21, 0.21 | 0.993 | 1.21, 2.18 (1.36 - 2.07) |
| Lysine | 1.31 (0.034) (1.22 - 1.41) | 1.28 (0.040) (1.25 - 1.32) | 0.033 (0.052) (-0.030 - 0.057) | -0.13, 0.20 | 0.573 | 1.02, 1.90 (1.20 - 1.68) |

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**Table H-3. Statistical Summary of Site MBNW Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302 Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Amino Acid (% dw)** | | | | | | |
| Methionine | 0.41 (0.012) (0.40 - 0.45) | 0.42 (0.014) (0.40 - 0.44) | -0.0029 (0.019) (-0.038 - 0.0034) | -0.063, 0.057 | 0.887 | 0.30, 0.65 (0.36 - 0.57) |
| Phenylalanine | 0.91 (0.025) (0.84 - 0.97) | 0.91 (0.029) (0.90 - 0.93) | 0.0043 (0.038) (-0.053 - 0.019) | -0.12, 0.12 | 0.916 | 0.77, 1.26 (0.84 - 1.25) |
| Proline | 1.27 (0.038) (1.20 - 1.35) | 1.24 (0.044) (1.20 - 1.29) | 0.029 (0.058) (-0.030 - 0.036) | -0.16, 0.21 | 0.659 | 0.90, 2.01 (1.12 - 1.78) |
| Serine | 0.96 (0.029) (0.87 - 1.03) | 0.95 (0.033) (0.94 - 0.97) | 0.0032 (0.044) (-0.077 - 0.051) | -0.14, 0.14 | 0.945 | 0.81, 1.32 (0.88 - 1.30) |
| Threonine | 0.94 (0.022) (0.88 - 0.98) | 0.94 (0.025) (0.92 - 0.96) | 0.0053 (0.034) (-0.044 - 0.058) | -0.10, 0.11 | 0.884 | 0.82, 1.20 (0.84 - 1.22) |
| Tryptophan | 0.21 (0.017) (0.17 - 0.26) | 0.21 (0.020) (0.19 - 0.25) | -0.0040 (0.026) (-0.037 - -0.0025) | -0.088, 0.080 | 0.889 | 0.13, 0.35 (0.17 - 0.32) |

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**Table H-3. Statistical Summary of Site MBNW Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Amino Acid (% dw)** | | | | | | |
| Tyrosine | 0.64 (0.015) (0.59 - 0.66) | 0.63 (0.017) (0.63 - 0.64) | 0.0046 (0.022) (-0.037 - 0.016) | -0.066, 0.075 | 0.850 | 0.57, 0.81 (0.60 - 0.84) |
| Valine | 1.12 (0.036) (1.04 - 1.21) | 1.10 (0.041) (1.05 - 1.15) | 0.021 (0.054) (-0.032 - 0.014) | -0.15, 0.19 | 0.719 | 0.92, 1.55 (1.01 - 1.46) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 16:0 Palmitic | 4.10 (0.029) (4.02 - 4.16) | 4.00 (0.033) (3.97 - 4.06) | 0.10 (0.044) (0.0047 - 0.18) | -0.039, 0.24 | 0.105 | 2.84, 5.26 (3.55 - 4.69) |
| 16:1 Palmitoleic | 0.21 (0.0031) (0.20 - 0.21) | 0.23 (0.0036) (0.22 - 0.23) | -0.022 (0.0044) (-0.028 - -0.015) | -0.036, -0.0081 | 0.015 | 0.17, 0.30 (0.19 - 0.27) |
| 18:0 Stearic | 1.73 (0.039) (1.64 - 1.87) | 1.97 (0.045) (1.93 - 2.01) | -0.24 (0.060) (-0.35 - -0.059) | -0.43, -0.049 | 0.028 | 0.90, 3.05 (1.50 - 2.64) |
| 18:1 Oleic | 63.40 (0.19) (62.94 - 64.03) | 65.71 (0.22) (65.55 - 65.93) | -2.30 (0.29) (-3.00 - -1.52) | -3.24, -1.37 | 0.004 | 56.13, 70.69 (57.86 - 68.53) |

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**Table H-3. Statistical Summary of Site MBNW Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 18:2 Linoleic | 19.27 (0.16) (18.82 - 19.66) | 17.89 (0.19) (17.70 - 18.17) | 1.38 (0.25) (0.65 - 1.96) | 0.59, 2.17 | 0.011 | 12.60, 24.49 (14.12 - 22.57) |
| 18:3 Linolenic | 9.19 (0.091) (8.88 - 9.42) | 8.12 (0.10) (7.98 - 8.25) | 1.08 (0.14) (0.76 - 1.43) | 0.64, 1.52 | 0.004 | 6.96, 11.73 (7.99 - 10.94) |
| 20:0 Arachidic | 0.52 (0.010) (0.50 - 0.54) | 0.56 (0.012) (0.54 - 0.58) | -0.042 (0.015) (-0.081 - -0.0032) | -0.091, 0.0064 | 0.069 | 0.45, 0.80 (0.53 - 0.71) |
| 20:1 Eicosenoic | 1.08 (0.021) (1.06 - 1.15) | 1.03 (0.024) (1.00 - 1.08) | 0.055 (0.032) (-0.016 - 0.064) | -0.046, 0.16 | 0.180 | 0.83, 1.68 (1.04 - 1.56) |
| 22:0 Behenic | 0.25 (0.0058) (0.24 - 0.26) | 0.26 (0.0067) (0.24 - 0.27) | -0.010 (0.0089) (-0.030 - 0.00017) | -0.038, 0.018 | 0.337 | 0.19, 0.43 (0.27 - 0.38) |
| 24:0 Lignoceric | 0.15 (0.0053) (0.14 - 0.16) | 0.15 (0.0061) (0.15 - 0.15) | 0.0020 (0.0081) (-0.0041 - 0.0063) | -0.024, 0.028 | 0.824 | 0.033, 0.25 (0.044 - 0.21) |

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**Table H-3. Statistical Summary of Site MBNW Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fatty Acid (% Total FA)** | | | | | | |
| 24:1 Nervonic | 0.096 (0.027)<br>(0.046 - 0.12) | 0.090 (0.031)<br>(0.046 - 0.12) | 0.0065 (0.041)<br>(-0.069 - 0.072) | -0.13, 0.14 | 0.884 | 0.041, 0.18<br>(0.044 - 0.20) |
| **Mineral** | | | | | | |
| Calcium (g/100g dw) | 0.48 (0.010)<br>(0.45 - 0.51) | 0.44 (0.012)<br>(0.43 - 0.46) | 0.038 (0.016)<br>(0.017 - 0.081) | -0.012, 0.089 | 0.095 | 0.16, 0.61<br>(0.25 - 0.53) |
| Copper (mg/kg dw) | 3.72 (0.040)<br>(3.61 - 3.83) | 3.41 (0.046)<br>(3.36 - 3.44) | 0.32 (0.061)<br>(0.22 - 0.40) | 0.12, 0.51 | 0.013 | 2.00, 4.43<br>(2.52 - 4.93) |
| Iron (mg/kg dw) | 42.22 (1.46)<br>(40.55 - 43.60) | 46.51 (1.66)<br>(41.65 - 51.30) | -4.30 (1.90)<br>(-8.99 - -1.10) | -10.33, 1.74 | 0.108 | 23.39, 86.23<br>(39.16 - 77.92) |
| Magnesium (g/100g dw) | 0.34 (0.010)<br>(0.31 - 0.35) | 0.33 (0.012)<br>(0.31 - 0.35) | 0.0078 (0.016)<br>(0.0036 - 0.039) | -0.042, 0.057 | 0.651 | 0.32, 0.43<br>(0.30 - 0.45) |
| Manganese (mg/kg dw) | 39.62 (1.70)<br>(35.28 - 43.84) | 39.54 (1.96)<br>(37.35 - 41.11) | 0.078 (2.60)<br>(-3.95 - 6.49) | -8.18, 8.34 | 0.977 | 14.85, 61.05<br>(25.00 - 54.11) |

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**Table H-3. Statistical Summary of Site MBNW Canola Seed Nutrient Content for MON 88302 vs the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Mineral** | | | | | | |
| Phosphorus (g/100g dw) | 0.72 (0.041)<br>(0.60 - 0.78) | 0.72 (0.047)<br>(0.61 - 0.79) | 0.0064 (0.062)<br>(-0.034 - 0.16) | -0.19, 0.20 | 0.925 | 0.38, 1.06<br>(0.44 - 0.87) |
| Potassium (g/100g dw) | 0.56 (0.0096)<br>(0.54 - 0.57) | 0.56 (0.011)<br>(0.54 - 0.58) | -0.0054 (0.015)<br>(-0.0098 - 0.021) | -0.052, 0.041 | 0.734 | 0.39, 0.96<br>(0.50 - 0.92) |
| Zinc (mg/kg dw) | 34.91 (1.09)<br>(32.40 - 37.15) | 30.24 (1.26)<br>(28.46 - 32.84) | 4.66 (1.66)<br>(-0.44 - 7.72) | -0.63, 9.95 | 0.067 | 20.19, 48.23<br>(22.18 - 47.61) |
| **Vitamin (mg/100g dw)** | | | | | | |
| Vitamin E (α-tocopherol) | 13.06 (0.31)<br>(12.22 - 13.47) | 9.36 (0.36)<br>(8.89 - 10.15) | 3.70 (0.48)<br>(3.07 - 4.46) | 2.17, 5.23 | 0.004 | 3.88, 17.28<br>(2.62 - 14.84) |

