Safety Assessment of Glyphosate-tolerant Canola

Safety, Compositional, and Nutritional Aspects of Glyphosate-tolerant Canola: Conclusion Based on Studies and Information Evaluated According to FDA's Consultation Process

by

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I. LIST OF ABBREVIATIONS

Ag Canada - Agriculture and Agri-Food Canada
bp - base pair
CP4 EPSPS - 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4
CTP - chloroplast transit peptide
EPA - United States Environmental Protection Agency
ELISA - enzyme-linked immunosorbent assay
EPSP - 5-enolpyruvylshikimate-3-phosphate
EPSPS - 5-enolpyruvylshikimate-3-phosphate synthase
FDA - Food and Drug Administration
FFDCA - Federal Food, Drug, and Cosmetics Act
GLP - United States Good Laboratory Practices
GOX - glyphosate oxidoreductase from *Achromobacter* sp. strain LBAA
GOXv247 - a variant of glyphosate oxidoreductase from *Achromobacter* sp. strain LBAA
GT73 - glyphosate-tolerant canola line 73 also known as 17237-73
GT200 - glyphosate-tolerant canola line 200 also known as 17209-200
GTC - glyphosate-tolerant canola
mg - milligram
PAGE - polyacrylamide gel electrophoresis
PEP - phosphoenolpyruvate
PCR - polymerase chain reaction
POS - Protein, Oil, Starch Pilot Plant Corporation
RAL - Ralston Analytical Laboratories
RBDO - refined, bleached, deodorized oil
S3P - shikimate-3-phosphate
SSU - small subunit
SDS - sodium dodecyl sulfate
TAS - Technical Assessment Systems, Inc.
U - unit of enzymatic activity
μg - microgram
US - United States
II. INTRODUCTION

The Agricultural Group of Monsanto Company (Monsanto) is providing the Food and Drug Administration (FDA) with a summary of studies demonstrating that canola, modified by the addition of two genes which confer the glyphosate tolerance phenotype, is substantially equivalent, with respect to composition, safety, and any other relevant parameter, to canola currently on the market. Monsanto has performed analyses and studies to support this conclusion on glyphosate-tolerant canola (GTC) line 17237-73 (GT73). GT73 is a selection from Westar (1), a commercial Brassica napus canola variety, and produces a small amount of two proteins, CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) (2) and glyphosate oxidoreductase (GOX) (3,4), which provide tolerance to glyphosate, the active ingredient of Roundup® herbicide. The data from our studies demonstrate that GTC is substantially equivalent to canola varieties commercially registered today allowing for the presence of the CP4 EPSPS and GOX proteins.

The glyphosate tolerance locus associated with GT73 has been transferred to other canola varieties through traditional breeding methods. Seed company partners plan to commercialize progenies derived from these crosses. The assessment summarized in this document establishes the safety of GT73, and of any progenies which will be derived from crosses between GT73 and other canola varieties. Canola varieties derived from GT73 will be commercialized in Canada and food and feed products from these varieties will be imported into the United States.

The food use of refined oil from canola varieties with the glyphosate tolerance trait developed from GT73 has been approved by the Food Directorate, Health Protection Branch of Canada. The "Draft Guidelines for the Safety Assessment of Novel Foods" of the Food Directorate, Health Protection Branch, Health Canada (October, 1993), provided guidance for determining whether this new plant variety, produced using genetic modification, is as safe and nutritious as its parental variety. We carefully followed those Draft Guidelines (which are consistent with the criteria for "substantial equivalence" developed and currently used by the OECD [Organization for Economic Cooperation and Development] and were eventually finalized in September, 1994) in evaluating the safety of GTC. GTC is considered a novel food by the Canadian definition outlined in the guidelines; therefore, GTC requires notification prior to sale in Canada. Monsanto submitted notification to the Food Directorate, Health Protection Branch in August 1994 and received a no objection for food uses of refined oil from canola varieties with the glyphosate tolerance trait developed from GT73 in November 1994 (Appendix B).

Agriculture and Agri-Food Canada (Ag Canada), with authority under the Feeds Act, reviews animal feed safety and nutrition since canola meal is used
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as a feed ingredient. Draft Guidelines for assessment of plants with novel traits as Livestock Feed have recently been issued and Monsanto has submitted and received approval for such use on March 24, 1995.

Ag Canada with authority under the Seeds Act and Plant Protection Act reviews safety for unconfined environmental release. Draft guidelines for safety assessment for unconfined release of plants with novel traits have been issued. Monsanto provided information on November 1, 1994 on the environmental safety of the GT73 and Monsanto has received approval for unconfined environmental release of GTC in Canada on March 24, 1995.

Ag Canada with authority of the Seeds Act and Pest Control Products Act reviews safety and efficacy/benefit data for a new canola variety registration and amended herbicide use, respectively. Monsanto has petitioned for certification of GTC for seed sales and for extension of the use of Roundup in crop in GTC.

In anticipation of the need for regulatory oversight of the science of biotechnology and its products, The United States Office of Science and Technology Policy issued the “Coordinated Framework for Regulation of Biotechnology” on June 26, 1986. Based on the belief that a new agency or new legislation was not required, this coordinated framework assigned regulatory responsibility of biotechnology to existing Federal Agencies. Monsanto plans to discuss GTC line GT73 and any progenies derived from crosses between line GT73 and traditional canola varieties with the applicable US regulatory agencies.

We will complete the consultation process with FDA following the guidance outlined in FDA’s Statement of Policy “Foods Derived From New Plant Varieties” (“Food Policy”) (5). That policy discusses the safety and regulatory status of food/feed derived from new plant varieties, including plants developed by genetic modification.

FDA’s Food Policy (5) provides guidance for determining whether a new plant variety is as safe and nutritious as its parental variety. This guidance provides a mechanism for establishing the lack of a material difference between the modified product and its traditional counterpart. We have carefully followed this guidance (which is consistent with the criteria for “substantial equivalence” developed and currently used by the OECD) in demonstrating that GT73 canola is not materially different from other varieties of canola. To assess as thoroughly as possible whether a material difference might exist, we have conducted numerous compositional studies and thoroughly reviewed all relevant data and information. To focus the analysis on any effects of the introduced gene and protein, the canola from which the tested and analyzed seed were derived were not treated with Roundup.
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herbicide. Upon quantitatively and qualitatively evaluating all of the data available, we have been able to ensure that, in all instances, for every parameter there are no meaningful differences between the GT73 line of canola and its parental variety. In a few instances where the data were statistically analyzed and where a statistical difference was noted, we established that the values are within established and accepted ranges for the parental variety as well as for canola. On the basis of these evaluations, we confidently conclude, that except for the tolerance to glyphosate, GT73 is not materially different from, and is as safe and nutritious as, its parental variety and other canola varieties now being marketed.

We have held consultations with the FDA, starting in 1993, to define and discuss studies to assess the composition and safety of GTC. The concepts and approaches we have employed are derived from and consistent with the guidance presented in the flowcharts found in the FDA Food Policy (5). For each question, we have developed answers based on extensive studies or analyses. The thoroughness and detail of these studies are unprecedented for the typical introduction of foods or feeds from a new plant variety. Our data and findings in every case have led us to the conclusion of "no concern" as described in the relevant sections of the following summary. Under these circumstances, following the agency's Food Policy has provided us with a basis for concluding that GT73 is as safe and nutritious as its traditional counterparts.

In the FDA Food Policy, there are two main categories of questions to address regarding foods/feeds derived from new plant varieties: 1) unexpected or unintended effects; and, 2) expected or intended effects. Accordingly, the following data summary is organized in this manner. Preceding the data summary is background information regarding the development of GTC, including a description of CP4 EPSPS and GOX proteins.

III. BACKGROUND INFORMATION REGARDING GTC

A. Rationale for the Development of GTC
Glyphosate (N-phosphonomethyl-glycine) (CAS Registry #s 1071-83-6, 38841-94-0), the active ingredient in the non-selective, foliar-applied, broad-spectrum, post-emergent herbicide Roundup (6,7), is the world's most popular herbicide. This is primarily due to its excellent weed control capabilities and its well-known, favorable environmental and safety characteristics. However, the sensitivity of crop plants to glyphosate has prevented the in-season use of this herbicide over-the-top on crops. The extension of the use of Roundup herbicide to allow in-season application in major crops such as canola will provide new weed control options for farmers. Recent advances in plant biotechnology have made it possible to insert a gene into canola to provide crop tolerance specifically to the non-selective herbicide glyphosate, and bring the benefits of
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its use to weed management in canola (8-13).

Weed management is a critical step to maximize canola yields and retain a high-quality harvest, free of weed seeds. For effective weed control, the farmer typically selects a herbicide based on several factors: weed spectrum, lack of crop injury, cost, and environmental characteristics. Few herbicides available today deliver optimal performance in all of these areas. Several classes of herbicides are effective for broad-spectrum weed control, but many are either non-selective and kill crop plants or they significantly injure some crops at the application rates required for effective weed control. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds.

The use of GTC for canola production would enable the farmer to utilize Roundup herbicide for effective control of weeds. In addition, GTC affords the opportunity to take advantage of this herbicide's environmental and safety characteristics. Environmentally, glyphosate rapidly binds to soil (resistance to leaching) and biodegrades (which decreases persistence). It also has very low toxicity to mammals, birds, and fish (7). Recently, glyphosate was classified by the EPA as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739).

B. Development of GTC

Since the early 1980's, Monsanto has been developing GTC as a part of a broad program to offer specific glyphosate-tolerant crops to farmers (8-11). This research has resulted in the development of two distinct mechanisms of glyphosate tolerance (Figure 1), reduced sensitivity of the molecular site of herbicidal activity, the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), and introduction of the means by which the plant can degrade the herbicide, both of which are operative in GTC. The gene encoding the protein CP4 EPSPS was originally obtained from Agrobacterium sp. strain CP4. This protein, like other EPSPSs, catalyzes the reaction of shikimate-3-phosphate (S3P) with phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phosphate (EPSP), a step in the production of aromatic amino acids via the shikimate pathway (14,15). Unlike EPSPSs found in plants, CP4 EPSPS (2) is highly insensitive to inhibition by glyphosate (7,16), the active ingredient in Roundup herbicide. A plant that expresses CP4 EPSPS produces aromatic amino acids even when treated with glyphosate. The proteins GOX (4) and a variant of the same enzyme (GOXv247) (17) also impart glyphosate tolerance to GTC. These two enzymes are >99% identical, differing by 3 amino acids out of more than 400. The substitution of the histidine residue at position 334
with arginine effects a ten-fold lowering of the apparent $K_m$ for glyphosate in GOXv247 (17), and thus enhances the efficiency of glyphosate degradation. Because of this extremely high identity, both are referred to simply as GOX in general discussions. GOX was isolated from *Achromobacter* sp. strain LBAA, and catalyzes the breakdown of glyphosate into aminomethylphosphonic acid (AMPA) and glyoxylate (3). This degradation effectively inactivates the herbicide and enables GTC to grow when treated with Roundup herbicide.

**Figure 1. Mechanisms of glyphosate tolerance in GTC**

C. **CP4 EPSPS: One of the Two Proteins which Confer Glyphosate Tolerance to GTC Line GT73**

1. **Kinetic characteristics**

   The EPSPS enzyme from *Agrobacterium* sp. strain CP4 was identified from a screen of microorganism cell extracts as having very favorable glyphosate tolerance kinetic parameters, namely high glyphosate tolerance.

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1 The Michaelis-Menton constant, $K_m$, is equal to that concentration of substrate, which in this case is glyphosate, expressed in moles per liter that gives half the numerical initial maximal velocity. The $K_m$ is a measure of the affinity of a particular substrate for an enzyme. The lower the $K_m$, the higher the affinity for the enzyme.
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(K_i [glyphosate]= 2.7 mM)^2 and tight binding of PEP (appK_m[PEP]=12 μM) (2,18). The appK_m(PEP) of CP4 EPSPS is only about 2-fold greater than the appK_m(PEP) of the wild-type petunia EPSPS (5 μM) (19). In fact, CP4 EPSPS exhibits the lowest appK_m(PEP) constant of any highly glyphosate-tolerant EPSPS identified to date. The constant k_cat^3•appK_i(glyphosate) / appK_m(PEP), which is a measure of the catalytic efficiency of the EPSPS in the presence of glyphosate, is approximately 10-fold higher for CP4 EPSPS than for the petunia G101A (glycine to alanine substitution at position 101) petunia EPSPS (20), and results from the 17-fold reduction in appK_m(PEP) for the CP4 enzyme relative to G101A petunia EPSPS. Based on these kinetic parameters, and thus the suitability for use in conferring glyphosate tolerance to crops, the gene for CP4 EPSPS was cloned from Agrobacterium sp. strain CP4, and expressed in Escherichia coli for further characterization (2).

2. Cloning of the CP4 EPSPS gene
The EPSPS gene from Agrobacterium sp. strain CP4 was identified and cloned by two parallel approaches: cloning based on the expected phenotype for a glyphosate-tolerant EPSPS, and purification of the enzyme to provide material to raise antibodies and to obtain amino acid sequences from the protein to facilitate the verification of clones (2). A cosmid bank was constructed using DNA from Agrobacterium sp. strain CP4, and the cosmids were transformed into E. coli and selected for the EPSPS gene using inhibitory concentrations of glyphosate. The EPSPS gene was then cloned from one of the resulting cosmids imparting glyphosate tolerance, and the nucleotide sequence was determined (2). The deduced amino acid sequence from the resulting cloned EPSPS gene from Agrobacterium sp. strain CP4 is shown in Figure 2. CP4 EPSPS is a 47.6 kD protein consisting of a single polypeptide of 455 amino acids (2). The identification of codons in the gene encoding four peptide sequences obtained directly from the purified enzymatically-active CP4 EPSPS conclusively demonstrated that the gene cloned was the EPSPS gene from Agrobacterium sp. strain CP4 (Figure 2).

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2 The inhibition constant, K_i, of a particular inhibitor of an enzymatic reaction is defined as the dissociation constant of an enzyme-inhibitor complex. The K_i of a particular inhibitor is a measure of that inhibitors ability to inhibit an enzymatic reaction. When comparing inhibitors, the lower the value for K_i, the better the inhibitor.

3 The catalytic rate constant, k_cat, is a measure of the catalytic efficiency of an enzymatic reaction. The higher the value of k_cat, the more efficient the enzyme is.
Figure 2. Deduced amino acid sequence of the Agrobacterium sp. strain CP4 EPSPS gene from pMON17081. The underlined sequences represent amino acid sequences obtained from peptides of the purified enzyme.

1 MSHGASSRPA TARKSSGSLG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
51 LEGEDVINTG KAMQAMGARI RKEGDTWIID GIDGNGDAPL GQVSKEDGD
101 TGCRLTGMLV GYDFDSTFI GDASLTKRPM GRIINPLREM GVQKSEDGD
151 RLPVTLRGPK TPTFITYRVP MASAQVSAV LLACLNTFGT TTVEFINTF
201 DHKMEMLQGF GANLTVETDA DGVTIRLEG RGGLTQVTO VPGDPSSTAF
251 PTVAVLLEFG SVTITLAVVL MPCLTVGLLIL TVEMSSADEL TVPPLAGGGG
301 VADLRSVTST LKVVPVTDGR APEQMDKXPF LAVAVWAFSG ATVMGDLRL
351 RPSQESRLSA VANGKLVNGL IGLQGGLYTV VRPGPDQKGL GMNAGAVAT
401 HVMLKLAMSF LVMGLVSENP VTVDDATMIA TSSFPMDEM AGGKARTEL
451 DTKAA

3. Sequence homology of EPSPS proteins

There is considerable divergence in the EPSPSs which are typically present in foods. The divergence of the CP4 EPSPS sequence from typical food EPSPS sequences is of the same order. All crops and microbial food sources such as Baker's yeast (S. cerevisiae) or B. subtilis (21) contain EPSPS proteins. These proteins, which are GRAS (generally regarded as safe) substances, show a wide range of amino acid compositions as deduced from their DNA sequences. The CP4 EPSPS shows the same range of similarity to the EPSPS enzymes from foods. Comparing the deduced amino acid sequences of CP4 EPSPS with EPSPS from R. napus, soybean, corn, petunia, F. coli, R. subtilis, and S. cerevisiae (Baker's yeast) yields similarities of 48.8%, 51.2%, 48.5%, 50.1%, 52.2%, 59.3%, and 53.5%, respectively, and identities of 23.0%, 26.0%, 24.1%, 23.3%, 26.0%, 41.1%, and 29.9%, respectively (2).