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

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**Table H-4. Statistical Summary of Site MBNW Canola Seed Anti-nutrients for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Anti-nutrient** | | | | | | |
| Alkyl Glucosinolate (µmole/g dw) | 5.19 (0.28)<br>(4.47 - 5.87) | 5.43 (0.31)<br>(4.85 - 6.16) | -0.23 (0.28)<br>(-0.38 - 0.091) | -1.13, 0.67 | 0.472 | 0, 29.02<br>(2.32 - 28.33) |
| Indolyl Glucosinolate (µmole/g dw) | 4.23 (0.57)<br>(2.92 - 5.75) | 4.01 (0.65)<br>(2.92 - 5.31) | 0.22 (0.86)<br>(-1.32 - 2.83) | -2.53, 2.97 | 0.817 | 1.37, 6.62<br>(1.84 - 7.18) |
| Phytic Acid (% dw) | 2.06 (0.17)<br>(1.73 - 2.46) | 2.27 (0.20)<br>(1.77 - 2.56) | -0.21 (0.26)<br>(-0.67 - 0.68) | -1.05, 0.63 | 0.489 | 0.70, 3.52<br>(1.10 - 2.71) |
| Sinapic Acid (% dw) | 1.02 (0.014)<br>(0.99 - 1.06) | 0.92 (0.014)<br>(0.92 - 0.94) | 0.095 (0.0093)<br>(0.076 - 0.11) | 0.066, 0.12 | 0.001 | 0.57, 1.13<br>(0.48 - 0.99) |
| Total Glucosinolate (µmole/g dw) | 9.60 (0.77)<br>(7.60 - 11.42) | 9.61 (0.88)<br>(8.44 - 11.56) | -0.014 (1.05)<br>(-1.54 - 2.98) | -3.35, 3.32 | 0.990 | 0, 32.20<br>(5.52 - 31.98) |

[1]dw = dry weight.
[2] MON 88302 treated with glyphosate
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

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**Table H-5. Statistical Summary of Site MBPL Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Proximate (% dw)** | | | | | | |
| Ash | 4.15 (0.070) (3.99 - 4.38) | 4.28 (0.080) (4.17 - 4.38) | -0.14 (0.089) (-0.21 - 0.020) | -0.38, 0.11 | 0.201 | 3.32, 4.66 (2.98 - 4.52) |
| Carbohydrates | 27.51 (0.48) (26.55 - 28.81) | 28.11 (0.56) (26.87 - 28.73) | -0.59 (0.74) (-2.18 - 1.94) | -2.64, 1.45 | 0.466 | 23.12, 30.77 (22.53 - 29.96) |
| Moisture (% fw) | 5.68 (0.12) (5.45 - 5.93) | 5.24 (0.14) (4.93 - 5.47) | 0.44 (0.19) (0.12 - 0.67) | -0.078, 0.95 | 0.077 | 4.33, 6.91 (4.09 - 8.48) |
| Protein | 23.70 (0.26) (23.17 - 24.33) | 23.46 (0.30) (23.03 - 24.12) | 0.23 (0.40) (-0.95 - 1.30) | -0.87, 1.33 | 0.590 | 17.20, 30.08 (18.68 - 28.32) |
| Total Fat | 44.66 (0.36) (43.96 - 45.72) | 44.20 (0.41) (43.65 - 44.85) | 0.46 (0.49) (-0.84 - 1.11) | -0.91, 1.83 | 0.405 | 39.65, 51.24 (40.71 - 50.26) |
| **Fiber (% dw)** | | | | | | |
| Acid Detergent Fiber | 16.75 (0.70) (15.17 - 18.19) | 14.19 (0.73) (12.59 - 16.16) | 2.55 (0.46) (2.03 - 3.57) | 1.28, 3.83 | 0.005 | 6.95, 23.92 (9.75 - 21.22) |

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**Table H-5. Statistical Summary of Site MBPL Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fiber (% dw)** | | | | | | |
| Neutral Detergent Fiber | 19.45 (0.50)<br>(18.35 - 20.02) | 16.87 (0.57)<br>(15.44 - 18.06) | 2.58 (0.66)<br>(1.50 - 4.43) | 0.74, 4.43 | 0.017 | 10.07, 25.94<br>(10.93 - 22.75) |
| Total Dietary Fiber | 22.61 (1.30)<br>(18.67 - 24.98) | 17.91 (1.50)<br>(14.58 - 20.42) | 4.70 (1.99)<br>(3.52 - 9.96) | -0.82, 10.23 | 0.077 | 13.97, 24.85<br>(12.64 - 26.47) |
| **Amino Acid (% dw)** | | | | | | |
| Alanine | 1.06 (0.013)<br>(1.04 - 1.08) | 1.04 (0.015)<br>(1.02 - 1.09) | 0.017 (0.020)<br>(-0.049 - 0.059) | -0.040, 0.073 | 0.460 | 0.77, 1.34<br>(0.87 - 1.27) |
| Arginine | 1.57 (0.033)<br>(1.51 - 1.64) | 1.54 (0.039)<br>(1.48 - 1.65) | 0.031 (0.051)<br>(-0.14 - 0.15) | -0.11, 0.17 | 0.578 | 1.10, 1.93<br>(1.23 - 1.96) |
| Aspartic Acid | 1.84 (0.024)<br>(1.81 - 1.89) | 1.79 (0.027)<br>(1.73 - 1.85) | 0.047 (0.029)<br>(-0.023 - 0.086) | -0.034, 0.13 | 0.179 | 1.33, 2.12<br>(1.42 - 2.23) |
| Cystine | 0.55 (0.016)<br>(0.50 - 0.59) | 0.57 (0.019)<br>(0.53 - 0.60) | -0.013 (0.025)<br>(-0.054 - 0.052) | -0.082, 0.056 | 0.625 | 0.38, 0.83<br>(0.45 - 0.79) |

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**Table H-5. Statistical Summary of Site MBPL Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Glutamic Acid | 4.26 (0.084)<br>(4.15 - 4.41) | 4.24 (0.097)<br>(4.05 - 4.53) | 0.017 (0.13)<br>(-0.37 - 0.36) | -0.34, 0.37 | 0.903 | 2.73, 5.89<br>(3.26 - 5.43) |
| Glycine | 1.23 (0.013)<br>(1.21 - 1.24) | 1.21 (0.015)<br>(1.19 - 1.26) | 0.013 (0.020)<br>(-0.047 - 0.044) | -0.043, 0.070 | 0.542 | 0.96, 1.47<br>(1.01 - 1.50) |
| Histidine | 0.65 (0.0098)<br>(0.63 - 0.67) | 0.65 (0.011)<br>(0.62 - 0.68) | 0.0039 (0.015)<br>(-0.041 - 0.044) | -0.037, 0.045 | 0.806 | 0.47, 0.86<br>(0.54 - 0.80) |
| Isoleucine | 0.99 (0.011)<br>(0.95 - 1.01) | 0.98 (0.012)<br>(0.96 - 1.00) | 0.0090 (0.016)<br>(-0.050 - 0.034) | -0.036, 0.054 | 0.609 | 0.70, 1.22<br>(0.78 - 1.15) |
| Leucine | 1.73 (0.021)<br>(1.70 - 1.76) | 1.71 (0.024)<br>(1.66 - 1.78) | 0.027 (0.032)<br>(-0.076 - 0.086) | -0.063, 0.12 | 0.448 | 1.21, 2.18<br>(1.36 - 2.07) |
| Lysine | 1.41 (0.019)<br>(1.37 - 1.45) | 1.40 (0.022)<br>(1.36 - 1.45) | 0.0064 (0.029)<br>(-0.067 - 0.086) | -0.073, 0.086 | 0.833 | 1.02, 1.90<br>(1.20 - 1.68) |

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**Table H-5. Statistical Summary of Site MBPL Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Methionine | 0.46 (0.0083)<br>(0.43 - 0.48) | 0.47 (0.0095)<br>(0.45 - 0.49) | -0.0073 (0.013)<br>(-0.024 - 0.030) | -0.042, 0.028 | 0.593 | 0.30, 0.65<br>(0.36 - 0.57) |
| Phenylalanine | 1.05 (0.011)<br>(1.04 - 1.05) | 1.03 (0.013)<br>(1.01 - 1.07) | 0.014 (0.017)<br>(-0.032 - 0.039) | -0.032, 0.060 | 0.443 | 0.77, 1.26<br>(0.84 - 1.25) |
| Proline | 1.39 (0.033)<br>(1.32 - 1.47) | 1.36 (0.038)<br>(1.30 - 1.45) | 0.021 (0.050)<br>(-0.12 - 0.17) | -0.12, 0.16 | 0.696 | 0.90, 2.01<br>(1.12 - 1.78) |
| Serine | 1.08 (0.016)<br>(1.05 - 1.09) | 1.07 (0.018)<br>(1.04 - 1.12) | 0.012 (0.018)<br>(-0.037 - 0.052) | -0.039, 0.063 | 0.559 | 0.81, 1.32<br>(0.88 - 1.30) |
| Threonine | 1.06 (0.013)<br>(1.04 - 1.06) | 1.02 (0.015)<br>(0.99 - 1.07) | 0.031 (0.016)<br>(-0.0054 - 0.065) | -0.013, 0.075 | 0.119 | 0.82, 1.20<br>(0.84 - 1.22) |
| Tryptophan | 0.25 (0.0075)<br>(0.24 - 0.25) | 0.26 (0.0087)<br>(0.25 - 0.27) | -0.011 (0.011)<br>(-0.014 - -0.0041) | -0.043, 0.021 | 0.388 | 0.13, 0.35<br>(0.17 - 0.32) |