To gain further insight into the relatedness of CP4 EPSPS to other known EPSPSs, the amino acid sequence of CP4 EPSPS was aligned against a consensus sequence of previously-identified EPSPSs (2). Several residues which have been previously identified as important for EPSPS function are conserved in CP4 EPSPS (2). These residues include lysine at position 28 of CP4 EPSPS, which corresponds to lysine at position 22 in E. coli EPSPS and likely interacts with PEP (22); arginine at position 33, which corresponds to arginine at position 27 of E. coli EPSPS and is involved in S3P binding (23); and arginine at position 128, which corresponds to arginine at position 124 of E. coli.
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EPSPS, and is believed to be involved in PEP binding (23). The homology of catalytically critical amino acids in CP4 EPSPS to those in other known EPSPSs demonstrates the relatedness of the CP4 EPSPS active site to other plant EPSPSs.

Additional evidence which supports the high degree of relatedness between CP4 EPSPS and other EPSPSs was recently found in the 3-dimensional X-ray crystal structure of CP4 EPSPS (Monsanto unpublished results). These data show that, on a molecular level, the CP4 EPSPS has the same overall folding patterns as the E. coli EPSPS. The structural similarity of CP4 EPSPS to the E. coli EPSPS (which is more similar to plant EPSPSs than CP4 EPSPS in amino acid sequence) allows the inference that the CP4 EPSPS is structurally similar to plant EPSPSs.

We conclude that the information summarized above demonstrates that based on 1) the reaction catalyzed; 2) the amino acid sequence similarity; 3) the homology of active site residues; and 4) the 3-dimensional structure, CP4 EPSPS is functionally and structurally similar to the EPSPS proteins typically present in food and feed derived from plant and microbial sources.

4. pH and temperature dependence of CP4 EPSPS

One aspect of CP4 EPSPS of interest was the temperature dependence of the stability of the enzyme. One reason for this interest was to attempt to project whether glyphosate-tolerant raw agricultural commodities expressing CP4 EPSPS would be likely to be inactivated upon heat processing. Virtually 100% of canola is heated prior to use as an animal feed (24,25). Upon incubation of CP4 EPSPS at 55°C for 15 minutes, more than half of the activity observed during incubation at 25°C was lost; enzymatic activity was completely eliminated after 15 minutes incubation at 65°C (2). These results indicate that the enzymatic activity of CP4 EPSPS will be lost or significantly decreased upon processing seeds using heat treatment. For instance, canola seed, after flaking, are heated approximately 20 to 40 minutes at 75-85°C in order to inactivate the enzyme myrosinase (24,25) which catalyzes the breakdown of glucosinolates into degradation products. In a later processing step, the meal is desolventized and toasted at 103-107°C for 30 to 40 minutes (24,25).

Similarly, it was of interest to determine whether CP4 EPSPS would be enzymatically active in the acidic environment of a mammalian stomach, in the unlikely event that heat processing did not denature and inactivate all of the enzyme prior to consumption, and that digestion did not occur virtually instantaneously. The pH dependence of CP4 EPSPS was therefore measured from pH 4 to pH 11 (2). The maximal activity of CP4 EPSPS under these conditions was observed to be in the range of pH 9 to 9.5. No enzymatic activity was detectable at pH values less than pH 5. These results indicate
that CP4 EPSPS would not be enzymatically active in the acidic environment of a mammalian stomach.

D. GOX Protein: The Second Protein Which Confers Glyphosate Tolerance to GTC Line GT73

1. Kinetic and enzymatic properties
The GOX enzyme from *Achromobacter* sp. strain LBAA was identified from a collection of glyphosate-degrading bacteria (26) by screening for bacteria that showed the most rapid degradation of glyphosate (4). The GOX enzyme expressed in *E. coli* has been found to have an appKm(glyphosate) of approximately 19-25 mM (3). The enzyme has been extensively characterized (3). GOX catalyzes the reaction of glyphosate with oxygen to yield aminomethylphosphonic acid (AMPA) and glyoxylate (Figure 1). The stoichiometry of the reaction has been determined to be 1 mole of glyphosate reacting with 0.5 mole of oxygen to yield 1 mole each of AMPA and glyoxylate. In the absence of external electron acceptors, GOX is dependent on the presence of oxygen for its activity.

The substrate specificity of GOX was examined using an oxygen polarographic electrode, which measures the GOX plus substrate dependent consumption of oxygen in a GOX assay solution. Iminodiacetic acid (IDA), an analog of glyphosate where a carboxylic acid replaces the phosphonate moiety, was found to be a substrate for GOX, with an appKm(IDA) of 2.8 mM. However, the amino acids glycine, D- and L-glutamic acid, D- and L-aspartic acid, D- and L-phenylalanine, D- and L-alanine, D- and L-methionine, and sarcosine were not substrates for GOX. No evidence of activity was detected when the carbon analogue of IDA, glutaric acid, the oxalamide derivative of glyphosate, and N-methyl glyphosate were exposed to GOX (3). Furthermore, enzymatic activity significantly diminished when a methyl group was placed at the 2-position in glyphosate (next to the carboxylate moiety) (3). These studies indicate a narrow specificity of the GOX enzyme for secondary amines with specific steric and electronic properties similar to IDA and glyphosate.

2. GOXv247
Due to the relatively high appKm(glyphosate) exhibited by GOX (19-25 mM), efforts were made to identify variants of GOX which exhibit reduced appKm(glyphosate) constants. Presumably, a GOX variant with a reduced appKm(glyphosate) would more efficiently metabolize glyphosate in planta, assuming its appVmax is not significantly reduced relative to wild-type GOX. Utilizing a random mutagenesis approach, a variant of GOX denoted GOXv247

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4 The apparent Vmax is defined as the limiting maximal velocity that would be observed in an enzyme catalyzed reaction when the concentration of substrate is high enough to convert all enzyme into enzyme-substrate complex.
was identified which had an app\(K_m\) (glyphosate) of approximately 2.5 mM, which is 8-10 times lower than that for wild-type GOX (17). Nucleotide sequencing of GOXv247 revealed that GOXv247 has three amino acid substitutions relative to GOX: serine for glycine at position 84, lysine for arginine at position 153, and histidine for arginine at position 334. Site-directed mutagenesis of GOX demonstrated that only the histidine for arginine substitution at position 334 lowered the app\(K_m\) for glyphosate, and hence is the critical substitution in GOXv247 leading to increased GOX-mediated glyphosate metabolism. Estimates indicate that the app\(V_{\max}\) of GOXv247 is not reduced relative to wild type GOX.

3. Cloning of the \textit{gox} gene and amino acid sequence

The glyphosate-to-AMPA pathway appears to be the predominant degradation route for glyphosate in soil (26-28) and has been identified in a number of gram negative and gram positive bacteria (27,28). Prior to cloning the gene encoding GOX, efficient glyphosate degrading bacteria were first isolated from a collection of putative glyphosate-to-AMPA bacteria (26). The \textit{Achromobacter} sp. strain LBAA was chosen for further study. This microorganism is capable of using glyphosate as a carbon or phosphorus source or as a source for both (4). A direct approach to the cloning of the gene(s) responsible for the glyphosate-to-AMPA activity approach was taken (4,18) and was based on the ability of certain \textit{E. coli} strains (Mpu+, methylphosphonic acid utilizing) (29-31) to utilize AMPA or other phosphonic acids as phosphorus sources through the action of a C-P lyase activity. A cosmid library of LBAA genomic DNA was screened in an Mpu+ \textit{E. coli} host on the basis of growth on glyphosate as a phosphorus source. The ability to use glyphosate was linked to tolerance to glyphosate (growth of wild-type bacteria is inhibited by glyphosate).

An amino acid sequence comparison of GOX with other proteins shows the greatest homology to D-amino acid dehydrogenases of \textit{E. coli} (46.7% similar, and 28.6% identical), and to a lesser extent to sarcosine oxidases and D-amino acid and monoamine oxidases (4,32,33). Of particular note is a conserved motif at the N-terminus homologous to a flavin binding site. The involvement of a flavin co-factor in the GOX catalyzed oxidation of glyphosate has been proposed (3). Studies detailing the substrate specificity of the GOX enzyme show that the D-amino acids investigated and sarcosine are not substrates for the GOX reaction (see Section III.D.1).

The amino acid sequence of GOXv247 is compared to that of GOX in Figure 3. It is readily seen that the two are >99% identical. The differences between the two forms of GOX are shown in bold in Figure 3.
Figure 3. Amino acid sequences of the GOX (lower sequence) and GOXv247 (top sequence) as introduced into plant transformation vectors.

```
1 MAENHKKVGIAGACGIGCVCVVWLTIDPHNPQGKGVGAGSFGNAGC 50
1 MAENHKKVGIAGACGIGCVCVVWLTIDPHNPQGKGVGAGSFGNAGC 50
51 FNGSSVPMSPGNLTSVPLLDPMGLSLRFGYFPTIMWLIRPLL 100
51 FNGSSVPMSPGNLTSVPLLDPMGLSLRFGYFPTIMWLIRPLL 100
101 RPNKVEQAKALRNKLKSTIALPLSKLRKALKHDLPTDAG 150
101 RPNKVEQAKALRNKLKSTIALPLSKLRKALKHDLPTDAG 150
151 FADFRGWWLKNLNGTVRVSADALRDVDPNLHATRKLLEIIEN 200
151 FADFRGWWLKNLNGTVRVSADALRDVDPNLHATRKLLEIIEN 200
201 NPQGLYTLTFFRFINQGGSARVGFETEGRALKGITTNGVLABD 250
201 NPQGLYTLTFFRFINQGGSARVGFETEGRALKGITTNGVLABD 250
251 VVAGAHSKGSLGDIPLTERGTVHIVNPEAAPPITTDGKFI 300
251 VVAGAHSKGSLGDIPLTERGTVHIVNPEAAPPITTDGKFI 300
301 ATPMKRLVAGCMAQAFAIATRKRAHLRTHLRPPAPASEER 350
301 ATPMKRLVAGCMAQAFAIATRKRAHLRTHLRPPAPASEER 350
351 YSDKKMFQSTIPDSLPYRGRATTPFDVYAFSGHPLGHTGAMTPATLVS 400
351 YSDKKMFQSTIPDSLPYRGRATTPFDVYAFSGHPLGHTGAMTPATLVS 400
401 LLAGEKTSIDSPIPAAGIIGKSKQGTGPAS 431
401 LLAGEKTSIDSPIPAAGIIGKSKQGTGPAS 431
```

4. pH and temperature dependence

We evaluated the temperature stability of GOX activity to determine whether GOX activity in glyphosate-tolerant raw agricultural commodities expressing GOX may be inactivated upon heat processing. Virtually 100% of canola is heated prior to consumption as animal feed. Upon incubation of GOX at 60°C for 15 minutes, 83% of the 25°C incubation activity was lost (3). For comparison, canola flakes are heated approximately 30 to 40 minutes at 103
Safety Assessment of Glyphosate-tolerant Canola

to 107°C during the preparation of toasted meal for animal feed (24,25). These results indicate that the enzymatic activity of GOX will be lost or significantly decreased upon heat processing of glyphosate-tolerant canola seeds.

In addition, the pH dependence of GOX was measured from pH 5 to pH 9 (3). The maximal activity of GOX under these conditions was observed to be in the range of pH 6.5-7. At the high end of the pH titration, pH 9, GOX activity was reduced approximately four-fold from the maximal activity. At the low end of the pH titration, GOX activity was reduced approximately 1.6-fold from the maximal activity. These results indicate that GOX has enzymatic activity over a fairly broad pH range. Importantly, in other experiments (see Section IV.B.1.d.iii), the enzymatic activity of GOX proteins at very low pH in simulated gastric fluids is eliminated in less than 0.25 minutes.

E. Chloroplast Transit Peptides (CTP)
Results from early experiments showed that it was critical to target glyphosate-tolerant EPSPSs to the chloroplast, the site of aromatic acid biosynthesis, to obtain the highest levels of in planta tolerance (34). The CP4 EPSPS gene was engineered for plant expression by fusing the 5'-end of the CP4 EPSPS gene to the N-terminal chloroplast transit peptide (CTP) sequence derived from the Arabidopsis EPSPS gene [(35-37); AEPSPS/CTP1]. Likewise, the GOXv247 gene was fused to the N-terminal chloroplast transit peptide sequence of the small subunit 1A ribulose-1,5-bisphosphate carboxylase gene from Arabidopsis [(38); Arab-SSULAKTPl]. As discussed below, the current literature on transit peptides supports a model whereby the CTP is degraded rapidly and completely by proteases after transport of the precursor protein has occurred. Thus, after a "pre-" protein (containing the CTP amino-terminal extension) reaches the chloroplast or plastid stroma, the CTP is cleaved and degraded (39) leaving only a "mature" protein. This is the basis for our conclusion that the "mature" (not containing the CTP) CP4 EPSPS and GOX proteins are the only introduced proteins present in GTC.

Transport of proteins into chloroplasts, mitochondria, and microbodies has been well-studied (40-42), and the necessity of transit peptides has been demonstrated by experiments showing that precursor proteins lacking transit peptides cannot be imported into chloroplasts (41). In plants, most chloroplast-targeted proteins are synthesized on cytosolic ribosomes as larger precursors containing an amino terminal CTP extension (41). Although different types of membranes and energetics may be involved, the mechanisms for transport of proteins into different organelles appear to be similar. This is especially true for chloroplasts and mitochondria. Chloroplast targeting of proteins is known to be composed of at least two steps: a specific binding of precursor proteins to the surface of the chloroplastic envelope, followed by translocation of the precursor protein across the envelope (43,44). Then, the signal sequence is removed by a signal peptidase, also called a transit peptidase (40).
As of 1991, 260 different transit peptide amino acid sequences were known and available in a database of CTPs (45). Although sequence similarities generally exist among CTPs of the same precursor protein derived from different plant species, few similarities are found among different precursors, even when they are derived from the same plant species (41). Extensive experimental studies do not support the hypothesis that specific transit peptide amino acid sequences have specific essential functions; i.e. the uptake function of the CTP does not appear to reside in the primary amino acid sequence. Rather, the results suggest that the essential feature of a transit peptide is some secondary or higher-order structure (41).

We conclude that the chloroplast transit peptides, like the other transit peptides, are rapidly degraded after cleavage in vivo by cellular proteases. This conclusion is based mainly on analogy with the results obtained with other transport systems (46-49), which are similar to chloroplastic transport. More specifically, we conclude that the chloroplast transit peptides removed from CP4 EPSPS (50,51) and GOX (52) proteins are degraded, and that the only introduced proteins present in line GT73 are the “mature” CP4 EPSPS and GOX proteins.

F. GTC Line GT73: Plasmid Utilized, Transformation, DNA Insert Analysis, and Insert Stability
1. Plant transformation vector, PV-BNGT04
The plant transformation vector used to produce line GT73 (17237-73) was PV-BNGT04. It is a double border vector, and encodes CP4 EPSPS and GOXv247 genes optimized for plant expression. It contains the following elements between the right and left borders which were specifically incorporated into the Westar chromosome.

P-CMoVb/CTP1 GOXv247 E9 3', P-CMoVb/CTP2 CP4 EPSPS E9 3'

The plasmid map is shown in Figure 4, and Table 1 lists all the genetic elements present in PV-BNGT04 and their function with literature references. The same constitutive promoter, P-CMoVb, was used to drive expression of both genes. A chloroplast transit peptide (CTP) was fused upstream of the N-terminus of CP4 EPSPS and GOX to facilitate import of the newly translated protein into chloroplasts (34,53). The Arab-SSU1A/CTP1 (CTP1), fused to the GOX proteins, is a chloroplast transit signal peptide derived from the small subunit of ribulose bisphosphate carboxylase of Arabidopsis thaliana. The CTP1 DNA sequence encodes an 89 amino acid peptide fused to the N-terminus of mature GOXv247. The amino acid sequence of CTP1 contains 2 potential Cys-Met (cysteine-methionine) cleavage sites upstream of the fusion. The AEPSPS/CTP2 (CTP2), fused to CP4 EPSPS, is the A. thaliana EPSPS CTP. The CTP2 DNA sequence encodes a 77 amino acid peptide fused to the
N-terminus of mature CP4 EPSPS. The amino acid sequence of CTP2 contains only one Cys-Met cleavage site at the point of the fusion. Vector PV-BNGT04 (Figure 4) contains bacterial origins of replication and the spc/str selectable marker outside the borders (54). These elements were not transferred to GTC (55). Lastly, well-defined restriction sites are present in PV-BNGT04 (Figure 4). These sites enable characterization of the genetic elements in GT73.

Table 1 is a list of the sequences of each of the genetic elements used to assemble plasmid PV-BNGT04. All of the clonings performed to construct plasmid PV-BNGT04 were done in non-pathogenic E. coli strains derived from E. coli K-12 that are commonly used in molecular biology research (56) (E. coli LE392, JM101, and MM294).

Figure 4. Plasmid map of PV-BNGT04 also referred to as pMON17237.
### Table 1. Summary of the genetic elements in PV-BNGT04.

<table>
<thead>
<tr>
<th>Genetic Element</th>
<th>Function (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right Border</td>
<td>A 25 nucleotide direct repeat that acts as the initial point of DNA transfer into plant cells, originally isolated from pTiT37 (57). Only the DNA segments from the right border clockwise to the left border sequence in Figure 5 was transferred to GT73 (55).</td>
</tr>
<tr>
<td>P-CMoVb</td>
<td>The 35S promoter from a modified figwort mosaic virus (35,36,58)</td>
</tr>
<tr>
<td>Arab-SSU1A/CTP1</td>
<td>The N-terminal of the small subunit 1A of the ribulose-1,5-bisphosphate carboxylase chloroplast transit peptide from <em>Arabidopsis</em> (38).</td>
</tr>
<tr>
<td>GOXv247</td>
<td>The C-terminal of variant number 247 of the glyphosate oxidoreductase (gox) gene isolated from <em>Achromobacter</em> sp. strain LBAA (4,17).</td>
</tr>
<tr>
<td>E9 3'</td>
<td>The 3’ end of the pea rbcS E9 gene which provides the polyadenylation sites for the GOXv247 and CP4 EPSPS genes (59,60).</td>
</tr>
<tr>
<td>AEPSPS/CTP2</td>
<td>The N-terminal chloroplast transit peptide sequence from the <em>Arabidopsis</em> EPSPS gene (35-37).</td>
</tr>
<tr>
<td>CP4 EPSPS</td>
<td>The C-terminal 5-enolpyruvylshikimate-3-phosphate (CP4 EPSPS) gene from <em>Agrobacterium</em> sp. strain CP4 (2).</td>
</tr>
<tr>
<td>Left Border</td>
<td>This was isolated from the octopine Ti plasmid, pTiA6 and contains the 25 bp direct repeat sequence that delimits the T-DNA transferred (61). All DNA located clockwise from the Left Border to the Right Border was not transferred to GT73 (55).</td>
</tr>
<tr>
<td>ori-V</td>
<td>The vegetative origin of replication that permits plasmid replication in <em>Agrobacterium</em>. It was originally isolated from plasmid RK2 and its function in binary plasmid vectors such as PV-BNGT04 has been described (62).</td>
</tr>
<tr>
<td>ori-322</td>
<td>A plasmid replication origin which permits propagation of DNA in bacterial hosts such as <em>E. coli</em>. (63).</td>
</tr>
<tr>
<td>SPC/Str</td>
<td>The bacterial gene encoding the Tn7 AAD 3&quot; adenyllytransferase conferring spectinomycin and streptomycin resistance on bacterial cells that carry the plant vector (64).</td>
</tr>
</tbody>
</table>
2. **Recipient canola variety, Westar**

GTC was selected from plants of the well-known Westar variety of canola (*Brassica napus* L.) (1). Since 1982, this variety has had a history of safe use in the commercial production and breeding of canola. Its pedigree has been published along with 6 year performance data (1). In 1985, Westar was used to plant over 80% of all *B. napus* canola acres in Canada, and has been used as a standard in the Western Canadian Cooperative Rapeseed Test (Co-Op Test) until 1994. Due to the development of varieties that are higher yielding and more tolerant to blackleg disease (*Phoma lingam*), the Westar variety has steadily lost market share to less than 1% in 1993. Because Westar canola has been used commercially since 1982, and has been a standard as well as a source of breeding germplasm for many other registered varieties of canola, there are no safety concerns related to the host plant for GTC. The commercialization strategy for GTC is to use traditional backcrossing and breeding to transfer the glyphosate tolerance locus from this cultivar to a wide range of varieties and maturity groups of canola.

3. **Agrobacterium transformation method**

The disarmed *Agrobacterium tumefaciens* plant transformation system was used to produce GTC (54). This delivery system is well documented to transfer and stably integrate T-DNA into a plant nuclear chromosome (65,66). Vector PV-BNGT04 was mobilized into disarmed *A. tumefaciens* strain ABI and selected on spectinomycin and chloramphenicol. Five to six week-old leaves and buds of Westar canola were used as explant sources, and were infected with the *Agrobacterium* culture. Explants were later placed on glyphosate selection medium. Developing shoots were excised from an R₀ plant, 17237-73 (GT73) (54). The positive shoots were grown to maturity, selfed to produce seed and the resulting progeny plants were screened for glyphosate tolerance (by Roundup herbicide spray test) and gene expression.

4. **Molecular description and stability of the DNA insert in GT73**

GTC line 73 is directly derived from an original R₀ transformant, 17237-73, which was obtained by *Agrobacterium* transformation of the variety Westar with vector PV-BNGT04. This vector (Figure 4) contains two functional segments: the T-DNA, containing the CP4 EPSPS and gox genes bounded by the Right and Left Borders and the plasmid backbone containing the bacterial origins of replication and selectable marker (Figure 4). Analyses performed on DNA derived from leaves of R₃ GT73 plants demonstrate that only a single copy of the T-DNA was inserted into the genomic DNA of Westar at a single location to produce GT73 and that the plasmid backbone sequences, including the bacterial marker gene, were absent from DNA of GT73. PCR analysis was conducted to demonstrate that the border sequences of the T-DNA were the endpoints of the DNA insert as further evidence that only the T-DNA sequences are present in the DNA of GT73. The presence of the single insert was confirmed by inheritance data showing the glyphosate tolerance
phenotype was inherited as a single dominant Mendelian trait. Finally, Southern blot analysis performed on DNA from the R3 and R5 generations showed the same patterns demonstrating structural stability of the inserted DNA (67). Details of these analyses follow.

Figure 5. Schematic diagram of PV-BNGT04 Showing the T-DNA present in GT73.

Only the genetic elements responsible for the glyphosate tolerance proteins and resultant phenotype were detected in GT73 (55). This insert (Figure 5) contains the CMoVb promoter, the Arabidopsis small subunit CTP, the gox variant gene, the pea E9 3' terminator, a second copy of the CMoVb promoter, the Arabidopsis EPSPS CTP, the CP4 EPSPS gene, and a second copy of the pea E9 3' terminator. Therefore, the only proteins encoded by PV-BNGT04 DNA present in line GT73 are the CP4 EPSPS and GOX variant proteins. This conclusion was drawn from the following types of molecular data obtained using the hybridization probes and DNA fragments shown in Figure 6: 1) the positive detection of the two CMoVb and two E9 3' fragments and fragments containing the CP4 EPSPS and gox genes by Southern analysis; 2) the lack of ori-322 and ori-V signals by Southern analysis; and 3) the lack of PCR fragments produced from a pair of PCR primers, one of which is located within the T-DNA paired with one located just beyond either of the border sequences.
Figure 6. Schematic linear diagram of PV-BNGT04. This diagram shows the probes used to generate the Southern blot data and the predicted size of that genetic element if it were present in GT73 DNA.

Diagram is not to scale.

The boxes under each genetic element represent the probe used to identify that element.

The lines with the numbers under them indicate the size band observed on the Southern blots when the genomic or plasmid DNA is cut with EcoRI and probed with the DNA indicated above the line. RB - Right Border; LB - Left Border.

Using the PCR method, the ends of the inserted DNA of GT73 have been mapped. It was concluded that the Right Border end of PV-BNGT04 DNA incorporated into the GT73 genome falls between nucleotide 9188 and nucleotide 9212 (Figure 4) (55). The Left Border end of the PV-BNGT04 DNA falls between nucleotide 3975 and nucleotide 3998 (Figure 4) (55). To illustrate, the method for analysis of the Left Border follows.

**Left border**: The Left Border sequence is located from map position 3975 to 3998 (Figure 4). Primers #5 and #6 are both located within the T-DNA and would be predicted to produce a product when used in a PCR experiment with GT73 genomic DNA as template. Primer #7 is located just before the Left Border sequence. When primers #5 and #7 are used in the PCR experiment with GT73 genomic DNA as template, a product may or may not be produced depending on whether the end of the T-DNA occurs before the beginning of the Left Border sequence or not. Primer #8 is located outside the T-DNA beyond the border and when used in a PCR experiment with GT73 genomic DNA as template paired with primer #5, no product would be expected to be produced if transfer terminated at the Left Border. Below are the primers that were used in the characterization of the Left Border area. A schematic diagram follows.
Primer #5. 3404 to 3425 >
CCATTATGCTATTACACTAGGC
This primer is located at the 3' end of the E9 3' element.

Primer #6. 3656 to 3635<
CGATTTGATCTAGTTATGCAATC
This primer lays just 3' of the E9 3' element.

Primer #7. 3963 to 3941 <---
CCGGAATACTACATGGAATCACGC
This primer is just 5' of the beginning of the left border sequence and ends 3' of the E9 3' sequence.

Primer #8. 4065 to 4044
GCTAACCAGCTACGTACG <---
This primer starts and ends 3' of the left border sequence.

Using PV-BNGT04 DNA as template, the primers should give the following size products: 5 + 6 = 252bp; 5 + 7 = 559bp; and 5 + 8 = 661bp. If the functionality of the Left Border has been maintained, no product should be produced when using GT73 DNA as template with primers 5 + 8. The results of the PCR using the above primer combinations with PV-BNGT04, GT73, GT200 (another GTC line), and Westar templates, are shown in Figure 7. The PV-BNGT04 template shows products of 252bp, 559bp, and 661bp as predicted. GT73 has a product of 252bp with primers 5 + 6 and a product of 559bp with primers 5 + 7. GT200 has a product of 252bp with primers 5 + 6. Neither GT73 nor GT200 DNAs has a product with primers 5 + 8. No bands are observed in the Westar control with any of the combinations of the primers. These results establish that integration of the plasmid DNA did not proceed outside of the Left Border.
**Figure 7. PCR Analysis of the Left Border in Canola Lines GT73 and GT200 (another GT line).** Genomic DNA from Canola Westar control, lines GT73 and GT200 were analyzed by PCR to determine the integrity of the left border. The positive control was pMON17209 plasmid DNA which is equivalent to PV-BNGT04 in that it also contains the E9 3' adjacent to the Left Border and plasmid backbone sequences.

Four oligonucleotides were used in this analysis:

- **Primer 5:** CCATTATGTTATCTAGGCC (22 mer) [3404 to 3425 base pairs in Figure 4].
- **Primer 6:** CGATTGATGCTGGTGTCAATC (22 mer) [3656 to 3635 base pairs in Figure 4].
- **Primer 7:** CCGGGAAATCTACATGGATCAGC (23 mer) [3963 to 3941 base pairs in Figure 4].
- **Primer 8:** GCCTAACCGCTCAGTTCTCG (22 mer) [4065 to 4044 base pairs in Figure 4].

The predicted product sizes are shown below:

- **Primer 5 + primer 6 = 252bp.**
- **Primer 5 + primer 7 = 559bp.**
- **Primer 5 + primer 8 = 661bp.**

Lanes 1, 2, and 3 have pMON17209 plasmid DNA as template. Lanes 4, 5, and 6 have genomic Westar control DNA as template. Lanes 7, 8, and 9 have genomic GT73 DNA as template. Lanes 10, 11, and 12 have genomic GT200 DNA as template. Lanes 1, 4, 7, and 10 have primers 5 + 6. Lanes 2, 5, 8, and 11 have primers 5 + 7. Lanes 3, 6, 9, and 12 have primers 5 + 8.

Reactions were done in 100μl total volume containing 100pg of each primer, 500ng template DNA (50 ng plasmid DNA), dNTP's at 200 μM, 10 units of Taq Polymerase (Perkin-Elmer Norwalk, CT). The PCR amplification cycle consisted of 94°C denaturation for 1.5 min, 65°C annealing for 1.5 min., and a 72°C extension for 1.5 min. The cycle was repeated 24 times. Products were separated on a 3% agarose gel and visualized by ethidium bromide. The lower bands at the bottom of the gel are unused oligonucleotides.
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Figure 8. Southern blot analysis of R₃ and R₅ generation DNAs. Digestions with EcoRI and probed with CP4 EPSPS, GOX, or E9 3'.

The pMON17237 plasmid DNA (lane 1 in all panels) was digested with EcoRI. Canola Westar control genomic DNA (lane 2 in all panels), line GT73 R₃ genomic DNA (lane 3 in all panels), and GT73 R₅ genomic DNA (lane 4 in all panels) were digested with EcoRI. Each lane represents 100 pg plasmid DNA or 5 µg (10 µg in Panel C) of genomic DNA. The digests were subjected to electrophoresis in a 0.8% agarose gel and transferred to a nylon membrane. The membranes were probed with ³²P labelled CP4 EPSPS coding region for Panel A, ³²P labelled GOX coding region for Panel B, or ³²P labelled E9 3' region for Panel C and then subjected to autoradiography.

* = bands common to all three DNAs.
Stability of Inserted DNA - Southern blot analysis was performed on DNA from the R₃ generation and R₅ generation of GT73. Both showed the same patterns demonstrating structural stability of the inserted DNA (67). The results of a Southern blot analysis after EcoRI digestion of Westar and GT73 R₃ generation or R₅ generation DNAs (Figure 8) exhibits identical banding patterns showing physical stability of the inserted and surrounding canola genomic DNA. The physical stability is consistent with inheritance data in F₂ progenics of crosses between other canola lines and GTC line GT73 consistently segregate 3 tolerant to 1 sensitive, establishing that the GT73 insert behaves as a single dominant gene inherited in a Mendelian fashion (68). The glyphosate tolerance phenotype and Mendelian transmission have been consistent for every generation of line GT73 canola tested to date which spans more than five generations.

G. Field Testing History
Tests with GTC have been conducted in 4 provinces in Canada for the past 3 years (1992-4) at approximately 10 locations per year and 1-6 trials per location. All field trials of genetically engineered canola in Canada have been conducted under the approval and supervision of the Agriculture and Agri-food Canada, Plant Industry Directorate.

Previously approvals for testing (as of September 1994):
- 1992 Agriculture Canada approvals of field trials with transformed canola containing glyphosate (Roundup) herbicide tolerance (reference letter 3625-6-9M1, 29 April 1992).
- 1993 Agriculture Canada approvals of field trials with transformed canola containing glyphosate (Roundup) herbicide tolerance (reference letter 3625-6-9M2, 5 May 1993).
- 1994 Agriculture Canada approvals of field trials with transformed canola containing glyphosate (Roundup) herbicide tolerance (reference letter 3625-6-9M1, 29 April 1994).