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**Table H-5. Statistical Summary of Site MBPL Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Amino Acid (% dw)** | | | | | | |
| Tyrosine | 0.72 (0.0065)<br>(0.72 - 0.73) | 0.71 (0.0069)<br>(0.69 - 0.73) | 0.017 (0.0052)<br>(0.0078 - 0.028) | 0.0030, 0.032 | 0.028 | 0.57, 0.81<br>(0.60 - 0.84) |
| Valine | 1.26 (0.014)<br>(1.21 - 1.29) | 1.25 (0.016)<br>(1.23 - 1.27) | 0.016 (0.021)<br>(-0.058 - 0.054) | -0.041, 0.074 | 0.474 | 0.92, 1.55<br>(1.01 - 1.46) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 16:0 Palmitic | 4.25 (0.031)<br>(4.20 - 4.29) | 4.34 (0.035)<br>(4.23 - 4.41) | -0.090 (0.047)<br>(-0.22 - 0.030) | -0.22, 0.040 | 0.126 | 2.84, 5.26<br>(3.55 - 4.69) |
| 16:1 Palmitoleic | 0.23 (0.0035)<br>(0.22 - 0.23) | 0.25 (0.0040)<br>(0.24 - 0.26) | -0.026 (0.0053)<br>(-0.031 - -0.015) | -0.040, -0.011 | 0.008 | 0.17, 0.30<br>(0.19 - 0.27) |
| 18:0 Stearic | 1.58 (0.023)<br>(1.55 - 1.59) | 1.87 (0.026)<br>(1.79 - 1.93) | -0.29 (0.028)<br>(-0.34 - -0.22) | -0.37, -0.22 | <0.001 | 0.90, 3.05<br>(1.50 - 2.64) |
| 18:1 Oleic | 62.06 (0.11)<br>(61.82 - 62.35) | 64.30 (0.13)<br>(64.19 - 64.56) | -2.24 (0.13)<br>(-2.40 - -1.84) | -2.59, -1.88 | <0.001 | 56.13, 70.69<br>(57.86 - 68.53) |

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**Table H-5. Statistical Summary of Site MBPL Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fatty Acid (% Total FA)** | | | | | | |
| 18:2 Linoleic | 20.43 (0.088)<br>(20.13 - 20.66) | 19.18 (0.099)<br>(19.01 - 19.26) | 1.25 (0.11)<br>(0.92 - 1.41) | 0.95, 1.55 | <0.001 | 12.60, 24.49<br>(14.12 - 22.57) |
| 18:3 Linolenic | 9.28 (0.085)<br>(9.12 - 9.43) | 7.74 (0.091)<br>(7.52 - 7.92) | 1.54 (0.073)<br>(1.35 - 1.67) | 1.34, 1.74 | <0.001 | 6.96, 11.73<br>(7.99 - 10.94) |
| 20:0 Arachidic | 0.53 (0.0046)<br>(0.52 - 0.54) | 0.60 (0.0049)<br>(0.59 - 0.61) | -0.070 (0.0042)<br>(-0.079 - -0.063) | -0.082, -0.059 | <0.001 | 0.45, 0.80<br>(0.53 - 0.71) |
| 20:1 Eicosenoic | 1.09 (0.0059)<br>(1.08 - 1.10) | 1.08 (0.0068)<br>(1.06 - 1.09) | 0.011 (0.0089)<br>(-0.013 - 0.042) | -0.014, 0.035 | 0.298 | 0.83, 1.68<br>(1.04 - 1.56) |
| 22:0 Behenic | 0.27 (0.0027)<br>(0.26 - 0.27) | 0.30 (0.0032)<br>(0.29 - 0.31) | -0.040 (0.0039)<br>(-0.047 - -0.030) | -0.050, -0.029 | <0.001 | 0.19, 0.43<br>(0.27 - 0.38) |
| 24:0 Lignoceric | 0.16 (0.0046)<br>(0.16 - 0.17) | 0.19 (0.0054)<br>(0.18 - 0.19) | -0.023 (0.0068)<br>(-0.024 - -0.022) | -0.042, -0.0038 | 0.029 | 0.033, 0.25<br>(0.044 - 0.21) |

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**Table H-5. Statistical Summary of Site MBPL Canola Seed Nutrient content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fatty Acid (% Total FA)** | | | | | | |
| 24:1 Nervonic | 0.13 (0.0066)<br>(0.12 - 0.13) | 0.16 (0.0076)<br>(0.15 - 0.17) | -0.033 (0.010)<br>(-0.052 - -0.014) | -0.061, -0.0047 | 0.031 | 0.041, 0.18<br>(0.044 - 0.20) |
| **Mineral** | | | | | | |
| Calcium (g/100g dw) | 0.36 (0.0051)<br>(0.35 - 0.37) | 0.34 (0.0059)<br>(0.32 - 0.34) | 0.021 (0.0078)<br>(0.0015 - 0.035) | -0.00057, 0.043 | 0.053 | 0.16, 0.61<br>(0.25 - 0.53) |
| Copper (mg/kg dw) | 3.47 (0.082)<br>(3.35 - 3.56) | 3.97 (0.094)<br>(3.68 - 4.18) | -0.50 (0.12)<br>(-0.83 - -0.23) | -0.84, -0.15 | 0.016 | 2.00, 4.43<br>(2.52 - 4.93) |
| Iron (mg/kg dw) | 44.13 (0.64)<br>(42.80 - 45.09) | 51.01 (0.73)<br>(49.75 - 52.89) | -6.87 (0.90)<br>(-9.20 - -4.80) | -9.38, -4.37 | 0.001 | 23.39, 86.23<br>(39.16 - 77.92) |
| Magnesium (g/100g dw) | 0.41 (0.0070)<br>(0.39 - 0.42) | 0.41 (0.0081)<br>(0.40 - 0.42) | -0.0029 (0.011)<br>(-0.022 - 0.021) | -0.033, 0.027 | 0.797 | 0.32, 0.43<br>(0.30 - 0.45) |
| Manganese (mg/kg dw) | 39.33 (0.90)<br>(37.24 - 41.46) | 37.78 (1.03)<br>(36.29 - 39.99) | 1.55 (1.37)<br>(-1.82 - 4.38) | -2.25, 5.34 | 0.321 | 14.85, 61.05<br>(25.00 - 54.11) |

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**Table H-5. Statistical Summary of Site MBPL Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Mineral** | | | | | | |
| Phosphorus (g/100g dw) | 0.78 (0.0092) (0.75 - 0.80) | 0.81 (0.011) (0.80 - 0.82) | -0.031 (0.014) (-0.033 - -0.0099) | -0.069, 0.0077 | 0.090 | 0.38, 1.06 (0.44 - 0.87) |
| Potassium (g/100g dw) | 0.70 (0.025) (0.63 - 0.76) | 0.77 (0.027) (0.77 - 0.81) | -0.068 (0.019) (-0.097 - -0.017) | -0.12, -0.015 | 0.023 | 0.39, 0.96 (0.50 - 0.92) |
| Zinc (mg/kg dw) | 31.25 (0.49) (30.45 - 32.05) | 33.88 (0.56) (32.82 - 35.76) | -2.63 (0.75) (-4.50 - -1.02) | -4.70, -0.56 | 0.024 | 20.19, 48.23 (22.18 - 47.61) |
| **Vitamin (mg/100g dw)** | | | | | | |
| Vitamin E (α-tocopherol) | 11.50 (0.24) (10.70 - 12.20) | 7.63 (0.27) (7.50 - 7.72) | 3.88 (0.36) (3.20 - 4.23) | 2.87, 4.89 | <0.001 | 3.88, 17.28 (2.62 - 14.84) |

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

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**Table H-6. Statistical Summary of Site MBPL Canola Seed Anti-nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Anti-nutrient** | | | | | | |
| Alkyl Glucosinolate (µmole/g dw) | 2.98 (0.64)<br>(1.91 - 4.03) | 5.03 (0.74)<br>(3.06 - 6.50) | -2.05 (0.98)<br>(-2.68 - -1.15) | -4.76, 0.66 | 0.103 | 0, 29.02<br>(2.32 - 28.33) |
| Indolyl Glucosinolate (µmole/g dw) | 3.90 (0.69)<br>(1.67 - 5.76) | 4.29 (0.79)<br>(3.26 - 5.89) | -0.39 (0.93)<br>(-1.59 - 0.96) | -2.98, 2.20 | 0.697 | 1.37, 6.62<br>(1.84 - 7.18) |
| Phytic Acid (% dw) | 2.36 (0.066)<br>(2.19 - 2.58) | 2.39 (0.076)<br>(2.35 - 2.41) | -0.027 (0.10)<br>(-0.15 - 0.17) | -0.31, 0.25 | 0.803 | 0.70, 3.52<br>(1.10 - 2.71) |
| Sinapic Acid (% dw) | 0.97 (0.0080)<br>(0.95 - 0.99) | 0.86 (0.0090)<br>(0.86 - 0.86) | 0.10 (0.010)<br>(0.085 - 0.12) | 0.076, 0.13 | <0.001 | 0.57, 1.13<br>(0.48 - 0.99) |
| Total Glucosinolate (µmole/g dw) | 7.01 (1.30)<br>(3.66 - 9.77) | 9.40 (1.49)<br>(6.42 - 12.59) | -2.39 (1.83)<br>(-2.82 - -0.44) | -7.45, 2.68 | 0.261 | 0, 32.20<br>(5.52 - 31.98) |