GTC line GT73 has also been field tested at sites in the United States (USDA permits 92-049-02, 93-060-03 and 94-055-04R), South America and Europe. Data collected from these trials (including yield, agronomic characteristics, vigor, disease and insect susceptibility), literature references, and expert opinion letters demonstrate that the GTC line GT73: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than the non-modified parental varieties; 3) has not revealed any potential to increase the weediness of any other cultivated plant or native wild species; 4) does not negatively impact processed agricultural commodities; and 5) has not revealed any potential to harm other organisms that are beneficial to agriculture. Therefore, the Agricultural Group of Monsanto Company provided information on the environmental safety of the GTC lines and requested from Agriculture and Agri-Food Canada, under their authority from the Seeds Act and Plant
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Protection Act, permission for unconfined environmental release of the GT73 line in Canada on November 15, 1994.

H. Canola-based Products and Human/Animal Consumption
To design a relevant food/feed safety assessment program for GTC, it was crucial to understand the uses of canola. Based on an understanding of this information, combined with the extensive safety assessment data summarized here, we conclude that the presence of the CP4 EPSPS and GOX proteins and the accompanying glyphosate tolerance trait in GTC will not change the commercial utility or safety of canola and canola products. Summarized below are the key aspects of canola food and feed utilization.

1. Canola processing and end uses
Canola today is a major export crop for Canada and is second only to wheat as a crop revenue producer. With its superior fatty acid composition, canola oil is well suited for human consumption. Canola oil contains a very low level of saturated fatty acids, which have been shown to increase blood cholesterol levels; a high level (58 percent) of the monounsaturated fatty acid, oleic acid, which has been shown to reduce serum cholesterol levels; a moderate level (26 percent) of linoleic acid, and an appreciable amount (10 percent) of alphalinolenic acid (69). Linoleic acid and alpha-linolenic acid are fatty acids that are essential to human health and must be supplied in the diet. Not only is canola oil an excellent product, but canola meal, with its low levels of glucosinolates, is a good source of protein for animal nutrition (69-71). Canola is derived from oilseed rape which was not suitable for human or animal nutrition due to high levels (>50%) of erucic acid (C22:1) and glucosinolates. The processed oil derived from rapeseed was used extensively as an industrial lubricant (72) particularly for marine engines. High erucic acid rapeseed (HEAR) oil has been shown to have cardiopathic potential in experimental animals. Canola oil is defined to contain less than 2% erucic acid and lacks cardiopathogenicity except in certain strains of rats (80). Also, it is believed that the high levels of glucosinolates made the meal unsuitable for use in animal nutrition because of antinutritional, goitrogenic, reproductive, and palatability problems (73,74). Only after years of breeding rapeseed varieties to canola quality (<30 μmole/g defatted meal of alkyl glucosinolates and <2% erucic acid), have the meal and oil become accepted as an animal feed and for human nutrition, respectively. Further discussion of erucic acid and glucosinolates is presented in Sections IV.A.1.b.v. and vi.

In calendar year 1992 Canada planted nearly eight million acres (7.89 million) and produced 3.68 million tonnes of canola (75). These figures are consistent with the five-year averages (1988-1992) of 7.65 million acres and 3.72 million tonnes of canola.

Once harvested, canola is delivered to grain companies, farmer-owned
cooperatives or crushing plants. The canola is graded according to standards set by the Canadian Grain Commission. These grades range from the most superior grade, Canada No. 1, to a Sample grade.

The first step in producing canola oil and meal is oil extraction. Rolling or flaking the seed fractures the seed coat and ruptures the oil cells. Canola seed contains greater than 40 percent oil (69). To extract this oil, the seed is flaked and then cooked at 75-85°C (25). The cooking deactivates the endogenous enzyme myrosinase which catalyzes the breakdown of glucosinolates (24,25). Cooking also ruptures any intact cells that remain after the flaking compresses the tiny flakes into cake fragments. These cake fragments are then solvent extracted to remove most of the remaining oil. Heat treatment of the meal, toasting, is important for removing volatile components which often are toxicants (25). The solvent is also removed from the oil fraction which subsequently undergoes a degumming process and produces a semi-refined oil (24,25).

The oil is refined and chlorophyll pigments and other minor impurities are removed with bleaching clays (76). The final steps of canola oil processing depend on the end product, i.e., salad oils, margarines, etc. The cake fragments remaining after oil extraction are further processed and dried, producing a high protein feed supplement for livestock and poultry (69,71).

Canola is exported from Canada in three forms: seed, meal, and oil. Production from crop year (August 1 to July 31) 1991-92 measured 4.2 million tonnes of canola seed; 742,000 tonnes of canola oil; and one million tonnes of canola meal (75). Exports from crop year 1991-92 measured 1.89 million tonnes of canola seed; 285,000 tonnes of canola oil; and 740,000 tonnes of canola meal (75). Japan is Canada's major export market for canola seed, accounting for approximately half of Canada's production. Japan imported 1.82 million of the 1.89 million tonnes of 1991-92 crop year exports (seed) (75). In fact, canola oil production in Japan now exceeds soybean oil production (69). The United States continues to lead the list of Canada's canola oil export markets. For the 1991-92 crop year, Canada exported 285,000 tonnes of oil, of which the United States imported 232,000 tonnes. In addition, the United States imported 545,000 of Canada's 740,000 tonnes of exported canola meal. Japan is Canada's second largest importer of meal, importing 119,000 tonnes of canola meal for 1991-92 crop year (75).

2. Animal consumption
The primary use of canola protein is as a heat-processed meal for supplementation of animal feeds. Canola meal has major uses in livestock feed, poultry feed, pet food, fish food, and is also used as a fertilizer (69). Canola meal is regarded as an good source of the sulfur amino acids methionine and cystine in formulating animal diets (71).
3. Human consumption
Currently, edible oil is the sole product from canola with direct human consumption. Canola oil, which contains negligible quantities of protein (77), is extensively used in the food industry in products such as cooking and salad oils, salad dressings, shortening, and oleo margarine. Canola oil is currently a major edible oil used in Canada, Japan, and the U.S. (69).

IV. SAFETY ASSESSMENT OF NEW VARIETIES
Detailed below is a summary of the safety assessment studies performed on GT73. The safety assessment program was designed to address the specific questions detailed in the applicable FDA Food Policy flowcharts (5), and the data will be discussed accordingly. The pathway leading to “no concern” for GTC is highlighted with bold arrows in the flowcharts reproduced below.

A. Absence of Unexpected or Unintended Effects
1. Safety assessment of the host plant, canola
Shown in Figure 9 is the flowchart from the FDA Food Policy concerning the safety assessment of the host plant.

a. Canola
Canola is the name given to varieties of rapeseed bred to contain < 30 μmol glucosinolates/g defatted meal and <2% erucic acid in the final oil (69). The name is a trademark owned by the Canola Council of Canada.

Due to a critical shortage of rapeseed oil in World War II, the Canadian government initiated an aggressive program to produce large quantities (78). Two species, *Brassica napus* or “Argentine” and *B. campestris* or “Polish”, have been developed over the years into canola quality, and the yearly Canadian production is approximately a 1:1 mix of these two species. Several US Federal Agencies have identified standards for canola grain, meal and oil in their regulation. Additionally, the FDA has granted canola oil the classification of “Generally Regarded as Safe” (21 CFR§ 184.1555).

The safe use of canola has been well characterized. The characteristics of canola, or the specific progenitor line, therefore do not warrant analytical or toxicological tests beyond analyses for the antinutrients: glucosinolates and erucic acid. Typically, canola breeders make genetic crosses to generate new cultivars with enhanced commercial value, and they evaluate new varieties based on yield, disease susceptibility (*Phoma lingam*), as well as protein and oil content. However, the FDA Food Policy (5) recommends that key compositional components of genetically-modified plant varieties be assessed prior to commercial introduction. Monsanto has therefore performed extensive analytical studies to compare the compositional quality of GT73 to the
Safety Assessment of Glyphosate-tolerant Canola

Figure 9. Safety assessment of new varieties: the host plant (taken from Food Policy, Figure 2) (5). The pathway leading to “no concern” for GTC is highlighted with bold arrows.

New canola varieties are not typically subjected to extensive analytical or feeding tests. However, compositional analyses to verify levels of nutrients and antinutrients, as well as feeding studies to ensure the wholesomeness of GTC line GT73, were performed as discussed in the Food Policy.

b. Compositional analyses of GT73 seeds
Analyses show that GTC seeds are not materially different from other canola in essential nutrients or antinutrients (77,79). The strategy taken for measurement of compositional parameters was to focus on the raw agricultural commodity, the canola seed. Extensive analyses were performed
on a single sample each of toasted meal and refined, bleached, deodorized (RBD) oil to verify that processing of GTC yielded products that were consistent with those produced from other canola varieties. It is reasonable to infer that if the GTC seeds are not materially different from parental control canola seeds, then meal and oil derived from the GTC seeds will also not be materially different from those derived from parental control seeds. In order to provide test material for these analyses, GT73 and the parental variety, Westar were generated in field trials conducted in Canada over two years. The first test was conducted in 1992 (77) at 7 locations distributed across the primary canola production areas (Manitoba, Saskatchewan, and Ontario). A trial had been initiated in Alberta, but was destroyed due to a hail storm. Seed from these trials were sent to the laboratories of Ag Canada Research Station in Saskatoon for analysis of oil, protein, chlorophyll, sinapines (total choline esters), fatty acid and glucosinolate compositions. This lab was chosen because of their experience in analysis of canola and since they generated all the data for the new canola variety registration test (Co-Op Test). Additionally, these researchers (Drs. Keith Downey, Philip Raney, Gerhard Rakow, and Ian McGregor) have extensive experience in the development and analysis of Westar canola (1). Samples of seed were also transported under USDA APHIS permit to St. Louis for analysis of protein expression levels at the Agricultural Group of Monsanto Company (MAG), and for proximate and amino acid compositional analyses at Ralston Analytical Laboratories (RAL) in St. Louis, Missouri. The latter two laboratories conducted all analyses under US EPA GLP compliance.

The 1993 field trial (79) was also conducted in Canada at 4 locations in the provinces of Manitoba and Saskatchewan. Seed was shipped to Ag Canada for analyses as in 1992 with the exception of chlorophyll analysis which was now conducted at the Grain Research Laboratory (GRL) in Winnipeg, Manitoba. Seed of GT73 and Westar were again shipped to St. Louis under USDA APHIS permits for the same analyses conducted under GLP at MAG and RAL as in 1992.

Initially no data were analyzed statistically since the Westar genotype is known to be heterogeneous (see Appendix C), and we could establish substantial equivalence using the extensive database that exists for Westar from several years of official trials required for registration of all new canola varieties (Co-Op Tests). However, upon request from Ag Canada Feeds Division, we have statistically analyzed the key nutritional components in the seed: protein and oil values generated at Ag Canada; protein, oil, fiber, ash, and gross energy determined at RAL, the aromatic amino acids phenylalanine, tyrosine and tryptophan both on a per seed and per unit protein basis. We determined at the 95% confidence levels that the alkyl glucosinolates and erucic acid levels will not exceed the established limits of 30 μmoles/g defatted meal and 2% of the oil composition, respectively. These results are
summarized below, and demonstrate that there is no material difference between GT73 and Westar in composition.

i. Proximate analysis:
Proximate analyses were performed at RAL on the canola seeds from GT73 and the Westar canola (see Appendix A for additional information on the analytical methods used). The results from 1992 and 1993 (77,79), expressed on a dry-weight basis, are shown in Figures 10 and 11. Components measured were protein, fat, moisture, fiber, and ash, and, with the exception of moisture, are reported on a dry weight basis. The carbohydrate content was derived by calculation. The data summarized in Figures 10 and 11 establish that the levels of these components in GT73 are not materially different from the levels in Westar. Furthermore, there was no statistically significant difference between GT73 and Westar (p=0.05) for any component in either year.

Figure 10. Proximate analysis of canola seeds. Bars represent the means of seeds from single plots at seven field sites, and the thin lines represent the ranges of experimental values obtained.
Figure 11. Proximate analysis of canola seeds from second year field tests. Bars represent the means of seeds from four field sites, and the thin lines represent the ranges of experimental values obtained.

ii. Ag Canada analyses:
Protein and oil analyses were also conducted at Ag Canada Research Station in Saskatoon in 1992 and 1993 (77,79). This was done to ensure that GT73 seed would be directly comparable to a much larger data set of values for Westar obtained from the Co-Op Test. The larger data set was needed to better estimate the variation observed in the Westar genotype. The results, depicted in Figure 12, show no meaningful difference between GT73 and Westar in fat content (whole seed, dry wt. basis) and protein (defatted meal basis). Statistical analysis was conducted on these data and two significant differences were noted. In 1992, the protein value in GT73 was significantly higher compared to Westar; while, in 1993, the fat level was significantly higher in GT73 than in Westar. Since these results were not consistently noted in the proximate analyses or from year-to-year, they may be due to random variation and these values are within the range reported for canola.
iii. Amino acid analysis:

No meaningful differences were detected over two years of amino acid compositional analyses conducted on a per dry weight of seed and per protein basis for GT73. Figures 13 and 14 depict the results of analysis of 18 individual amino acids in 1992 and 1993 (77,79), respectively. These data establish that there are no meaningful difference between GT73 and Westar in terms of amino acid composition. Upon statistical analysis of the levels of phenylalanine, tyrosine, and tryptophan, the tryptophan level in GT73 was
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significantly lower (p=0.05) on both a per seed dry weight and per protein basis in 1993. The actual values for all the aromatic amino acids in both years are listed in Tables 2a and 2b. Since the level of tryptophan was not statistically significantly different in the 1992 field test and in 1993 was within the range for Westar as well as other canola varieties, this difference was not considered meaningful.

Figure 13. Amino acid analysis of canola seed in 1992. Bars represent the means of seeds from four field sites, and the thin lines represent the ranges of experimental values obtained.
Figure 14. Amino acid analysis of canola seeds in 1993. Bars represent the means of seeds from four field sites, and the thin lines represent the ranges of experimental values obtained.
Table 2a. Values of the aromatic amino acids in GT73 and Westar on a per seed and per protein basis from 1992.

<table>
<thead>
<tr>
<th>amino acid (per seed basis(^1))</th>
<th>GT73</th>
<th>Westar</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenylalanine</td>
<td>0.97</td>
<td>0.93</td>
</tr>
<tr>
<td>range</td>
<td>0.91 - 1.03</td>
<td>0.79 - 1.01</td>
</tr>
<tr>
<td>tryptophan</td>
<td>0.25</td>
<td>0.24</td>
</tr>
<tr>
<td>range</td>
<td>0.23 - 0.27</td>
<td>0.21 - 0.26</td>
</tr>
<tr>
<td>tyrosine</td>
<td>0.65</td>
<td>0.62</td>
</tr>
<tr>
<td>range</td>
<td>0.60 - 0.69</td>
<td>0.52 - 0.69</td>
</tr>
</tbody>
</table>

(values are given as g/100 g of seed on a dry weight basis, n=3 in 1992 and n=4 in 1993.

Table 2b. Values of the aromatic amino acids in GT73 and Westar on a per seed and per protein basis from 1993.

<table>
<thead>
<tr>
<th>amino acid (per seed basis(^1))</th>
<th>GT73</th>
<th>Westar</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenylalanine</td>
<td>0.88</td>
<td>0.91</td>
</tr>
<tr>
<td>range</td>
<td>0.82 - 1.01</td>
<td>0.86 - 1.01</td>
</tr>
<tr>
<td>tryptophan</td>
<td>0.24 ((^*))</td>
<td>0.26</td>
</tr>
<tr>
<td>range</td>
<td>0.23 - 0.28</td>
<td>0.24 - 0.29</td>
</tr>
<tr>
<td>tyrosine</td>
<td>0.59</td>
<td>0.62</td>
</tr>
<tr>
<td>range</td>
<td>0.56 - 0.66</td>
<td>0.59 - 0.67</td>
</tr>
</tbody>
</table>

(values are given as g/100 g of protein, n=3 in 1992 and n=4 in 1993.

* Significantly different from the value for Westar (p=0.05)

iv. Fatty acid analysis:
The fatty acid composition of oil extracted from seeds was measured in both GT73 and Westar in 1992 and 1993 (77,79). The means and ranges of the most abundant fatty acids are presented in Figures 15 and 16. The values for all fatty acids were compared with values obtained from the Westar control as well as data obtained for Westar from the Co-Op Test for each particular year. The data clearly establish that there are no meaningful differences between GT73 and Westar in terms of their fatty acid composition. Based on these same data, Monsanto received a letter of no objection for food use of the oil derived from GTC (Appendix B)
Figure 15. Fatty acid analysis of canola seeds from 1992. Bars represent the means of seeds from 7 field sites, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low values for Westar from the official Co-Op trials in 1992.

A.
B. 1992 Fatty Acid Composition Results

- GT73 (MEAN)
- Co-Op Highest Value
- Co-Op Lowest Value
- WESTAR (MEAN)
- Range of Experimentally Determined Values
Figure 16. Fatty acid analysis of canola seeds from 1993. Bars represent the means of seeds from four field sites, and the thin lines represent the ranges of experimental values obtained in 1993. Triangles represent high and low values for Westar from the official Co-Op trials in 1993.
v. Erucic Acid:
Erucic acid is a mono-unsaturated, 22-carbon fatty acid (C22:1) which is a natural constituent of rapeseed. High erucic acid rapeseed (HEAR) oil has been shown to have cardiopathic potential in experimental animals. Canola oil, which is defined by less than 2 percent erucic acid, is free of cardiopathogenicity except in certain strains of rats (80). This improvement coupled with its low saturated fatty acid content has led to the wide use of canola oil in human nutrition. We have closely monitored the level of erucic acid in oil from GT73 to ensure the quality of the human food product. Data obtained in 1992 and 1993 (77,79) on the levels of erucic acid have been statistically analyzed to ensure, 95% confidence level, that they will not exceed the 2% maximum level allowed in the oil. The means and ranges of values for erucic acid in GT73 were: 0.24% (0.1 to 0.5, n=7) in 1992, and 0.04% (0 to 0.1%, n=4) in 1993. As is evident from the data for GT73 over two years, the levels of erucic acid are well below the limits allowed for human use and
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equivalent to the Westar control.

vi. Glucosinolate analysis:
Canola is one example of the Brassica crops of the crucifer family which are known to produce glucosinolates. To date, over 100 structural types of glucosinolates have been identified. All are derived biosynthetically from amino acids (81). The key structural features are the sulfonated oximes which is in a syn orientation to a thioglycoside moiety and an aliphatic, aromatic, or heteroaromatic sidechain which is always oriented anti to the sulfate group.

In canola (B. napus and B. rapa) breeding programs and variety registration tests, 9 unique glucosinolates are closely monitored (Table 3). Of these, only two account for >70% of the total glucosinolates measured.

Table 3. List of commonly detected glucosinolates in canola.

<table>
<thead>
<tr>
<th>Structure name</th>
<th>Trivial Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>allylglucosinolate</td>
<td>sinigrin</td>
</tr>
<tr>
<td>but-3-enylglucosinolate</td>
<td>gluconapin</td>
</tr>
<tr>
<td>2-hydroxybut-3-enylglucosinolate</td>
<td>progoitrin</td>
</tr>
<tr>
<td>pent-4-enylglucosinolate</td>
<td>glucobrassicanapin</td>
</tr>
<tr>
<td>2-hydroxypent-4-enylglucosinolate</td>
<td>napoleiferin</td>
</tr>
<tr>
<td>4-methylthiobutylglucosinolate</td>
<td>glucorucin</td>
</tr>
<tr>
<td>5-methyliophenylglucosinolate</td>
<td>glucobrassicin</td>
</tr>
<tr>
<td>4-hydroxyindol-3-ylmethylglucosinolate</td>
<td>4-hydroxyglucobrassicin</td>
</tr>
<tr>
<td>Indol-3-ylmethylglucosinolate</td>
<td></td>
</tr>
</tbody>
</table>

* One of the glucosinolates which accounts for >70% of the total in canola.

Glucosinolates are closely monitored due to reported antinutritional properties (81,82), and the sum concentration of four (but-3-enyl, 2-hydroxybut-3-enyl, pent-4-enyl, and 2-hydroxypent-4-enyl) must be less than a total of 30 μmole per grams of oil-free meal for the seed to be classified as canola quality (69). The indol glucosinolates now account for approximately 50% of the glucosinolates in canola meal as a result of the success achieved by rapeseed breeders in reducing the levels of the aliphatic glucosinolates. They have been exempt from standards since they have only recently been discovered, and there is no evidence to merit a concern given their levels in meal. Within a few years, both Europe and Canada will change the definition of low glucosinolate rapeseed/canola to 20 μmole/g seed. This will include the levels of the indol glucosinolates. The standard for glucosinolates was arbitrary; it was established as a target for plant breeders to use in their rapeseed breeding programs and not for any reasons of safety.

Glucosinolates become goitrogenic upon hydrolysis by myrosinase, and enzyme localized within Brassica seed cells. When the seed is crushed, the enzyme acts upon the glucosinolate to yield isothiocyanates, thiocyanates, and
possibly nitriles, depending upon moisture and temperature conditions. However, in commercial processing a cooking step inactivates myrosinase leaving glucosinolates intact (74,76). Some destruction and reduction of glucosinolates normally occurs in a desolventizing-toaster phase of commercial crushing by the prepress solvent method (76). Intestinal microflora may achieve some hydrolysis of glucosinolates and it is largely for this reason that nutritionists have encouraged plant breeders to work toward the elimination of glucosinolates. In general, it is agreed that present canola levels of glucosinolates in meal are of minor concern compared to fiber levels which depress the digestible and metabolizable energy levels (83). Numerous feeding studies with high and low glucosinolate varieties of rapeseed in swine, cattle, poultry, and rats have noted a correlation between toxic effects (as indicated by growth performance, reproduction, goitrogenicity, liver hypertrophy and hemorrhage, and palatability) and the levels of glucosinolates in the meal (74). Based on studies where canola meal was incorporated into test diets at varying levels, recommendation for inclusion into animal rations have been established (71,84). Any assessment of canola meal in animals has been complicated by a poor understanding of the specific effect the toxicants (glucosinolates and derived metabolites), other components of canola (tannins, phytates, and sinapines), and the metabolizable and digestible energies of the meal have on the growth parameters measured.

Because of the importance of maintaining canola quality, GT73 samples were analyzed for these glucosinolates using standard methods of the Co-Op Test in both 1992 and 1993 (77,79). The results of these analyses were compared to the commercial limits and to values for Westar from the Co-Op Test (n=13). Means determined for the total alkyl and indolyl glucosinolates are plotted in Figure 17 for both years. While it is apparent that the average level of alkyl glucosinolates in GT73 is consistently higher than the mean value for Westar, all individual values are well below the 30 µmole limit. In addition, statistical analysis was conducted on these data and demonstrate that, at the 95% confidence level, the values of glucosinolates in GT73 will not exceed the 30 µmole limit. To gain better insight into the variation in the level of alkyl glucosinolates in the Westar variety, we asked Dr. [Name] of Ag Canada, a leading canola breeder and developer of the Westar variety, to look at our data. He concluded that the variation observed is expected for a line, such as GT73, which was selected from the Westar genotype (see letter from Dr. [Name] in Appendix C). It is also important to note that, while this value of alkyl glucosinolates in GT73 may be on the higher side of the range observed for Westar, it is well below the harvest survey value (17 µmol/g in 1992 and 14 µmole/g in 1993) for commercially produced No. 1 Canada canola (85,86).

We conclude that there is no meaningful difference between GT73 and Westar canola based on levels of glucosinolates. Furthermore, the levels of the alkyl
glucosinolates are well below the limits established for the safe use of meal derived from canola seed as an animal feed.

**Figure 17. Glucosinolates in canola seeds in 1992 and 1993.** Bars represent the means of seeds from seven field sites in 1992 and four field sites in 1993. The thin lines represent the ranges of experimental values obtained. Triangles represent high and low values for Westar from the official Co-Op trials in each year.

![Graph showing glucosinolates in canola seeds]

vii. *Sinapine analysis.* Sinapine is used here as a general term for a family of choline esters naturally occurring in canola. Their impact to the poultry feed industry is significant since sinapines are known to render an off-odor to chicken eggs (87). These analyses were performed on samples from both 1992 and 1993 (77,79), and the results (reported in mg/g defatted meal) are depicted in Figure 18.

The data establish that there are no meaningful difference between GT73 and Westar based on the levels of choline esters in the seed.
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Figure 18. Sinapine analysis of canola seeds in 1992 and 1993. Bars represent the means of seeds from nine field sites in 1992 and four field sites in 1993. The thin lines represent the ranges of experimental values obtained.

<table>
<thead>
<tr>
<th>mg/g defatted meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
</tr>
<tr>
<td>18</td>
</tr>
<tr>
<td>16</td>
</tr>
<tr>
<td>14</td>
</tr>
<tr>
<td>12</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>


GT73 (MEAN)       WESTAR (MEAN)

Range of Experimentally Determined Values

c. Compositional analyses of GT73 processing fractions

Two processing fractions are currently used from commercial production of canola. Toasted meal is used extensively as a feedstuff for livestock, poultry, hogs and fish (69,71,84). Because of the levels of glucosinulates, it is not used for human nutrition (81,82). Canola meal differs from soybean meal in that it contains lower levels of total protein and certain essential amino acids, but it is a good source of sulfur amino acids (70). The processed, toasted meal fraction is currently the only commercial product from canola used in animal feed (69). The second fraction is refined, bleached, deodorized (RBD) oil which is widely used in human nutrition because of its favorable fatty acid composition (69).

Both toasted meal and RBD oil fractions were manufactured from the GT73 and the Westar canola at Protein Oil and Starch (POS) Pilot Plant Corporation of Saskatoon, Saskatchewan in 1993 (79). The toasted meal was analyzed for proximate analysis (% protein, fat, fiber, ash, moisture, carbohydrate and energy by calculation); glucosinolate, amino acid and mineral composition; phytic acid and nitrogen solubility. The RBD oil was evaluated to ensure that
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it met the CODEX specification (88) which include a series of 18 analyses that define canola oil. All analyses except for glucosinolates in toasted meal were conducted at RAL under GLP. The results, which are tabulated or graphed below, show that there are no meaningful differences between processed fractions from GT73 and canola that is currently used in commerce.

i. Proximate analysis and nitrogen solubility of toasted meal
The results of proximate and nitrogen solubility analyses for toasted meal derived from GT73 and Westar seed are tabulated in Table 4. Since they are based on analysis of single samples, no statistical analysis can be performed. However, the values from GT73 and Westar are in excellent agreement with each other as well as with literature values (71,89). These results establish that there is no meaningful difference between meal from GT73 and meal derived from Westar canola based on proximate and nitrogen solubility.

### Table 4. Summary of proximate analysis of GT73 meal

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Westar</th>
<th>GT73</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein, % DW</td>
<td>41.3</td>
<td>41.4</td>
</tr>
<tr>
<td>ash, % DW</td>
<td>7.24</td>
<td>7.33</td>
</tr>
<tr>
<td>moisture, %2</td>
<td>10.8</td>
<td>10.0</td>
</tr>
<tr>
<td>fat, % DW</td>
<td>4.5</td>
<td>3.4</td>
</tr>
<tr>
<td>fiber, % DW</td>
<td>14.5</td>
<td>14.6</td>
</tr>
<tr>
<td>carbohydrates, %</td>
<td>47.0</td>
<td>47.8</td>
</tr>
<tr>
<td>calories, kcal/100 g4</td>
<td>332</td>
<td>330</td>
</tr>
<tr>
<td>nitrogen solubility5</td>
<td>28.3</td>
<td>29.1</td>
</tr>
</tbody>
</table>

Dry weight (DW) basis:
1. Analysis on composite sample from 4 sites.
2. This is the equilibrium moisture value.
3. Carbohydrates determined by subtraction method.
4. Calories are calculated from the protein, fat, and carbohydrate values.
5. Nitrogen solubility determined from water extraction at neutral pH. No literature database is available. The value for nitrogen solubility is dependent on the processing operation (90).

ii. Amino acid analysis of toasted meal
Amino acid composition is important for the use of canola meal as a protein source in a diet formulation. We have therefore determined the amino acid composition of GT73 meal, compared it to Westar, and established that there is no meaningful difference between the two. The results, which are given on a per protein basis, are depicted in Figure 19. We have also compared these results to literature ranges (71,84,89,91,92) and shown that the amino acid composition of meal from GT73 is within the variation seen in commercial canola meal (89). This comparison with literature is given below in Table 5.
Figure 19. Amino acid compositional analysis of toasted meal from GT73. Values are from single analysis of a single toasted meal sample. All analyses were conducted at RAL.

1993 Amino Acid Composition in Meal

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/100g protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>arginine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aspartic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cystine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glutamic acid</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>glycine</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>histidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>isoleucine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leucine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lysine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>methionine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phenylalanine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>threonine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tryptophan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tyrosine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>valine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each amino acid value is from a single sample.
Table 5. Summary of amino acid analysis of GT3 canola meal

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Westar</th>
<th>GT3</th>
<th>Literature Range²</th>
</tr>
</thead>
<tbody>
<tr>
<td>alanine</td>
<td>1.83</td>
<td>1.86</td>
<td>1.71-2.05</td>
</tr>
<tr>
<td>arginine</td>
<td>2.36</td>
<td>2.42</td>
<td>2.32-2.93</td>
</tr>
<tr>
<td>aspartic acid</td>
<td>2.89</td>
<td>2.94</td>
<td>2.85-3.64</td>
</tr>
<tr>
<td>cysteine</td>
<td>0.96</td>
<td>1.01</td>
<td>0.47-1.32</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>6.78</td>
<td>6.99</td>
<td>6.34-9.68</td>
</tr>
<tr>
<td>glycine</td>
<td>2.00</td>
<td>2.04</td>
<td>1.88-2.39</td>
</tr>
<tr>
<td>histidine</td>
<td>1.05</td>
<td>1.10</td>
<td>1.07-1.59</td>
</tr>
<tr>
<td>isoleucine</td>
<td>1.61</td>
<td>1.64</td>
<td>1.51-2.07</td>
</tr>
<tr>
<td>leucine</td>
<td>2.93</td>
<td>2.99</td>
<td>2.65-3.30</td>
</tr>
<tr>
<td>lysine</td>
<td>2.19</td>
<td>2.30</td>
<td>2.27-2.64</td>
</tr>
<tr>
<td>methionine</td>
<td>0.77</td>
<td>0.79</td>
<td>0.68-0.96</td>
</tr>
<tr>
<td>phenylalanine</td>
<td>1.64</td>
<td>1.66</td>
<td>1.52-1.88</td>
</tr>
<tr>
<td>proline</td>
<td>2.5</td>
<td>2.56</td>
<td>1.96-2.82</td>
</tr>
<tr>
<td>serine</td>
<td>1.75</td>
<td>1.79</td>
<td>1.67-2.21</td>
</tr>
<tr>
<td>threonine</td>
<td>1.77</td>
<td>1.80</td>
<td>1.71-2.11</td>
</tr>
<tr>
<td>tryptophan</td>
<td>0.46</td>
<td>0.47</td>
<td>0.44-0.57</td>
</tr>
<tr>
<td>tyrosine</td>
<td>1.06</td>
<td>1.10</td>
<td>0.93-1.38</td>
</tr>
<tr>
<td>valine</td>
<td>2.05</td>
<td>2.08</td>
<td>1.94-2.53</td>
</tr>
</tbody>
</table>

¹ Values are g/100 g meal dry wt.; analysis on composite sample from 4 sites.
² The samples analyzed were mixtures of several varieties. Samples analyzed in references (84,89,92) were from commercial crushing facilities. The data are given to note representative amino acid compositions for commercial canola meal on dry matter basis.

iii. Analysis of alkyl glucosinolates in toasted canola meal
All canola seed is rated based on a glucosinolate value determined on the seed. In addition to the standard analysis of the seed, we measured the glucosinolate content of single meal samples from GT3 and Westar obtained from POS. The value for alkyl glucosinolates in GT3 was 9.9 µmole/g oil-free meal (butenyl, pentenyl, hydroxybutenyl, and hydroxypentenyl) and in Westar it was 4.4 µmole/g oil-free meal. Despite the difference between these values, the meal from GT3 would be acceptable as an animal feed because it is significantly lower than the limit of 30 µmoles/g oil-free meal defined for canola.

iv. Phytate and minerals in toasted meal
Canola meal is rich in many of the essential minerals; however, soil minerals and fertility, and other environmental factors can influence the mineral content of canola meal. Additionally phytic acid has been demonstrated to adversely affect the uptake of phosphorus, calcium, magnesium, and zinc in animal diets (93). High levels of phytic acid, a hexaphosphorylated inositol, reduce the availability of these essential minerals in meal. Since, there is a relationship between phytic acid and mineral content in canola, analyses were conducted to compare the levels of various essential minerals and phytic acid
Safety Assessment of Glyphosate-tolerant Canola

in toasted meal derived from GT73 to those from Westar and the literature.