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero

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**Table H-7. Statistical Summary of Site MNCA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Proximate (% dw)** | | | | | | |
| Ash | 4.35 (0.23) (4.20 - 4.45) | 4.18 (0.20) (3.76 - 5.10) | 0.17 (0.30) (0.33 - 0.64) | -0.60, 0.94 | 0.591 | 3.32, 4.66 (2.98 - 4.52) |
| Carbohydrates | 27.31 (0.35) (26.27 - 27.90) | 25.99 (0.30) (25.57 - 26.55) | 1.32 (0.46) (-0.29 - 1.92) | 0.13, 2.51 | 0.035 | 23.12, 30.77 (22.53 - 29.96) |
| Moisture (% fw) | 5.52 (0.13) (5.37 - 5.61) | 6.69 (0.11) (6.33 - 6.98) | -1.17 (0.17) (-1.53 - -0.72) | -1.60, -0.74 | <0.001 | 4.33, 6.91 (4.09 - 8.48) |
| Protein | 22.00 (0.70) (21.51 - 22.03) | 23.23 (0.61) (21.50 - 24.27) | -1.23 (0.81) (-2.29 - 0.53) | -3.30, 0.85 | 0.189 | 17.20, 30.08 (18.68 - 28.32) |
| Total Fat | 46.04 (0.72) (45.76 - 47.55) | 46.59 (0.69) (45.26 - 48.05) | -0.55 (0.46) (-0.78 - -0.21) | -1.72, 0.62 | 0.280 | 39.65, 51.24 (40.71 - 50.26) |
| **Fiber (% dw)** | | | | | | |
| Acid Detergent Fiber | 17.89 (0.87) (15.99 - 20.24) | 17.66 (0.75) (16.11 - 18.71) | 0.23 (1.15) (-2.71 - 2.09) | -2.73, 3.20 | 0.847 | 6.95, 23.92 (9.75 - 21.22) |

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**Table H-7. Statistical Summary of Site MNCA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fiber (% dw)** | | | | | | |
| Neutral Detergent Fiber | 19.55 (0.82) (17.90 - 21.19) | 18.78 (0.71) (17.83 - 19.58) | 0.76 (1.08) (-1.56 - 3.36) | -2.01, 3.54 | 0.511 | 10.07, 25.94 (10.93 - 22.75) |
| Total Dietary Fiber | 20.18 (1.55) (16.91 - 22.24) | 19.75 (1.35) (17.40 - 23.00) | 0.44 (2.06) (-0.49 - 3.79) | -4.85, 5.72 | 0.839 | 13.97, 24.85 (12.64 - 26.47) |
| **Amino Acid (% dw)** | | | | | | |
| Alanine | 0.98 (0.027) (0.97 - 0.98) | 1.05 (0.023) (0.98 - 1.10) | -0.070 (0.032) (-0.12 - -0.014) | -0.15, 0.014 | 0.084 | 0.77, 1.34 (0.87 - 1.27) |
| Arginine | 1.40 (0.054) (1.38 - 1.39) | 1.53 (0.048) (1.40 - 1.65) | -0.13 (0.063) (-0.27 - -0.010) | -0.29, 0.029 | 0.089 | 1.10, 1.93 (1.23 - 1.96) |
| Aspartic Acid | 1.59 (0.067) (1.57 - 1.60) | 1.79 (0.058) (1.61 - 1.97) | -0.20 (0.082) (-0.37 - -0.045) | -0.41, 0.0098 | 0.057 | 1.33, 2.12 (1.42 - 2.23) |
| Cystine | 0.57 (0.025) (0.53 - 0.58) | 0.55 (0.022) (0.52 - 0.61) | 0.015 (0.031) (0.012 - 0.035) | -0.064, 0.094 | 0.650 | 0.38, 0.83 (0.45 - 0.79) |

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**Table H-7. Statistical Summary of Site MNCA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Glutamic Acid | 3.89 (0.15) (3.84 - 3.87) | 4.25 (0.13) (3.98 - 4.45) | -0.36 (0.17) (-0.59 - -0.11) | -0.80, 0.083 | 0.091 | 2.73, 5.89 (3.26 - 5.43) |
| Glycine | 1.12 (0.034) (1.11 - 1.12) | 1.22 (0.030) (1.13 - 1.30) | -0.099 (0.044) (-0.18 - -0.0061) | -0.21, 0.014 | 0.073 | 0.96, 1.47 (1.01 - 1.50) |
| Histidine | 0.62 (0.018) (0.61 - 0.62) | 0.65 (0.016) (0.61 - 0.67) | -0.031 (0.022) (-0.058 - -0.00063) | -0.087, 0.025 | 0.214 | 0.47, 0.86 (0.54 - 0.80) |
| Isoleucine | 0.90 (0.027) (0.88 - 0.90) | 0.97 (0.024) (0.89 - 1.03) | -0.076 (0.031) (-0.13 - -0.0068) | -0.16, 0.0044 | 0.059 | 0.70, 1.22 (0.78 - 1.15) |
| Leucine | 1.56 (0.052) (1.55 - 1.55) | 1.70 (0.045) (1.58 - 1.80) | -0.15 (0.064) (-0.25 - -0.034) | -0.31, 0.015 | 0.066 | 1.21, 2.18 (1.36 - 2.07) |
| Lysine | 1.37 (0.032) (1.35 - 1.36) | 1.40 (0.029) (1.35 - 1.44) | -0.033 (0.036) (-0.055 - -0.0095) | -0.13, 0.059 | 0.397 | 1.02, 1.90 (1.20 - 1.68) |

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**Table H-7. Statistical Summary of Site MNCA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Amino Acid (% dw)** | | | | | | |
| Methionine | 0.44 (0.020)<br>(0.43 - 0.45) | 0.44 (0.017)<br>(0.42 - 0.48) | 0.0058 (0.024)<br>(0.011 - 0.019) | -0.056, 0.067 | 0.819 | 0.30, 0.65<br>(0.36 - 0.57) |
| Phenylalanine | 0.93 (0.030)<br>(0.92 - 0.93) | 1.02 (0.026)<br>(0.94 - 1.08) | -0.089 (0.036)<br>(-0.17 - -0.011) | -0.18, 0.0043 | 0.057 | 0.77, 1.26<br>(0.84 - 1.25) |
| Proline | 1.36 (0.045)<br>(1.29 - 1.39) | 1.40 (0.040)<br>(1.30 - 1.46) | -0.040 (0.053)<br>(-0.16 - 0.076) | -0.18, 0.096 | 0.479 | 0.90, 2.01<br>(1.12 - 1.78) |
| Serine | 0.98 (0.038)<br>(0.98 - 0.99) | 1.08 (0.033)<br>(1.02 - 1.16) | -0.095 (0.050)<br>(-0.17 - -0.037) | -0.22, 0.033 | 0.114 | 0.81, 1.32<br>(0.88 - 1.30) |
| Threonine | 0.95 (0.027)<br>(0.94 - 0.94) | 1.02 (0.024)<br>(0.97 - 1.06) | -0.073 (0.031)<br>(-0.12 - -0.027) | -0.15, 0.0074 | 0.067 | 0.82, 1.20<br>(0.84 - 1.22) |
| Tryptophan | 0.22 (0.020)<br>(0.18 - 0.24) | 0.24 (0.017)<br>(0.22 - 0.27) | -0.022 (0.026)<br>(-0.063 - 0.024) | -0.089, 0.044 | 0.426 | 0.13, 0.35<br>(0.17 - 0.32) |

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**Table H-7. statistical Summary of Site MNCA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Tyrosine | 0.65 (0.019) (0.64 - 0.65) | 0.71 (0.017) (0.66 - 0.75) | -0.061 (0.024) (-0.11 - -0.0055) | -0.12, 0.0017 | 0.054 | 0.57, 0.81 (0.60 - 0.84) |
| Valine | 1.15 (0.031) (1.13 - 1.15) | 1.24 (0.027) (1.15 - 1.31) | -0.091 (0.035) (-0.16 - -0.017) | -0.18, -0.00082 | 0.048 | 0.92, 1.55 (1.01 - 1.46) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 16:0 Palmitic | 4.27 (0.051) (4.27 - 4.28) | 4.14 (0.045) (4.07 - 4.19) | 0.13 (0.061) (0.087 - 0.21) | -0.029, 0.28 | 0.090 | 2.84, 5.26 (3.55 - 4.69) |
| 16:1 Palmitoleic | 0.21 (0.0030) (0.21 - 0.21) | 0.24 (0.0026) (0.23 - 0.25) | -0.026 (0.0039) (-0.039 - -0.020) | -0.036, -0.016 | 0.001 | 0.17, 0.30 (0.19 - 0.27) |
| 18:0 Stearic | 1.67 (0.044) (1.65 - 1.71) | 1.86 (0.038) (1.78 - 1.92) | -0.19 (0.057) (-0.26 - -0.074) | -0.33, -0.039 | 0.022 | 0.90, 3.05 (1.50 - 2.64) |
| 18:1 Oleic | 61.67 (0.59) (61.70 - 61.87) | 64.86 (0.52) (63.72 - 65.52) | -3.19 (0.69) (-3.81 - -3.11) | -4.98, -1.41 | 0.005 | 56.13, 70.69 (57.86 - 68.53) |

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**Table H-7. Statistical Summary of Site MNCA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 18:2 Linoleic | 20.20 (0.24) (20.00 - 20.32) | 18.35 (0.21) (17.90 - 19.29) | 1.85 (0.30) (1.86 - 2.42) | 1.07, 2.63 | 0.001 | 12.60, 24.49 (14.12 - 22.57) |
| 18:3 Linolenic | 9.79 (0.58) (9.76 - 9.79) | 8.40 (0.50) (8.16 - 8.64) | 1.39 (0.75) (1.31 - 1.60) | -0.54, 3.32 | 0.122 | 6.96, 11.73 (7.99 - 10.94) |
| 20:0 Arachidic | 0.53 (0.013) (0.52 - 0.54) | 0.57 (0.012) (0.55 - 0.60) | -0.042 (0.018) (-0.081 - -0.019) | -0.088, 0.0027 | 0.060 | 0.45, 0.80 (0.53 - 0.71) |
| 20:1 Eicosenoic | 1.08 (0.012) (1.06 - 1.09) | 1.07 (0.011) (1.05 - 1.09) | 0.016 (0.016) (-0.024 - 0.041) | -0.025, 0.058 | 0.354 | 0.83, 1.68 (1.04 - 1.56) |
| 22:0 Behenic | 0.27 (0.0056) (0.27 - 0.28) | 0.27 (0.0049) (0.26 - 0.29) | 0.0022 (0.0074) (-0.021 - 0.016) | -0.017, 0.021 | 0.776 | 0.19, 0.43 (0.27 - 0.38) |
| 24:0 Lignoceric | 0.11 (0.033) (0.049 - 0.16) | 0.14 (0.029) (0.049 - 0.19) | -0.032 (0.044) (-0.14 - 0.069) | -0.15, 0.081 | 0.499 | 0.033, 0.25 (0.044 - 0.21) |