Table 6 lists the values of the minerals and phytic acid on a dry weight basis and presents a list of literature values (89,94,95). The values for all of the minerals and phytic acid levels in meal from GT73 fall within the literature ranges and correlate well with Westar. The results support the conclusion that there is no meaningful difference between meal derived from GT73 and meal from Westar canola in the phytic acid and mineral composition.

Table 6. Summary of mineral and phytic acid analysis of GT73 meal.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Westar</th>
<th>GT73</th>
<th>Literature Range2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (% DW)</td>
<td>0.76</td>
<td>0.82</td>
<td>0.57-0.82</td>
</tr>
<tr>
<td>Copper (µg/g)</td>
<td>7.06</td>
<td>7.56</td>
<td>4.0-8.0</td>
</tr>
<tr>
<td>Iron (µg/g)</td>
<td>194</td>
<td>160</td>
<td>116-204</td>
</tr>
<tr>
<td>Magnesium (% DW)</td>
<td>0.57</td>
<td>0.64</td>
<td>0.49-0.64</td>
</tr>
<tr>
<td>Manganese (µg/g)</td>
<td>48.5</td>
<td>53.2</td>
<td>30.0-62.9</td>
</tr>
<tr>
<td>Phosphorus (% DW)</td>
<td>1.19</td>
<td>1.23</td>
<td>1.08-1.33</td>
</tr>
<tr>
<td>Potassium (% DW)</td>
<td>1.38</td>
<td>1.42</td>
<td>1.20-1.46</td>
</tr>
<tr>
<td>Zinc (µg/g)</td>
<td>55.0</td>
<td>64.7</td>
<td>59.0-80.9</td>
</tr>
<tr>
<td>Phytic Acid (% DW)</td>
<td>3.09</td>
<td>3.33</td>
<td>2.0-5.0</td>
</tr>
</tbody>
</table>

Dry weight (DW) basis

1 Analysis on composite sample from 4 sites. Samples were dried prior to analysis.
2 The samples analyzed were mixtures of several varieties. Samples analyzed in references (81,86,87) were from commercial crushing facilities. The data are given to note representative mineral components of commercial canola meal on dry matter basis.

v. Analysis of refined, bleached, deodorized oil

The purpose of this experiment (96) was to demonstrate that the values obtained from the analyses of GT73 were within acceptable ranges for CODEX standards (88) for food quality of RBD oil from canola. Data for Westar was also used in comparisons. The results for the fatty acid analysis are listed in Table 7 while the other test results are shown in Table 8.

RBD oil from GT73 meets the CODEX definition for canola oil. While the results for C24:1, C20:0, C22:0 and C24:0 exceed the specifications, they do for both the GT73 and Westar samples. Additionally, since the heavy metals test is a crude method, this was run 4 times on each sample. The GT73 sample passed 3 of the 4 times and the Westar sample passed in all 4 tests. More importantly, the levels of specific heavy metals arsenic and lead are below the CODEX limits.
Table 7. Fatty acid ester profile for GT73 vs Westar\(^1,2\)

<table>
<thead>
<tr>
<th>Fatty Acid Type</th>
<th>Westar</th>
<th>GT73</th>
<th>Codex Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.05</td>
<td>0.05</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>16:0</td>
<td>4.01</td>
<td>3.99</td>
<td>&lt;6.0</td>
</tr>
<tr>
<td>16:1</td>
<td>0.18</td>
<td>0.20</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>18:1</td>
<td>2.05</td>
<td>2.10</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>18:2</td>
<td>63.3</td>
<td>64.0</td>
<td>&gt;50.0</td>
</tr>
<tr>
<td>18:3</td>
<td>19.8</td>
<td>19.0</td>
<td>&lt;40.0</td>
</tr>
<tr>
<td>20:0</td>
<td>6.27</td>
<td>6.74</td>
<td>&lt;14.0</td>
</tr>
<tr>
<td>20:1</td>
<td>1.02</td>
<td>1.06</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>22:0</td>
<td>1.82</td>
<td>1.67</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>22:1</td>
<td>0.51</td>
<td>0.52</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>24:0</td>
<td>0.39</td>
<td>0.19</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>24:1</td>
<td>0.24</td>
<td>0.23</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>24:2</td>
<td>0.30</td>
<td>0.31</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

\(^1\) All analyses were performed by Ralston Analytical Laboratories.
\(^2\) Values are % of fatty acid ester profile. This method does not measure free fatty acids.

Table 8. Food Chemical Codex tests for GT73 vs Westar\(^1\)

<table>
<thead>
<tr>
<th>Test</th>
<th>Westar</th>
<th>GT73</th>
<th>Codex Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Value</td>
<td>0.03</td>
<td>0.03</td>
<td>≤6</td>
</tr>
<tr>
<td>Arsenic(^2)</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>≤0.5 mg/kg</td>
</tr>
<tr>
<td>Cold Test</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
</tr>
<tr>
<td>Lovibond Color</td>
<td>0.2 red; 2 yellow</td>
<td>0.2 red; 2 yellow</td>
<td>1.5 red; 15 yellow</td>
</tr>
<tr>
<td>Erucic Acid</td>
<td>0.39</td>
<td>0.19</td>
<td>≤2.0%</td>
</tr>
<tr>
<td>Free Fatty Acids(^3)</td>
<td>0.02</td>
<td>0.19</td>
<td>≤0.05%</td>
</tr>
<tr>
<td>Heavy Metals(^4)</td>
<td>&lt;5</td>
<td>3; &lt;5; 1; &gt;5</td>
<td>≤5 mg/kg</td>
</tr>
<tr>
<td>Iodine Value</td>
<td>111</td>
<td>110</td>
<td>110-126</td>
</tr>
<tr>
<td>Lead</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>≤0.1 mg/kg</td>
</tr>
<tr>
<td>Linolenic Acid</td>
<td>6.27</td>
<td>6.74</td>
<td>≤15.0%</td>
</tr>
<tr>
<td>Peroxide Value</td>
<td>0.7</td>
<td>0.7</td>
<td>≤10 meq/kg</td>
</tr>
<tr>
<td>Refractive Index</td>
<td>1.4658</td>
<td>1.4657</td>
<td>1.465-1.467</td>
</tr>
<tr>
<td>Saponifiable Value</td>
<td>189.3</td>
<td>190.1</td>
<td>178-193</td>
</tr>
<tr>
<td>Stability</td>
<td>17</td>
<td>17</td>
<td>≥ 7 hrs</td>
</tr>
<tr>
<td>Sulfur</td>
<td>4.04</td>
<td>2.12</td>
<td>≤10 mg/kg</td>
</tr>
<tr>
<td>Unsaponifiable</td>
<td>0.400</td>
<td>0.460</td>
<td>≤1.5%</td>
</tr>
<tr>
<td>Matter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>≤0.1%</td>
</tr>
</tbody>
</table>

\(^1\) All analyses were performed by Ralston Analytical Laboratories.
\(^2\) This method measures both inorganic and organic arsenicals.
\(^3\) Free fatty acids were calculated as oleic acid.
\(^4\) Values were calculated as lead sulfate.
d. **Summary of compositional analysis**

A summary of the results of the compositional analyses performed with seeds and toasted meal is given in Table 9. The results of all analyses (> 1800 individual assays) show that GT73 seeds and the processed fractions (toasted meal and RDB oil) are not materially different from the control canola seeds or fractions. Furthermore, the levels of antinutrients in GT73 toasted meal are at or below levels currently found in commercial canola. Based upon this compositional information and the criteria provided in the Food Policy (5), we have established the identity and safety of GTC line GT73.

**Table 9. Summary of compositional analyses performed on GT73.**

<table>
<thead>
<tr>
<th>Component</th>
<th>GT73 seeds</th>
<th>GT73 toasted meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximate analysis</td>
<td>NMD</td>
<td>NMD</td>
</tr>
<tr>
<td>Amino acid composition</td>
<td>NMD</td>
<td>NMD</td>
</tr>
<tr>
<td>Fatty acid composition</td>
<td>NMD</td>
<td>NA*</td>
</tr>
<tr>
<td>Erucic Acid</td>
<td>NMD</td>
<td>NA*</td>
</tr>
<tr>
<td>Glucosinolates</td>
<td>NMD</td>
<td>NMD</td>
</tr>
<tr>
<td>Sinapines</td>
<td>NMD</td>
<td>NA</td>
</tr>
<tr>
<td>Phytate</td>
<td>NA</td>
<td>NMD</td>
</tr>
<tr>
<td>Minerals</td>
<td>NA</td>
<td>NMD</td>
</tr>
</tbody>
</table>

NMD = not materially different from the control  
NA = not analyzed  
*RBD oil meets CODEX definition for canola oil

2. **Confirmatory animal studies supporting the wholesomeness and safety of glyphosate-tolerant canola when used in animal feed**

Insertion of the CP4 EPSPS and GOX genes into canola was not expected to affect wholesomeness (ability to support typical growth and well-being). However, to confirm that glyphosate-tolerant canola (GTC) was substantially equivalent to control canola (parental variety Westar) as an animal feed, several animal feeding studies were undertaken under GLP guidelines.

The animal wholesomeness studies (184-188), which were not designed as toxicology tests, included a four week rat feeding study with unprocessed canola meal. This would be a worse case test of wholesomeness since the natural toxicants present in canola would not have been reduced or removed by processing (see Sections IV.A.1.b.v. and vi.). In this same study, separate groups of rats were also fed processed canola meal. Due to technical problems encountered in the original 4 week rat feeding study (184), the study with processed canola meal was repeated (185). A 10 week trout feeding study with processed canola meal was also completed (186) as well as a 5 day quail feeding studies with unprocessed canola meal (187,188). The quail study and
Safety Assessment of Glyphosate-tolerant Canola

the rat study with unprocessed canola meal have relevance for environmental risk assessment.

Presented below are summaries of the individual animal feeding studies performed to assess the wholesomeness of GTC.

a. rat feeding study with unprocessed and processed GTC meal (184)
Six week old male and female Sprague-Dawley rats (10/sex/group) were fed either 0, 5, or 15% w/w ground (unprocessed) canola seed incorporated in the diet for 4 weeks. Diets were balanced nutritionally at test diets based on proximate analysis of canola seed. All test diets were formulated to be isonitrogenous and as similar as possible in composition to commercial There were two lines of GTC tested (GT73, GT200) as well as the parental (Westar) line and a diet control (commercial rodent chow with no added canola meal). Groups of rats were separately fed ground canola seed that was added to diets as well as ground canola seed that was further processed (toasted, defatted).

Variable decreased weight gains were observed primarily in male rats fed unprocessed or processed GTC (Table 10). There were no differences in food consumption between any of the groups that would account for the variable weight gains.

Absolute and/or relative liver and kidney weights were increased approximately 5 to 20% for groups fed both GTC and parental line canola when compared to diet controls. Weights were generally increased more in males than females. However, there were no differences in absolute or relative organ weights between the GTC and parental line groups which is the most appropriate comparison.

Subsequent to the completion of the first study, it was discovered that the GTC lines tested were not discrete lines but were mixtures of both lines due to inadvertent commingling of seed. There was no commingling of parental line seed. For example, unprocessed GTC diets were found to contain 83-86% seed from the intended GTC line with the remaining seed from the other GTC line. Processed diets contained approximately 50/50 mixtures of seed from both GTC lines, eg. both GTC diets were similar composition. As a consequence of commingling seed, a second study was undertaken.

b. 2nd four week rat feeding study with processed GTC meal (185)
This feeding study was carried out with one GTC line (GT73) as commercial development of the other GTC line was discontinued. The feeding of unprocessed ground canola seed to rats was not repeated as this was considered to have little practical relevance to commercial practices since unprocessed ground canola seed is not fed to farm animals. There was no diet...
control group in this study. The rest of the experimental design was the same as the first study.

There were no variable decreases in weight gain in this study as body weights and body weight gains were comparable for groups fed GTC and parental line canola meal (Table 10). However, in contrast to the first study, relative liver weights were increased approximately 12-16% for males and females fed the 15% (but not 5%) GTC diet when compared to parental line controls. In this study as well as the first study, livers appeared normal at gross necropsy.

The occurrence of increased liver weights in the second rat feeding study but not the first study is attributed to two factors.

(1) Seed from GTC lines contained, in comparison to the parental line, a higher level of the natural glucosinolate toxicants. While the level of glucosinolates was higher in the GTC line fed to rats in the 2nd study (4 gm/kg compared to 1.8 gm/kg for parental line), they were well within limits (> 12 gm/kg seed which is equivalent to the 30 μmol/g defatted meals sited earlier) established for commercial canola cultivars that are considered to be low glucosinolate varieties.

In the 2nd rat feeding study, the level of total glucosinolates in the processed GTC meal rodent diet (15% incorporation rate of GTC meal) was approximately 0.6 gm/kg compared to 0.3 gm/kg for the parental line. Various animal species fed defatted canola meal have been observed to have increased liver weights which is attributed to the presence of glucosinolates and their degradation products in the meal (74). When rodent diets (no canola meal added) are spiked with individual glucosinolates at concentrations of 1 gm/kg and fed to rats for 29 days, relative liver weights may increase up to 7% relative to diet controls (not spiked with glucosinolates) (182). Relative liver weights are increased more (15%) at higher dietary levels (3-7 gm/kg) of glucosinolates (182,183). The degradation products of glucosinolates are more potent inducers of liver enlargement than the parent molecules. As mentioned earlier (Section IV.A.1.b.v.), an enzyme called myrosinase which is present in canola catalyzes the hydrolytic cleavage of glucosylying an unstable intermediate that rearranges into a variety of degradation products such as alkyl, alkenyl and aromatic nitriles, isothiocyanates etc. When myrosinase was added to rat diets spiked with glucosinolates, liver enlargement was exacerbated over that observed without the addition of the enzyme (74). This is why canola meal is processed in commercial practice to inactivate the enzyme myrosinase and reduce the generation of more toxic glucosinolate degradation products.