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**Table H-7. Statistical Summary of Site MNCA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 24:1 Nervonic | 0.10 (0.025) (0.049 - 0.15) | 0.099 (0.021) (0.049 - 0.12) | 0.0042 (0.032) (-0.072 - 0.062) | -0.079, 0.088 | 0.902 | 0.041, 0.18 (0.044 - 0.20) |
| **Mineral** | | | | | | |
| Calcium (g/100g dw) | 0.47 (0.016) (0.46 - 0.47) | 0.45 (0.014) (0.42 - 0.49) | 0.018 (0.022) (-0.0037 - 0.053) | -0.038, 0.074 | 0.438 | 0.16, 0.61 (0.25 - 0.53) |
| Copper (mg/kg dw) | 4.40 (0.074) (4.16 - 4.57) | 4.11 (0.064) (4.06 - 4.18) | 0.28 (0.093) (0.056 - 0.39) | 0.046, 0.52 | 0.027 | 2.00, 4.43 (2.52 - 4.93) |
| Iron (mg/kg dw) | 42.57 (1.72) (40.56 - 44.18) | 50.64 (1.53) (46.23 - 54.03) | -8.07 (1.87) (-12.92 - -4.82) | -12.89, -3.25 | 0.007 | 23.39, 86.23 (39.16 - 77.92) |
| Magnesium (g/100g dw) | 0.38 (0.0074) (0.36 - 0.40) | 0.37 (0.0067) (0.36 - 0.38) | 0.014 (0.0076) (0.0074 - 0.018) | -0.0049, 0.034 | 0.113 | 0.32, 0.43 (0.30 - 0.45) |
| Manganese (mg/kg dw) | 38.70 (2.68) (37.83 - 39.93) | 40.94 (2.32) (33.70 - 46.19) | -2.24 (3.54) (-8.36 - -2.11) | -11.34, 6.86 | 0.554 | 14.85, 61.05 (25.00 - 54.11) |

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**Table H-7. Statistical Summary of Site MNCA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Mineral** | | | | | | |
| Phosphorus (g/100g dw) | 0.81 (0.039) (0.74 - 0.87) | 0.79 (0.034) (0.72 - 0.93) | 0.022 (0.044) (-0.011 - 0.098) | -0.090, 0.13 | 0.633 | 0.38, 1.06 (0.44 - 0.87) |
| Potassium (g/100g dw) | 0.65 (0.027) (0.58 - 0.70) | 0.64 (0.023) (0.60 - 0.72) | 0.012 (0.035) (-0.020 - 0.098) | -0.079, 0.10 | 0.746 | 0.39, 0.96 (0.50 - 0.92) |
| Zinc (mg/kg dw) | 39.18 (2.73) (35.19 - 45.56) | 35.29 (2.41) (32.63 - 36.66) | 3.90 (3.15) (-1.44 - 10.33) | -4.21, 12.00 | 0.271 | 20.19, 48.23 (22.18 - 47.61) |
| **Vitamin (mg/100g dw)** | | | | | | |
| Vitamin E (α-tocopherol) | 13.39 (0.48) (12.58 - 14.62) | 10.82 (0.42) (10.15 - 11.77) | 2.57 (0.57) (2.18 - 3.99) | 1.11, 4.03 | 0.006 | 3.88, 17.28 (2.62 - 14.84) |

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

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**Table H-8. Statistical Summary of Site MNCA Canola Seed Anti-nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Anti-nutrient** | | | | | | |
| Alkyl Glucosinolate (µmole/g dw) | 4.64 (0.62)<br>(4.35 - 4.88) | 4.88 (0.54)<br>(2.92 - 6.16) | -0.24 (0.79)<br>(-1.48 - 1.43) | -2.28, 1.80 | 0.775 | 0, 29.02<br>(2.32 - 28.33) |
| Indolyl Glucosinolate (µmole/g dw) | 4.17 (0.52)<br>(3.79 - 4.47) | 4.35 (0.45)<br>(3.28 - 5.66) | -0.19 (0.69)<br>(-1.42 - 0.13) | -1.96, 1.59 | 0.798 | 1.37, 6.62<br>(1.84 - 7.18) |
| Phytic Acid (% dw) | 2.28 (0.12)<br>(2.14 - 2.47) | 2.36 (0.10)<br>(2.15 - 2.77) | -0.078 (0.15)<br>(-0.011 - 0.12) | -0.47, 0.31 | 0.630 | 0.70, 3.52<br>(1.10 - 2.71) |
| Sinapic Acid (% dw) | 1.06 (0.015)<br>(1.02 - 1.08) | 0.96 (0.014)<br>(0.94 - 0.97) | 0.10 (0.015)<br>(0.082 - 0.12) | 0.063, 0.14 | 0.001 | 0.57, 1.13<br>(0.48 - 0.99) |
| Total Glucosinolate (µmole/g dw) | 9.08 (0.69)<br>(8.35 - 9.36) | 9.42 (0.61)<br>(7.15 - 10.65) | -0.33 (0.81)<br>(-1.29 - 1.19) | -2.41, 1.74 | 0.696 | 0, 32.20<br>(5.52 - 31.98) |

[1]dw = dry weight.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

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## Table H-9. Statistical Summary of Site NDVA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Proximate (% dw)** | | | | | | |
| Ash | 3.31 (0.31)<br>(3.31 - 3.31) | 3.25 (0.26)<br>(3.20 - 3.35) | 0.063 (0.40)<br>(0.11 - 0.11) | -1.22, 1.35 | 0.886 | 3.32, 4.66<br>(2.98 - 4.52) |
| Carbohydrates | 24.01 (0.95)<br>(21.83 - 26.20) | 25.49 (0.78)<br>(24.69 - 26.01) | -1.48 (1.22)<br>(-4.18 - 0.45) | -5.37, 2.42 | 0.314 | 23.12, 30.77<br>(22.53 - 29.96) |
| Moisture (% fw) | 5.72 (0.23)<br>(5.72 - 6.08) | 5.61 (0.20)<br>(5.24 - 6.18) | 0.12 (0.22)<br>(-0.10 - 0.090) | -0.58, 0.82 | 0.633 | 4.33, 6.91<br>(4.09 - 8.48) |
| Protein | 24.66 (0.74)<br>(23.33 - 25.98) | 26.12 (0.61)<br>(25.33 - 27.02) | -1.46 (0.96)<br>(-1.99 - -0.028) | -4.52, 1.60 | 0.226 | 17.20, 30.08<br>(18.68 - 28.32) |
| Total Fat | 48.04 (0.44)<br>(47.20 - 48.87) | 45.17 (0.36)<br>(44.77 - 45.78) | 2.87 (0.57)<br>(1.42 - 4.10) | 1.07, 4.67 | 0.014 | 39.65, 51.24<br>(40.71 - 50.26) |
| **Fiber (% dw)** | | | | | | |
| Acid Detergent Fiber | 15.20 (0.37)<br>(14.53 - 15.86) | 15.22 (0.31)<br>(14.88 - 15.35) | -0.016 (0.39)<br>(-0.62 - 0.52) | -1.26, 1.23 | 0.969 | 6.95, 23.92<br>(9.75 - 21.22) |

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**Table H-9. Statistical Summary of Site NDVA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fiber (% dw)** | | | | | | |
| Neutral Detergent Fiber | 17.32 (0.81) (15.91 - 18.74) | 17.74 (0.66) (17.16 - 18.65) | -0.42 (1.05) (-2.74 - 1.58) | -3.76, 2.93 | 0.718 | 10.07, 25.94 (10.93 - 22.75) |
| Total Dietary Fiber | 18.71 (1.39) (17.08 - 21.08) | 17.17 (1.15) (14.88 - 19.61) | 1.54 (1.65) (-0.30 - 1.47) | -3.70, 6.79 | 0.418 | 13.97, 24.85 (12.64 - 26.47) |
| **Amino Acid (% dw)** | | | | | | |
| Alanine | 1.08 (0.042) (1.01 - 1.15) | 1.14 (0.034) (1.11 - 1.19) | -0.061 (0.054) (-0.10 - 0.031) | -0.23, 0.11 | 0.344 | 0.77, 1.34 (0.87 - 1.27) |
| Arginine | 1.60 (0.076) (1.47 - 1.72) | 1.72 (0.062) (1.68 - 1.77) | -0.12 (0.098) (-0.21 - 0.030) | -0.43, 0.19 | 0.316 | 1.10, 1.93 (1.23 - 1.96) |
| Aspartic Acid | 1.79 (0.067) (1.65 - 1.93) | 1.83 (0.054) (1.79 - 1.89) | -0.040 (0.086) (-0.14 - 0.12) | -0.31, 0.23 | 0.675 | 1.33, 2.12 (1.42 - 2.23) |
| Cystine | 0.70 (0.040) (0.64 - 0.73) | 0.71 (0.033) (0.66 - 0.79) | -0.018 (0.046) (-0.028 - 0.037) | -0.16, 0.13 | 0.715 | 0.38, 0.83 (0.45 - 0.79) |

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**Table H-9. Statistical Summary of Site NDVA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Glutamic Acid | 4.66 (0.25) (4.25 - 5.06) | 5.04 (0.20) (4.92 - 5.26) | -0.38 (0.32) (-0.68 - 0.13) | -1.41, 0.64 | 0.319 | 2.73, 5.89 (3.26 - 5.43) |
| Glycine | 1.24 (0.048) (1.16 - 1.32) | 1.33 (0.039) (1.30 - 1.38) | -0.094 (0.062) (-0.15 - 0.0093) | -0.29, 0.10 | 0.229 | 0.96, 1.47 (1.01 - 1.50) |
| Histidine | 0.72 (0.034) (0.67 - 0.77) | 0.75 (0.027) (0.73 - 0.78) | -0.032 (0.043) (-0.065 - 0.032) | -0.17, 0.11 | 0.509 | 0.47, 0.86 (0.54 - 0.80) |
| Isoleucine | 1.01 (0.041) (0.94 - 1.08) | 1.07 (0.034) (1.04 - 1.12) | -0.067 (0.053) (-0.099 - 0.0095) | -0.24, 0.10 | 0.299 | 0.70, 1.22 (0.78 - 1.15) |
| Leucine | 1.77 (0.073) (1.64 - 1.90) | 1.88 (0.059) (1.83 - 1.95) | -0.11 (0.094) (-0.19 - 0.051) | -0.40, 0.19 | 0.336 | 1.21, 2.18 (1.36 - 2.07) |
| Lysine | 1.55 (0.066) (1.46 - 1.63) | 1.60 (0.054) (1.58 - 1.65) | -0.054 (0.085) (-0.12 - 0.052) | -0.32, 0.22 | 0.566 | 1.02, 1.90 (1.20 - 1.68) |

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**Table H-9. Statistical Summary of Site NDVA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Methionine | 0.52 (0.019) (0.49 - 0.54) | 0.53 (0.016) (0.50 - 0.56) | -0.0059 (0.020) (-0.013 - 0.0079) | -0.068, 0.056 | 0.782 | 0.30, 0.65 (0.36 - 0.57) |
| Phenylalanine | 1.04 (0.038) (0.97 - 1.11) | 1.11 (0.031) (1.08 - 1.15) | -0.066 (0.049) (-0.12 - 0.031) | -0.22, 0.091 | 0.272 | 0.77, 1.26 (0.84 - 1.25) |
| Proline | 1.59 (0.065) (1.46 - 1.71) | 1.68 (0.053) (1.62 - 1.73) | -0.086 (0.083) (-0.16 - 0.041) | -0.35, 0.18 | 0.377 | 0.90, 2.01 (1.12 - 1.78) |
| Serine | 1.09 (0.036) (1.05 - 1.14) | 1.16 (0.029) (1.13 - 1.18) | -0.064 (0.046) (-0.12 - 0.0094) | -0.21, 0.083 | 0.258 | 0.81, 1.32 (0.88 - 1.30) |
| Threonine | 1.05 (0.035) (0.99 - 1.11) | 1.09 (0.029) (1.06 - 1.12) | -0.037 (0.045) (-0.065 - 0.020) | -0.18, 0.11 | 0.470 | 0.82, 1.20 (0.84 - 1.22) |
| Tryptophan | 0.26 (0.023) (0.25 - 0.26) | 0.26 (0.019) (0.21 - 0.31) | 0.0021 (0.025) (0.0061 - 0.036) | -0.079, 0.083 | 0.938 | 0.13, 0.35 (0.17 - 0.32) |

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**Table H-9. Statistical Summary of Site NDVA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Amino Acid (% dw)** | | | | | | |
| Tyrosine | 0.71 (0.023) (0.67 - 0.75) | 0.74 (0.019) (0.72 - 0.77) | -0.031 (0.029) (-0.060 - 0.025) | -0.13, 0.062 | 0.364 | 0.57, 0.81 (0.60 - 0.84) |
| Valine | 1.29 (0.050) (1.21 - 1.37) | 1.36 (0.041) (1.32 - 1.41) | -0.073 (0.064) (-0.12 - 0.020) | -0.28, 0.13 | 0.340 | 0.92, 1.55 (1.01 - 1.46) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 16:0 Palmitic | 3.98 (0.065) (3.95 - 4.02) | 3.95 (0.053) (3.94 - 3.96) | 0.036 (0.084) (-0.016 - 0.083) | -0.23, 0.30 | 0.699 | 2.84, 5.26 (3.55 - 4.69) |
| 16:1 Palmitoleic | 0.20 (0.0057) (0.20 - 0.20) | 0.22 (0.0046) (0.22 - 0.23) | -0.025 (0.0068) (-0.029 - -0.023) | -0.047, -0.0031 | 0.036 | 0.17, 0.30 (0.19 - 0.27) |
| 18:0 Stearic | 1.77 (0.033) (1.71 - 1.84) | 2.11 (0.027) (2.10 - 2.12) | -0.34 (0.042) (-0.41 - -0.28) | -0.47, -0.20 | 0.004 | 0.90, 3.05 (1.50 - 2.64) |
| 18:1 Oleic | 65.14 (0.35) (64.90 - 65.20) | 68.38 (0.29) (68.11 - 68.44) | -3.24 (0.37) (-3.24 - -3.21) | -4.43, -2.05 | 0.003 | 56.13, 70.69 (57.86 - 68.53) |

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**Table H-9. Statistical Summary of Site NDVA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 18:2 Linoleic | 17.86 (0.31) (17.78 - 18.02) | 15.71 (0.25) (15.72 - 15.77) | 2.15 (0.36) (2.06 - 2.26) | 1.00, 3.29 | 0.009 | 12.60, 24.49 (14.12 - 22.57) |
| 18:3 Linolenic | 8.82 (0.070) (8.71 - 8.94) | 7.31 (0.057) (7.19 - 7.40) | 1.51 (0.091) (1.35 - 1.54) | 1.22, 1.80 | <0.001 | 6.96, 11.73 (7.99 - 10.94) |
| 20:0 Arachidic | 0.57 (0.0047) (0.56 - 0.57) | 0.65 (0.0046) (0.64 - 0.65) | -0.082 (0.0023) (-0.085 - -0.077) | -0.089, -0.074 | <0.001 | 0.45, 0.80 (0.53 - 0.71) |
| 20:1 Eicosenoic | 1.15 (0.011) (1.13 - 1.17) | 1.16 (0.0088) (1.15 - 1.18) | -0.0098 (0.014) (-0.042 - 0.020) | -0.054, 0.035 | 0.531 | 0.83, 1.68 (1.04 - 1.56) |
| 22:0 Behenic | 0.27 (0.0036) (0.27 - 0.27) | 0.30 (0.0029) (0.30 - 0.30) | -0.029 (0.0044) (-0.031 - -0.027) | -0.043, -0.015 | 0.007 | 0.19, 0.43 (0.27 - 0.38) |
| 24:0 Lignoceric | 0.17 (0.053) (0.16 - 0.18) | 0.16 (0.043) (0.049 - 0.22) | 0.012 (0.068) (-0.045 - 0.11) | -0.20, 0.23 | 0.873 | 0.033, 0.25 (0.044 - 0.21) |

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**Table H-9. Statistical Summary of Site NDVA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fatty Acid (% Total FA)** | | | | | | |
| 24:1 Nervonic | 0.12 (0.028)<br>(0.11 - 0.13) | 0.090 (0.023)<br>(0.049 - 0.12) | 0.031 (0.037)<br>(-0.0061 - 0.081) | -0.086, 0.15 | 0.460 | 0.041, 0.18<br>(0.044 - 0.20) |
| **Mineral** | | | | | | |
| Calcium (g/100g dw) | 0.34 (0.049)<br>(0.30 - 0.37) | 0.31 (0.040)<br>(0.28 - 0.34) | 0.030 (0.063)<br>(-0.038 - 0.068) | -0.17, 0.23 | 0.663 | 0.16, 0.61<br>(0.25 - 0.53) |
| Copper (mg/kg dw) | 3.80 (0.092)<br>(3.72 - 3.89) | 3.53 (0.076)<br>(3.33 - 3.67) | 0.28 (0.11)<br>(0.16 - 0.22) | -0.084, 0.64 | 0.092 | 2.00, 4.43<br>(2.52 - 4.93) |
| Iron (mg/kg dw) | 51.55 (4.68)<br>(46.78 - 56.32) | 63.44 (3.82)<br>(60.79 - 67.18) | -11.89 (6.04)<br>(-20.41 - -6.03) | -31.12, 7.34 | 0.143 | 23.39, 86.23<br>(39.16 - 77.92) |
| Magnesium (g/100g dw) | 0.36 (0.012)<br>(0.34 - 0.37) | 0.35 (0.0099)<br>(0.33 - 0.37) | 0.0074 (0.016)<br>(-0.012 - 0.043) | -0.043, 0.058 | 0.668 | 0.32, 0.43<br>(0.30 - 0.45) |
| Manganese (mg/kg dw) | 50.88 (6.56)<br>(46.85 - 51.55) | 50.07 (5.45)<br>(47.11 - 50.97) | 0.81 (7.57)<br>(-0.26 - 0.58) | -23.29, 24.90 | 0.921 | 14.85, 61.05<br>(25.00 - 54.11) |