(2) Processing of canola meal is another variable which can affect measured responses in rat wholesomeness studies. Processing of canola meal involves...
heating to inactivate myrosinase and solvent extraction to remove the oil. For our feeding studies, processing of canola seed was carried out on pilot scale (40-100 lbs seed) to provide sufficient material for testing. Each parental and GTC line of seed was processed as a separate lot. In the first rat feeding study, canola meal was processed in the United States and one GTC lot (identified as diet GT200 in Table 10 and as diet 47% GT200/53% GT73 in Tables 11 and 12) had a lower nitrogen solubility index (14.7) than the other GTC (20.0) and parental line (19.8) lot indicating it had received more heat treatment. This was corrected in the second study (canola meal processed in Canada) where both the GTC line and the parental line meal were processed very similarly (based on equivalent nitrogen solubilities of 28.3 and 29.1, respectively). However, the nitrogen solubility for these meal samples was higher than the first feeding study indicating these processed canola meal samples received less heat treatment. If the milder heat processing in the second study resulted in less inactivation of myrosinase, then the higher level of glucosinolates could have resulted in higher levels of degradation products in the GTC line. This could have caused the higher relative liver weights (16%) in the GTC line group compared to parental line controls. As indicated earlier, glucosinolate degradation products are more potent than the parent molecules for increasing liver weight.

In summary, there was nothing in the study results that was unexpected based on the known effects of feeding canola meal to animals. The two-fold difference in glucosinolate levels observed in seed from the glyphosate-tolerant strain is typical for the variable glucosinolate levels observed in the Westar variety from which it was derived. Insertion of glyphosate-tolerance genes into canola had no unintended effects on wholesomeness.

c. 10 week trout feeding study with processed GTC (186)
Processed canola meal was administered in the diet to trout for approximately 10 weeks. There were two lines of GTC tested as well as the parental (Westar) line and a diet control (no added canola meal). Incorporation rates for canola meal in trout diets were selected based on commercial aquaculture practices. The diets were formulated so that all nutritional requirements of the trout would be met. Trout were fed their respective diets at a rate of 3% of their body weight per day; this rate of feeding is near satiation for trout of this size.

Trout were approximately 10 grams in weight at study initiation and were grown in 120-L aquaria, 15 fish per tank. There were 3 replicates per dietary treatment or 45 trout per treatment group. Water in all aquaria was recirculated and filtered; environmental conditions were monitored on a regular basis to maintain acceptable conditions for growing trout.

Survival of fish in all groups was very good in the study (Table 11). There was
no statistically significant difference in body weight gain or feed efficiency between the canola fed groups although trout in one GTC group tended to have a lower weight gain than the other GTC and parental line groups fed 15 and 20% canola meal (Table 11). There were no statistically significant differences in feed efficiency between any of the groups although the same GTC group with the lower weight gain also had a slightly lower feed efficiency at the 20% incorporation rate (Table 11). (The same lot of processed canola meal fed to rats in the first feeding study was also used in this study. Therefore, the 2 processed GTC diets tested had similar composition due to commingling of seed. However, the growth response at higher dietary incorporation rates of canola meal was not the same for both GTC groups. The GTC group with the lower growth response had the lowest nitrogen solubility index (14.7) indicating it had received more heat treatment than the other diets tested—see discussion above).

From each tank, 3 average sized fish were used for proximate analysis determinations. There were no statistically significant differences in moisture content and ash levels between any group. A few statistically significant differences in protein and fat were observed between groups (Table 12). These differences were not related to dietary concentrations and occurred sporadically in all groups. They were considered to be due to variation inherent in the AOAC proximate analysis. Protein efficiency ratios (PER) were not statistically different between groups and followed a trend similar to feed efficiency since they are calculated similarly (Table 12). Protein retention (PR) values were different for the 5 and 10% GTC diets (47% GT200/53% GT73) but not at the 15 and 20% dietary rates indicating a lack of dose relationship for the changes (Table 12). Therefore, the statistical differences in PR values were attributed to random variation.

In summary, there were no adverse effects observed in the 10 week trout feeding study that were attributed to insertion of the glyphosate tolerance trait into canola.

d. 5 day quail feeding studies with unprocessed canola meal (187,188)
Thirty bobwhite quail of mixed sex (10 days of age) were assigned to each treatment group and housed in groups of ten birds/pen. Each control and treatment group was fed the test diet for 5 days, then switched to basal (unsupplemented) diets for the last 3 days of the study. Consumption of a diet containing 200,000 ppm (20% of the diet w/w) of unprocessed canola meal is equivalent to eating 9600 seeds/kg body weight per day. There was no mortality observed during the study and birds were normal in appearance and behavior. Body weight gain and food consumption were comparable for quail fed canola seed meal from the GTC lines or those fed the control (parental line) and basal diet.
The unprocessed canola meal used in this study was from the same lot used in the first rat feeding study and the trout study. Since seed from the GTC lines tested were commingled and contained 14-17% seed from the other GTC line tested (see discussion above), this study was repeated (188). The experimental design was the same in the repeat study except that only one GTC line was tested as the other line had been dropped from commercial development. The findings in the second study were similar to those in the first study.

In summary, there were no adverse effects observed in either study that were attributed to insertion of the glyphosate tolerant traits into canola. More discussion of the methods used in these studies is given in Appendix D.
Table 10. Male Rat Body Weight gms (Cumulative Body Weight Gain)\textsuperscript{a} from Rat Feeding Study.

<table>
<thead>
<tr>
<th>Diet Control</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet Control</td>
<td>242 (60.3)</td>
<td>299 (117)</td>
<td>337 (155)</td>
<td>380 (198)</td>
</tr>
<tr>
<td>Westar 5% Unprocessed</td>
<td>237 (55)</td>
<td>296 (115)</td>
<td>333 (151)</td>
<td>372 (190)</td>
</tr>
<tr>
<td>Westar 15%</td>
<td>242 (60)</td>
<td>299 (117)</td>
<td>333 (151)</td>
<td>369 (187)</td>
</tr>
<tr>
<td>Westar 5% Processed</td>
<td>243 (62)</td>
<td>301 (119)</td>
<td>336 (154)</td>
<td>376 (196)</td>
</tr>
<tr>
<td>Westar 15%</td>
<td>239 (57)</td>
<td>297 (115)</td>
<td>328 (146)</td>
<td>376 (186)</td>
</tr>
<tr>
<td>GT73 5% Unprocessed</td>
<td>239 (57)</td>
<td>294 (112)</td>
<td>327 (155)</td>
<td>367 (185)</td>
</tr>
<tr>
<td>GT73 15%</td>
<td>233 (51)*</td>
<td>256 (104)*</td>
<td>331 (149)</td>
<td>361 (180)* GT200</td>
</tr>
<tr>
<td>GT73 5% Processed</td>
<td>240 (58)</td>
<td>303 (121)</td>
<td>337 (155)</td>
<td>375 (192)</td>
</tr>
<tr>
<td>GT73 15%</td>
<td>239 (56)</td>
<td>293 (110)</td>
<td>332 (149)</td>
<td>365 (182) GT73</td>
</tr>
<tr>
<td>GT200 5% Unprocessed</td>
<td>243 (61)</td>
<td>298 (116)</td>
<td>346 (154)</td>
<td>397 (185) GT73</td>
</tr>
<tr>
<td>GT200 15%</td>
<td>237 (54)</td>
<td>297 (108)*</td>
<td>325 (143)</td>
<td>344* (162)** GT73</td>
</tr>
<tr>
<td>GT200 5% Processed</td>
<td>238 (56)*</td>
<td>290 (109)*</td>
<td>335 (154)</td>
<td>359 (178)* GT73</td>
</tr>
<tr>
<td>GT200 15%</td>
<td>239 (57)</td>
<td>288 (106)</td>
<td>330 (148)</td>
<td>352 (170)* GT73</td>
</tr>
</tbody>
</table>

\textsuperscript{a} mean values for 10 males/group.

\textsuperscript{**} p ≤ 0.01; \textsuperscript{*} p ≤ 0.05 from comparison to parental control only

\textsuperscript{b} actual composition of seed composite tested due to inadvertent commingling of seed
Safety Assessment of Glyphosate-tolerant Canola

Table 11: Results of 10 Week Trout Feeding Study with Processed Canola Meal.

<table>
<thead>
<tr>
<th>% Canola Meal</th>
<th>Weight Gain</th>
<th>FE*</th>
<th>Survival(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no canola)</td>
<td>593.7</td>
<td>1.01</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>568.9a</td>
<td>0.98a</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>497.6a,b</td>
<td>0.89a</td>
<td>97.8</td>
</tr>
<tr>
<td>15</td>
<td>509.9a,b</td>
<td>0.90a</td>
<td>97.8</td>
</tr>
<tr>
<td>20</td>
<td>508.0a,b</td>
<td>0.93a</td>
<td>97.8</td>
</tr>
<tr>
<td>53% GT200/47% GT73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>559.9a</td>
<td>0.97a</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>498.5a,b</td>
<td>0.90a</td>
<td>97.8</td>
</tr>
<tr>
<td>15</td>
<td>559.6a</td>
<td>0.98a</td>
<td>97.8</td>
</tr>
<tr>
<td>20</td>
<td>532.6a</td>
<td>0.94a</td>
<td>100</td>
</tr>
<tr>
<td>47% GT200/53% GT73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>552.3a</td>
<td>0.98a</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>516.1a,b</td>
<td>0.94a</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>484.0a,b</td>
<td>0.89a</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>428.5b</td>
<td>0.85a</td>
<td>100</td>
</tr>
</tbody>
</table>

* Feed efficiency

a Values in any column with the same letter designation were not significantly different.
Table 12. Mean whole body proximate composition of rainbow trout. Crude protein, fat and ash values are expressed as % dry matter. Values in any column with the same letter are not statistically different. The reference (0) diet was not included in the statistical analysis but was included in the table for comparative purposes.

<table>
<thead>
<tr>
<th>Canola % diet</th>
<th>Moisture</th>
<th>Crude Protein</th>
<th>Fat</th>
<th>Ash</th>
<th>PER1</th>
<th>PR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>71</td>
<td>53.3</td>
<td>39.7</td>
<td>7.9</td>
<td>2.8</td>
<td>37.2</td>
</tr>
</tbody>
</table>

Westar

<table>
<thead>
<tr>
<th>Canola % diet</th>
<th>Moisture</th>
<th>Crude Protein</th>
<th>Fat</th>
<th>Ash</th>
<th>PER1</th>
<th>PR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>71.2a</td>
<td>49.0a,b</td>
<td>41.7a,b</td>
<td>7.3a</td>
<td>2.7a</td>
<td>31.5a,b</td>
</tr>
<tr>
<td>10%</td>
<td>71.4a</td>
<td>50.7a,b</td>
<td>38.4b</td>
<td>7.3a</td>
<td>2.3a</td>
<td>28.0a,b</td>
</tr>
<tr>
<td>15%</td>
<td>70.4a</td>
<td>49.4a,b</td>
<td>42.3a,b</td>
<td>7.6a</td>
<td>2.5a</td>
<td>26.6a,b</td>
</tr>
<tr>
<td>20%</td>
<td>70.9a</td>
<td>49.4a,b</td>
<td>42.0a,b</td>
<td>7.3a</td>
<td>2.6a</td>
<td>29.4a,b</td>
</tr>
</tbody>
</table>

53% GT200/47% GT73

<table>
<thead>
<tr>
<th>Canola % diet</th>
<th>Moisture</th>
<th>Crude Protein</th>
<th>Fat</th>
<th>Ash</th>
<th>PER1</th>
<th>PR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>70.2a</td>
<td>50.1a,b</td>
<td>44.2a,b</td>
<td>7.1a</td>
<td>2.7a</td>
<td>32.3a,b</td>
</tr>
<tr>
<td>10%</td>
<td>70.1a</td>
<td>45.8a,b,c</td>
<td>42.1a,b</td>
<td>6.9a</td>
<td>2.3a</td>
<td>25.5a,b</td>
</tr>
<tr>
<td>15%</td>
<td>70.6a</td>
<td>43.3a,b,c</td>
<td>42.4a,b</td>
<td>7.3a</td>
<td>2.7a</td>
<td>27.9a,b</td>
</tr>
<tr>
<td>20%</td>
<td>70.8a</td>
<td>41.8b,c</td>
<td>40.4a,b</td>
<td>7.2a</td>
<td>2.6a</td>
<td>25.1a,b</td>
</tr>
</tbody>
</table>

47% GT200/53% GT73

<table>
<thead>
<tr>
<th>Canola % diet</th>
<th>Moisture</th>
<th>Crude Protein</th>
<th>Fat</th>
<th>Ash</th>
<th>PER1</th>
<th>PR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>70.8a</td>
<td>38.7c</td>
<td>42.0a,b</td>
<td>7.5a</td>
<td>2.7a</td>
<td>23.9b</td>
</tr>
<tr>
<td>10%</td>
<td>70.3a</td>
<td>52.9a</td>
<td>42.8a,b</td>
<td>7.1a</td>
<td>2.6a</td>
<td>34.2a</td>
</tr>
<tr>
<td>15%</td>
<td>71.1a</td>
<td>50.7a,b</td>
<td>42.3a,b</td>
<td>6.8a</td>
<td>2.5a</td>
<td>29.3a,b</td>
</tr>
<tr>
<td>20%</td>
<td>70.0a</td>
<td>50.6a,b</td>
<td>45.9a,b</td>
<td>7.5a</td>
<td>2.4a</td>
<td>28.2a,b</td>
</tr>
</tbody>
</table>

1 The protein efficiency ratio (PER) is calculated by dividing the weight gain by the protein intake (equivalent to protein fed). Protein intake is 36% of the total feed over the course of the experiment.

2 Protein retention (PR) is the percent protein retained. It is calculated by dividing the gain in protein over the experiment (final body protein minus the initial body protein) by the protein intake times a 100.

3. Safety assessment of the donor organisms

Shown in Figure 20 below is the flowchart from the FDA Food Policy concerning the safety assessment of the donor organism.

a. Agrobacterium species strain CP4

The safety of the donor organism, Agrobacterium sp. strain CP4, was considered. Agrobacterium sp. strain CP4 is not a food source but is related to microbes commonly present in the soil and in the rhizosphere of plants. Only one gene, the CP4 EPSPS gene, was transferred from CP4 to produce GTC line GT73. The sequence of the DNA transferred and of the protein produced are completely known. All of our plant, microbial, and fungal food sources contain
Safety Assessment of Glyphosate-tolerant Canola

EPSPS's; therefore, this enzyme and its activity are not novel to the food supply. Characteristics of the donor species, Agrobacterium, do not warrant analytical or toxicological tests since only the specific, sequenced gene encoding EPSPS was transferred to the host organism, canola (55). These points, taken with the properties and safety of the CP4 EPSPS protein discussed below (including lack of typical profile for protein allergens), led us to a conclusion of "no concern" for the source of the donor gene.

b. Achromobacter species strain LBAA
The safety of the donor organism of the gox gene, Achromobacter sp. strain LBAA, was also considered. Achromobacter sp. strain LBAA is also not a food source, but Achromobacter sp. is reported to be one of the most frequently occurring bacteria in the rhizosphere (97). Since only one gene, gox, was transferred from Achromobacter to canola (55) and the sequence of the DNA transferred to the host is completely known, characteristics of this donor species do not warrant further tests. After considering these facts with the properties and safety of the GOX protein discussed below, we conclude that there is no safety concern regarding the source of the gox gene.

4. Summary of the assessment of unintended effects
The absence of unexpected or unintended effects due to the CP4 EPSPS and GOX proteins and genes in GT73 was demonstrated by establishing that the recipient organism, canola, has a history of safe use and extensive compositional analysis of seed and processing fractions derived from GT73, focusing on both nutrients and antinutrient compounds. These data led to the conclusion of "no concern" using the flowcharts shown in Figures 9 and 20, via the pathways highlighted in bold type.
Safety Assessment of Glyphosate-tolerant Canola

Figure 20. Safety assessment of new varieties: the donor (taken from Food Policy Figure 3) (5). The pathway leading to "no concern" for GTC is highlighted with bold arrows.