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**Table H-9. Statistical Summary of Site NDVA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Mineral** | | | | | | |
| Phosphorus (g/100g dw) | 0.57 (0.077)<br>(0.56 - 0.59) | 0.57 (0.063)<br>(0.56 - 0.58) | 0.0026 (0.094)<br>(0.0027 - 0.014) | -0.30, 0.30 | 0.979 | 0.38, 1.06<br>(0.44 - 0.87) |
| Potassium (g/100g dw) | 0.48 (0.037)<br>(0.48 - 0.49) | 0.54 (0.030)<br>(0.53 - 0.54) | -0.053 (0.048)<br>(-0.053 - -0.048) | -0.20, 0.099 | 0.346 | 0.39, 0.96<br>(0.50 - 0.92) |
| Zinc (mg/kg dw) | 30.46 (1.73)<br>(29.81 - 31.08) | 32.41 (1.43)<br>(28.89 - 34.23) | -1.94 (2.07)<br>(-3.15 - 0.93) | -8.52, 4.63 | 0.416 | 20.19, 48.23<br>(22.18 - 47.61) |
| **Vitamin (mg/100g dw)** | | | | | | |
| Vitamin E ($\alpha$-tocopherol) | 15.89 (0.88)<br>(15.23 - 16.55) | 9.43 (0.72)<br>(8.46 - 10.20) | 6.45 (1.14)<br>(5.03 - 8.09) | 2.82, 10.08 | 0.010 | 3.88, 17.28<br>(2.62 - 14.84) |

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

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**Table H-10. Statistical Summary of Site NDVA Canola Seed Anti-nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Anti-nutrient** | | | | | | |
| Alkyl Glucosinolate (µmole/g dw) | 3.90 (0.65)<br>(3.74 - 3.88) | 4.22 (0.54)<br>(2.45 - 5.22) | -0.32 (0.76)<br>(-1.34 - 1.29) | -2.73, 2.10 | 0.705 | 0, 29.02<br>(2.32 - 28.33) |
| Indolyl Glucosinolate (µmole/g dw) | 4.51 (0.57)<br>(3.99 - 4.48) | 3.40 (0.49)<br>(1.83 - 4.23) | 1.11 (0.58)<br>(0.25 - 2.16) | -0.74, 2.96 | 0.152 | 1.37, 6.62<br>(1.84 - 7.18) |
| Phytic Acid (% dw) | 1.58 (0.097)<br>(1.41 - 1.57) | 1.59 (0.088)<br>(1.46 - 1.68) | -0.0050 (0.082)<br>(-0.052 - -0.012) | -0.27, 0.26 | 0.954 | 0.70, 3.52<br>(1.10 - 2.71) |
| Sinapic Acid (% dw) | 1.02 (0.027)<br>(1.00 - 1.04) | 0.83 (0.025)<br>(0.83 - 0.88) | 0.19 (0.018)<br>(0.18 - 0.21) | 0.14, 0.25 | 0.001 | 0.57, 1.13<br>(0.48 - 0.99) |
| Total Glucosinolate (µmole/g dw) | 8.59 (1.20)<br>(7.96 - 8.57) | 7.72 (1.00)<br>(4.38 - 9.61) | 0.87 (1.33)<br>(-1.04 - 3.58) | -3.37, 5.10 | 0.560 | 0, 32.20<br>(5.52 - 31.98) |

[1]dw = dry weight.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

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**Table H-11. Statistical Summary of Site SKSA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Proximate (% dw)** | | | | | | |
| Ash | 3.95 (0.18) (3.68 - 4.44) | 4.01 (0.17) (3.70 - 4.33) | -0.058 (0.10) (-0.20 - 0.12) | -0.34, 0.22 | 0.594 | 3.32, 4.66 (2.98 - 4.52) |
| Carbohydrates | 23.46 (0.51) (23.11 - 23.84) | 25.05 (0.44) (23.91 - 26.81) | -1.59 (0.59) (-2.97 - -0.39) | -3.23, 0.056 | 0.055 | 23.12, 30.77 (22.53 - 29.96) |
| Moisture (% fw) | 4.36 (0.21) (3.90 - 4.82) | 4.69 (0.18) (4.41 - 4.88) | -0.33 (0.28) (-0.75 - 0.010) | -1.12, 0.45 | 0.306 | 4.33, 6.91 (4.09 - 8.48) |
| Protein | 23.82 (0.55) (23.62 - 24.58) | 22.14 (0.50) (21.03 - 24.16) | 1.68 (0.55) (0.42 - 2.50) | 0.15, 3.21 | 0.038 | 17.20, 30.08 (18.68 - 28.32) |
| Total Fat | 48.83 (0.65) (47.91 - 49.22) | 48.81 (0.59) (46.96 - 50.24) | 0.022 (0.64) (-1.02 - 0.95) | -1.76, 1.80 | 0.974 | 39.65, 51.24 (40.71 - 50.26) |
| **Fiber (% dw)** | | | | | | |
| Acid Detergent Fiber | 10.40 (0.63) (9.19 - 11.50) | 9.85 (0.61) (8.94 - 10.78) | 0.54 (0.35) (-0.011 - 1.18) | -0.42, 1.51 | 0.193 | 6.95, 23.92 (9.75 - 21.22) |

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**Table H-11. Statistical Summary of Site SKSA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Fiber (% dw)** | | | | | | |
| Neutral Detergent Fiber | 11.61 (0.73) (9.48 - 12.75) | 12.59 (0.67) (11.56 - 13.91) | -0.98 (0.63) (-2.08 - 0.013) | -2.72, 0.77 | 0.194 | 10.07, 25.94 (10.93 - 22.75) |
| Total Dietary Fiber | 18.68 (0.54) (17.17 - 19.24) | 17.21 (0.48) (16.57 - 17.89) | 1.48 (0.56) (0.60 - 2.52) | -0.075, 3.03 | 0.057 | 13.97, 24.85 (12.64 - 26.47) |
| **Amino Acid (% dw)** | | | | | | |
| Alanine | 1.05 (0.033) (1.02 - 1.07) | 1.01 (0.028) (0.93 - 1.10) | 0.040 (0.043) (-0.031 - 0.069) | -0.080, 0.16 | 0.405 | 0.77, 1.34 (0.87 - 1.27) |
| Arginine | 1.33 (0.052) (1.27 - 1.38) | 1.39 (0.045) (1.29 - 1.55) | -0.062 (0.069) (-0.18 - -0.018) | -0.26, 0.13 | 0.420 | 1.10, 1.93 (1.23 - 1.96) |
| Aspartic Acid | 1.45 (0.037) (1.40 - 1.50) | 1.53 (0.032) (1.46 - 1.65) | -0.085 (0.049) (-0.15 - -0.084) | -0.22, 0.052 | 0.159 | 1.33, 2.12 (1.42 - 2.23) |
| Cystine | 0.55 (0.022) (0.53 - 0.57) | 0.55 (0.019) (0.49 - 0.62) | -0.0025 (0.025) (-0.050 - 0.053) | -0.072, 0.067 | 0.923 | 0.38, 0.83 (0.45 - 0.79) |

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**Table H-11. Statistical Summary of Site SKSA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Glutamic Acid | 3.84 (0.14)<br>(3.66 - 3.97) | 3.98 (0.12)<br>(3.65 - 4.39) | -0.14 (0.18)<br>(-0.50 - 0.094) | -0.64, 0.36 | 0.493 | 2.73, 5.89<br>(3.26 - 5.43) |
| Glycine | 1.05 (0.023)<br>(1.02 - 1.07) | 1.08 (0.020)<br>(1.01 - 1.15) | -0.026 (0.030)<br>(-0.084 - -0.016) | -0.11, 0.058 | 0.436 | 0.96, 1.47<br>(1.01 - 1.50) |
| Histidine | 0.59 (0.015)<br>(0.57 - 0.60) | 0.61 (0.013)<br>(0.57 - 0.65) | -0.016 (0.019)<br>(-0.058 - 0.0030) | -0.070, 0.037 | 0.443 | 0.47, 0.86<br>(0.54 - 0.80) |
| Isoleucine | 0.91 (0.021)<br>(0.89 - 0.93) | 0.90 (0.018)<br>(0.84 - 0.96) | 0.011 (0.028)<br>(-0.049 - 0.041) | -0.067, 0.088 | 0.716 | 0.70, 1.22<br>(0.78 - 1.15) |
| Leucine | 1.61 (0.045)<br>(1.55 - 1.65) | 1.58 (0.039)<br>(1.46 - 1.71) | 0.029 (0.059)<br>(-0.073 - 0.085) | -0.13, 0.19 | 0.644 | 1.21, 2.18<br>(1.36 - 2.07) |
| Lysine | 1.33 (0.037)<br>(1.27 - 1.37) | 1.37 (0.032)<br>(1.30 - 1.48) | -0.034 (0.050)<br>(-0.12 - 0.024) | -0.17, 0.10 | 0.527 | 1.02, 1.90<br>(1.20 - 1.68) |

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**Table H-11. Statistical Summary of Site SKSA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Methionine | 0.45 (0.015)<br>(0.44 - 0.47) | 0.44 (0.013)<br>(0.40 - 0.49) | 0.0082 (0.016)<br>(-0.022 - 0.034) | -0.037, 0.054 | 0.643 | 0.30, 0.65<br>(0.36 - 0.57) |
| Phenylalanine | 0.96 (0.023)<br>(0.93 - 0.98) | 0.94 (0.020)<br>(0.87 - 1.01) | 0.026 (0.031)<br>(-0.028 - 0.044) | -0.059, 0.11 | 0.443 | 0.77, 1.26<br>(0.84 - 1.25) |
| Proline | 1.39 (0.035)<br>(1.34 - 1.42) | 1.44 (0.030)<br>(1.35 - 1.52) | -0.050 (0.046)<br>(-0.13 - 0.0026) | -0.18, 0.077 | 0.335 | 0.90, 2.01<br>(1.12 - 1.78) |
| Serine | 0.97 (0.025)<br>(0.93 - 0.99) | 0.99 (0.022)<br>(0.94 - 1.06) | -0.022 (0.033)<br>(-0.067 - -0.0013) | -0.11, 0.070 | 0.546 | 0.81, 1.32<br>(0.88 - 1.30) |
| Threonine | 0.89 (0.022)<br>(0.86 - 0.92) | 0.93 (0.019)<br>(0.88 - 0.99) | -0.038 (0.029)<br>(-0.072 - -0.031) | -0.12, 0.043 | 0.263 | 0.82, 1.20<br>(0.84 - 1.22) |
| Tryptophan | 0.20 (0.0091)<br>(0.19 - 0.22) | 0.22 (0.0082)<br>(0.21 - 0.24) | -0.018 (0.0093)<br>(-0.035 - 0.0041) | -0.043, 0.0084 | 0.134 | 0.13, 0.35<br>(0.17 - 0.32) |

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**Table H-11. Statistical Summary of Site SKSA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Amino Acid (% dw)** | | | | | | |
| Tyrosine | 0.64 (0.014) (0.61 - 0.65) | 0.64 (0.012) (0.61 - 0.68) | -0.0084 (0.018) (-0.037 - 0.0079) | -0.060, 0.043 | 0.672 | 0.57, 0.81 (0.60 - 0.84) |
| Valine | 1.16 (0.025) (1.13 - 1.19) | 1.15 (0.022) (1.08 - 1.23) | 0.0090 (0.034) (-0.073 - 0.046) | -0.085, 0.10 | 0.801 | 0.92, 1.55 (1.01 - 1.46) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 16:0 Palmitic | 4.51 (0.047) (4.46 - 4.57) | 4.07 (0.042) (4.05 - 4.10) | 0.44 (0.049) (0.41 - 0.48) | 0.31, 0.58 | <0.001 | 2.84, 5.26 (3.55 - 4.69) |
| 16:1 Palmitoleic | 0.26 (0.0047) (0.25 - 0.26) | 0.25 (0.0043) (0.24 - 0.25) | 0.0062 (0.0044) (0.0043 - 0.0074) | -0.0061, 0.019 | 0.235 | 0.17, 0.30 (0.19 - 0.27) |
| 18:0 Stearic | 1.66 (0.055) (1.54 - 1.72) | 2.08 (0.049) (1.91 - 2.19) | -0.42 (0.057) (-0.48 - -0.35) | -0.58, -0.26 | 0.001 | 0.90, 3.05 (1.50 - 2.64) |
| 18:1 Oleic | 61.91 (0.55) (60.51 - 62.29) | 65.69 (0.51) (64.73 - 66.86) | -3.78 (0.47) (-4.30 - -2.59) | -5.08, -2.48 | 0.001 | 56.13, 70.69 (57.86 - 68.53) |

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**Table H-11. Statistical Summary of Site SKSA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Fatty Acid (% Total FA)** | | | | | | |
| 18:2 Linoleic | 18.49 (0.37) (18.08 - 19.48) | 17.22 (0.34) (16.64 - 18.01) | 1.27 (0.34) (0.40 - 1.90) | 0.33, 2.20 | 0.019 | 12.60, 24.49 (14.12 - 22.57) |
| 18:3 Linolenic | 10.78 (0.23) (10.39 - 11.23) | 8.38 (0.20) (7.94 - 8.99) | 2.40 (0.25) (2.08 - 2.64) | 1.72, 3.09 | <0.001 | 6.96, 11.73 (7.99 - 10.94) |
| 20:0 Arachidic | 0.54 (0.0079) (0.52 - 0.55) | 0.62 (0.0069) (0.59 - 0.63) | -0.082 (0.0090) (-0.091 - -0.070) | -0.11, -0.057 | <0.001 | 0.45, 0.80 (0.53 - 0.71) |
| 20:1 Eicosenoic | 1.24 (0.015) (1.22 - 1.26) | 1.13 (0.013) (1.10 - 1.17) | 0.11 (0.020) (0.050 - 0.14) | 0.052, 0.16 | 0.005 | 0.83, 1.68 (1.04 - 1.56) |
| 22:0 Behenic | 0.28 (0.0031) (0.28 - 0.29) | 0.29 (0.0027) (0.28 - 0.30) | -0.0088 (0.0037) (-0.014 - 0.0025) | -0.019, 0.0014 | 0.073 | 0.19, 0.43 (0.27 - 0.38) |
| 24:0 Lignoceric | 0.20 (0.029) (0.20 - 0.23) | 0.15 (0.026) (0.045 - 0.22) | 0.050 (0.030) (-0.016 - 0.062) | -0.032, 0.13 | 0.163 | 0.033, 0.25 (0.044 - 0.21) |

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**Table H-11. Statistical Summary of Site SKSA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| | | | Difference (MON 88302 minus Control) | | | |
|---|---|---|---|---|---|---|
| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
| **Fatty Acid (% Total FA)** | | | | | | |
| 24:1 Nervonic | 0.17 (0.020) (0.16 - 0.20) | 0.12 (0.018) (0.045 - 0.16) | 0.055 (0.021) (0.034 - 0.055) | -0.0021, 0.11 | 0.055 | 0.041, 0.18 (0.044 - 0.20) |
| **Mineral** | | | | | | |
| Calcium (g/100g dw) | 0.41 (0.015) (0.40 - 0.42) | 0.44 (0.013) (0.40 - 0.47) | -0.031 (0.020) (-0.068 - 0.0021) | -0.086, 0.025 | 0.199 | 0.16, 0.61 (0.25 - 0.53) |
| Copper (mg/kg dw) | 3.52 (0.16) (3.27 - 3.85) | 3.23 (0.15) (2.96 - 3.48) | 0.29 (0.18) (-0.21 - 0.57) | -0.21, 0.78 | 0.181 | 2.00, 4.43 (2.52 - 4.93) |
| Iron (mg/kg dw) | 63.21 (8.79) (55.62 - 69.61) | 59.66 (7.61) (50.11 - 77.74) | 3.56 (11.63) (-13.33 - 14.87) | -28.72, 35.84 | 0.774 | 23.39, 86.23 (39.16 - 77.92) |
| Magnesium (g/100g dw) | 0.36 (0.017) (0.31 - 0.39) | 0.36 (0.016) (0.34 - 0.39) | -0.0044 (0.014) (-0.032 - 0.026) | -0.042, 0.033 | 0.764 | 0.32, 0.43 (0.30 - 0.45) |
| Manganese (mg/kg dw) | 41.77 (2.44) (38.56 - 47.24) | 34.73 (2.12) (33.12 - 37.61) | 7.04 (3.03) (0.95 - 12.63) | -1.37, 15.45 | 0.080 | 14.85, 61.05 (25.00 - 54.11) |

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**Table H-11. Statistical Summary of Site SKSA Canola Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Mineral** | | | | | | |
| Phosphorus (g/100g dw) | 0.68 (0.051) (0.57 - 0.80) | 0.74 (0.048) (0.67 - 0.78) | -0.057 (0.043) (-0.095 - 0.040) | -0.18, 0.062 | 0.254 | 0.38, 1.06 (0.44 - 0.87) |
| Potassium (g/100g dw) | 0.82 (0.030) (0.77 - 0.90) | 0.71 (0.030) (0.67 - 0.80) | 0.11 (0.012) (0.084 - 0.14) | 0.077, 0.14 | <0.001 | 0.39, 0.96 (0.50 - 0.92) |
| Zinc (mg/kg dw) | 41.58 (2.06) (39.33 - 45.49) | 33.10 (1.90) (29.75 - 40.66) | 8.48 (1.84) (4.84 - 11.44) | 3.36, 13.60 | 0.010 | 20.19, 48.23 (22.18 - 47.61) |
| **Vitamin (mg/100g dw)** | | | | | | |
| Vitamin E (α-tocopherol) | 1.49 (1.08) (1.30 - 1.66) | 6.91 (0.94) (3.33 - 9.22) | -5.43 (1.43) (-6.92 - -1.67) | -9.40, -1.45 | 0.019 | 3.88, 17.28 (2.62 - 14.84) |

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

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**Table H-12. Statistical Summary of Site SKSA Canola Seed Anti-nutrient Content for MON 88302 vs. the Conventional Control**

| Analytical Component (Units)[1] | MON 88302[2] Mean (S.E.)[3] (Range) | Control[4] Mean (S.E.) (Range) | Difference (MON 88302 minus Control) Mean (S.E.) (Range) | 95% Confidence Interval[3] | Significance (p-Value) | Commercial Tolerance Interval[5] (Range) |
|---|---|---|---|---|---|---|
| **Anti-nutrient** | | | | | | |
| Alkyl Glucosinolate (µmole/g dw) | 1.61 (0.71) (1.19 - 2.17) | 5.82 (0.63) (4.87 - 8.28) | -4.21 (0.78) (-6.11 - -3.28) | -6.36, -2.06 | 0.005 | 0, 29.02 (2.32 - 28.33) |
| Indolyl Glucosinolate (µmole/g dw) | 0.86 (0.34) (0.49 - 1.31) | 3.30 (0.32) (2.68 - 4.35) | -2.44 (0.29) (-3.05 - -2.19) | -3.24, -1.64 | 0.001 | 1.37, 6.62 (1.84 - 7.18) |
| Phytic Acid (% dw) | 1.58 (0.20) (1.20 - 1.91) | 1.95 (0.18) (1.69 - 2.20) | -0.37 (0.24) (-0.62 - 0.22) | -1.03, 0.29 | 0.191 | 0.70, 3.52 (1.10 - 2.71) |
| Sinapic Acid (% dw) | 0.22 (0.059) (0.16 - 0.28) | 0.81 (0.051) (0.65 - 0.95) | -0.60 (0.075) (-0.76 - -0.49) | -0.80, -0.39 | 0.001 | 0.57, 1.13 (0.48 - 0.99) |
| Total Glucosinolate (µmole/g dw) | 2.53 (1.02) (1.73 - 3.51) | 9.22 (0.93) (7.85 - 12.72) | -6.69 (1.02) (-9.21 - -5.78) | -9.51, -3.86 | 0.002 | 0, 32.20 (5.52 - 31.98) |

[1]dw = dry weight.
[2] MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial substances. Negative limits set to zero.

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