1. Is food from the donor commonly allergenic?
   - Yes
     - Can it be demonstrated that the allergenic determinant has not been transferred to the new variety of host?
       - No
         - Consult FDA on protocols for allergenicity testing and/or labeling
       - Yes
         - New variety not acceptable
   - No
     - Do characteristics of the donor species, related species, or progenitor lines warrant analytical or toxicological tests?
       - Yes
         - Do test results provide evidence that toxicant levels in the new variety do not present a safety concern?
           - Yes
             - No concerns
           - No
             - New variety not acceptable
       - No
         - New variety not acceptable
Safety Assessment of Glyphosate-tolerant Canola

B. Expected or Intended Effects Due to the Expression of CP4 EPSPS in Canola

Shown in Figure 21 is the flowchart from the FDA Food Policy (5) concerning the safety assessment of the protein introduced from the donor organism.

Figure 21. Safety assessment of new varieties: proteins introduced from donor (taken from Food Policy Figure 4) (5). The pathways leading to "no concern" for GTC is highlighted with bold arrows.
1. Protein introduced from the donor
   
a. Expression levels of CP4 EPSPS and GOX in GT73
   To thoroughly characterize GTC, the levels of CP4 EPSPS and GOX proteins, were determined under the US Good Laboratory Practices (GLP) guidelines in leaf and seed tissue in 1992 (77) and in seed in 1993 (79). In 1992, seed used for planting GTC used in the safety assessment came from two separate sources. Thus, two distinct designations for seed were used: GT73H and GT73. Line GT73 corresponded exactly to GT73H except that the latter was homozygous for the glyphosate tolerance genes. Seed samples from GT73 and the control Westar were obtained from seven locations. Due to limited availability of pure homozygous seed for planting, samples of GT73H were obtained from only 3 of the seven sites. Leaf samples from GT73H were collected at three time points. Leaves from homozygous plants were collected and analyzed since they were expected to express the highest levels of the introduced proteins.

   Analysis of CP4 EPSPS and GOX proteins in leaf tissue from GT73H gave mean expression levels of 0.034 µg/mg tissue (fresh weight) and 0.108 µg/mg tissue (fresh weight), respectively (77). There was no evidence of an increase or decrease in leaf expression of CP4 EPSPS and GOX over time. Analyses of seed gave mean levels of CP4 EPSPS of 0.049 and 0.030 µg/mg tissue (fresh weight) in GT73H, and GT73, respectively (77). Mean levels of GOX in seed were 0.154 and 0.124 µg/mg tissue (fresh weight) for GT73H and GT73, respectively. Seed from plants homozygous for the glyphosate tolerance genes appear to express slightly higher levels of the introduced proteins as expected. These expression levels are low, accounting for less than 0.02% of the seed on a fresh weight basis.

   The data obtained in 1993 (79) for expression in seed was in good agreement with the values observed in the previous year (77). They show a range of expression for CP4 EPSPS in GT73 of 0.018 to 0.047 µg/mg tissue with a mean expression of 0.028 µg/mg tissue. The range of expression for GOX in GT73 was 0.108 to 0.334 µg/mg tissue with a mean expression level of 0.193 µg/mg tissue. In the 1993 field trial, only homozygous seed was used. No detectable CP4 EPSPS or GOX was measured in Westar seed tissue from either year.

b. Relationship between CP4 EPSPS and EPSPS enzymes found in food
   The information summarized above in Section III.C.3 supports the conclusion that CP4 EPSPS is functionally similar to the EPSPS proteins typically present in food and feed derived from plant and microbial sources, based on the reaction catalyzed. The structural relationship between CP4 EPSPS and other food EPSPSs is demonstrated by 1) the amino acid sequence comparison; 2) the homology of active site residues; and 3) the 3-dimensional structure.
Safety Assessment of Glyphosate-tolerant Canola

c. Assessment of the allergenic potential of GTC

Introduction of GTC varieties does not present allergenic concerns. Oil is the only human food product from canola (69) and analysis of oil derived from both the Westar control and GTC confirmed that there is no detectable protein in canola oil (77). Furthermore, using oil in direct food challenge of individuals allergic to proteins contained in the respective meal (soybean, peanut and sunflower), established that refined oil does not elicit an allergic response (98-100). This is consistent with the lack of detectable protein in the oil. This information provides the basis to conclude that glyphosate-tolerant canola varieties pose no significant allergenic concerns.

d. Studies demonstrating lack of toxicity associated with CP4 EPSPS and GOX proteins

i. Lack of homology of CP4 EPSPS and GOX to known protein toxins and allergens

CP4 EPSPS and GOX proteins show no meaningful amino acid sequence homology when compared to 1,935 known protein toxins present in the Pir protein, Swissprot, and Genpept protein databases (101). Patterns of amino acid sequence or regions of strong homology shared between two or more proteins may provide insight to the biological significance of a protein. For instance, amino acid sequences may provide information about structural properties, hydrophilicity/hydrophobicity, immunogenicity, stability, evolution, and the possible function or role of the identified protein. The use of protein databases has proven to be a useful tool for predicting biological function of an unknown protein. CP4 EPSPS and GOX proteins were compared to peptide sequences identified as *allergens* and *toxins* from all available protein databases, to identify if either protein has any meaningful sequence homology with known allergens or toxins. The FASTa-type algorithm, which is the standard method for database searching, was used to conduct the amino acid homology comparison between the test proteins and all available sequenced allergen and toxin proteins from all available electronic databases of protein sequences (102-105). The results show that no meaningful homologies exist between known allergens or toxins and CP4 EPSPS and GOX protein sequences. The lack of significance between the alignments was assessed by randomizing the CP4 EPSPS or GOX protein sequences, keeping the amino acid content identical, and comparing the randomized amino acid sequences to the identical database of known allergens and toxins. The output comparisons generated from the randomized CP4 EPSPS or GOX amino acid sequence closely resembles the output comparisons generated with the native, unrandomized CP4 EPSPS or GOX sequence. The evidence indicates, using the best methods available today, that neither the CP4 EPSPS nor the GOX proteins share any sequence similarity with the database of known sequenced protein allergens and toxins. This conclusion, that CP4 EPSPS and GOX proteins are not expected to be toxic, is supported by the digestion and acute mouse gavage studies discussed below. Further information on the homology
searches are provided in Appendix E.

ii. Acute mouse gavage study with CP4 EPSPS and GOX proteins

CP4 EPSPS
An acute mouse gavage study with mature (lacking the chloroplast transit peptide) CP4 EPSPS as the test material was performed in order to directly assess any potential toxicity associated with the protein (106). Results from this study demonstrated that the CP4 EPSPS protein is not toxic. The mature CP4 EPSPS protein was over-produced (107) and purified (108) from E. coli, demonstrated to be equivalent to the GTC canola seed-produced CP4 EPSPS (50,51), and administered by gavage to mice in an acute toxicity test. The criteria assessed for equivalence are shown in Table 10.

There were no adverse effects in mice administered CP4 EPSPS protein by oral gavage at dosages up to 572 mg/kg (106). This dose represents an approximate 1300-fold safety margin relative to the highest potential human consumption of CP4 EPSPS if the protein were expressed in canola, corn, tomato, and potato (assuming no loss of CP4 EPSPS due to processing) (109). Acute administration was considered sufficient to assess the safety of CP4 EPSPS, since proteins that are toxic act via acute mechanisms (110-112).

No treatment-related adverse effects were observed in animals dosed with CP4 EPSPS protein. There were no statistically significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and CP4 EPSPS protein-treated groups. In summary, there were no treatment-related adverse effects in mice administered CP4 EPSPS protein by oral gavage at dosages up to 572 mg/kg. This is not surprising since the CP4 EPSPS was demonstrated to be digested readily in gastric and intestinal fluid in vitro, as summarized below.

GOX
The appropriate test material for this gavage study was determined to be a mature GOX protein with the last 4 amino acids of the CTP fused to the N-terminus (52). This determination was based on experimental evidence (Western blot) which showed that plant-produced GOX was of slightly higher molecular weight than mature GOX produced in E. coli. In addition, N-terminal sequence analysis of a sample of GOX protein purified from tobacco chloroplast (53) showed that processing of the CTP in tobacco occurred at a Cys-Met site 4 residues upstream of the mature protein. Based on these facts, and since a GOX protein prepared with these 4 amino acid residues plus a terminal Met for expression in E. coli (pMON 21115) comigrated on Western blot with both tobacco- and GT73-derived GOX, we conclude that this protein was appropriate for use in the acute toxicity study in mice (52,53). The protein used in this study was termed GOXv247(M4-C1) to indicate the 4 amino acids
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from the CTP which were fused to the N-terminus of the mature protein (52). The unambiguous determination of the N-terminus of GOX produced in canola was unsuccessful despite numerous attempts at purification (52).

Both GOX and GOXv247 proteins, prepared as describe above, were administered separately by gavage to mice at targeted dose levels of 0, 1, 10 and 100 mg/kg body weight (113,114). The experimentally determined dose levels of GOXv247, the protein present in GT73, were 0, 1.08, 11.3, and 104 mg/kg body weight (115). There were no adverse effects noted. The doses administered were calculated to be approximately 5000-fold over a calculated exposure based on the hypothetical presence of GOX proteins in corn (115). Consumption of corn was used because canola meal is not consumed by humans, and the TAS (116) program “Exposure 1® Chronic Dietary Exposure Analysis”, used to calculate human exposure, requires a crop with human consumption in order to calculate an exposure. Furthermore, we assumed a maximum expression of GOX proteins in corn equivalent to that found in GT73. Hence, a maximum exposure level was calculated based on corn expressing GOX proteins. The GOX proteins were prepared by fermentation in *E. coli* and administered with minimum purification (117). Minimally purified GOX protein was used due to difficulties in preparing sufficient quantities of higher purity material. This protein preparation was characterized (118) and shown to be suitable substitutes for use in the gavage studies for GOX proteins produced in GT73 (52) (results summarized in Table 10). Acute administration was considered appropriate to assess the safety of GOX proteins, since proteins that are toxic act via acute mechanisms (110-112).

No adverse effects were observed in mice dosed with either GOX protein. There were no statistically significant differences in body weight, cumulative body weight or food consumption between the vehicle controls, extracts from *E. coli* carrying the vector only controls or extracts from *E. coli* producing GOX protein treated groups. No grossly observable pathologic changes were observed in mice at necropsy that were considered related to treatment (113,114).
### Table 13. Summary of equivalence analyses: GTC vs. *E. coli* Produced CP4 EPSPS and GOX proteins

<table>
<thead>
<tr>
<th>Analytical Method</th>
<th>Criteria</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE</td>
<td>Similar electrophoretic mobility</td>
<td>Similar apparent MW</td>
</tr>
<tr>
<td>Western blot</td>
<td>Similar electrophoretic mobility and immunological response</td>
<td>Similar apparent MW and immunological response</td>
</tr>
<tr>
<td>Glycosylation</td>
<td>Comparable response with glycosylation detection</td>
<td>No CP4 EPSPS or GOX specific carbohydrate moieties detected</td>
</tr>
<tr>
<td>CP4 EPSPS Amino Acid</td>
<td>Corresponds through 10 amino acid positions</td>
<td>Correct N-terminus through 12 positions (N-terminal methionine present on <em>E. coli</em>-produced CP4 EPSPS)</td>
</tr>
<tr>
<td>Sequence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GOX Amino Acid Sequence</td>
<td>Corresponds through 10 amino acid positions</td>
<td>Not Determined from canola</td>
</tr>
<tr>
<td>CP4 EPSPS enzymatic</td>
<td>Demonstration of unique functional activity</td>
<td>GTC 3.3 U*/mg CP4 EPSPS</td>
</tr>
<tr>
<td>enzymatic activity</td>
<td></td>
<td>* E. coli 3.6 U*/mg CP4 EPSPS</td>
</tr>
<tr>
<td>GOX enzymatic activity</td>
<td>Demonstration of unique functional activity</td>
<td>GTC 6.6 U*/mg GOXv247</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* E. coli 18.8 U*/mg GOXv247</td>
</tr>
<tr>
<td>ELISA</td>
<td>Comparable dose response</td>
<td>Dose response curves comparable for both CP4 EPSPS and GOX in their respective ELISAs.</td>
</tr>
</tbody>
</table>

* U (unit) is defined as 1 μmole of product (phosphate for CP4 EPSPS or glyoxylate for GOX) per minute at 25°C for CP4 EPSPS or 30°C for GOX.

### iii. Digestion of CP4 EPSPS and GOX proteins in simulated gastric and intestinal fluids

**CP4 EPSPS**

*In vitro*, simulated mammalian gastric and intestinal digestive mixtures were established and used to assess the susceptibility of CP4 EPSPS and GOX proteins to proteolytic digestion. The method of preparation of the simulated digestion solutions used is described in the United States Pharmacopoeia (119), a frequently cited reference for *in vitro* digestion. *In vitro* studies with simulated digestive solutions are widely used as models of animal digestion. They have been used to investigate the digestibility of plant proteins (120,121),
animal proteins (122) and food additives (123); to assess protein quality (124); to study digestion in pigs and poultry (125); to measure tablet dissolution rates to monitor biodegradation for pharmaceutical applications (124); and to investigate the controlled-release of experimental pharmaceuticals (126).

CP4 EPSPS was shown to be rapidly degraded \textit{in vitro} using simulated digestive fluids (127). The data demonstrated a half-life for CP4 EPSPS of less than 15 seconds in the gastric system and less than 10 minutes in the intestinal system, based on western blot analysis. To put the rapid degradation of the CP4 EPSPS protein in the simulated gastric system into perspective, solid food has been estimated to empty from the human stomach by about 50% in two hours, while liquid empties 50% in approximately 25 minutes (128). If some of the CP4 EPSPS protein did survive the gastric system, it would be rapidly degraded in the intestine. Greater than 50% of CP4 EPSPS protein was degraded in the simulated intestinal system in less than 10 minutes (western blot analysis). This compares with transit times through the intestine (for radiolabelled chromate, which is not absorbed) of 4 to 10 hours for the first products to appear in the feces and 68 to 165 hours for the last to be detected (129). Thus, using both simulated gastric fluids (SGF) and simulated intestinal fluid (SIF) model systems, CP4 EPSPS is predicted, as expected, to be readily digested in the mammalian digestive tract.

GOX and GOXv247 proteins were added to gastric and intestinal fluids as extracts from fermentations of \textit{E. coli}-produced material at defined concentrations, and were sampled at regular intervals (130). The levels of these proteins were measured by western blot and enzymatic activity assays. In gastric fluid, GOX and GOXv247 proteins degraded extremely rapidly; more than 90% of the initially added GOX and GOXv247 protein degraded after 0.25 min incubation in SGF as detected by western blot analysis (130). GOX enzymatic activity for both GOX and GOXv247 protein extracts also dissipated readily; more than 96% of the added GOX activity dissipated after 1 min incubation in SGF, the earliest time point (130).

In intestinal fluid, GOX and GOXv247 proteins degraded rapidly; more than 90% of the initially added GOX and GOXv247 protein degraded after 0.5 min incubation in SIF as detected by western blot analysis (130). GOX enzymatic activity for both GOX and GOXv247 protein extracts also dissipated readily; more than 95% of the added GOX enzymatic activity dissipated after 60 min incubation in SIF (130). A difference in dissipation of GOX activity at earlier incubation time periods was observed for the GOX and GOXv247 protein extracts (130). This difference is attributed to the introduction of a protease cleavage site upon production of the variant GOX protein (17) causing GOXv247 to be degraded more rapidly than GOX.
The results of this study establish that the GOX and GOXv247 proteins and their associated activities rapidly degrade in both gastric and intestinal models, suggesting that the GOX and GOXv247 proteins and their associated enzymatic activity will readily degrade in the mammalian digestive tract upon ingestion as a component of food or feed. The ready degradation of the GOX and GOXv247 proteins supports the safety of the GOX and GOXv247 proteins for animal consumption.

e. Consumption of the donor proteins and macroconstituent status
Expression studies on GTC seed show that the CP4 EPSPS and GOX proteins are expressed at approximately 0.02% (0.049 µg/mg) and 0.064% (0.154 µg/mg) of the fresh weight of canola seed (77). In the case of CP4 EPSPS protein, this is higher than the level of endogenous EPSPS, and thus the consumption of total EPSPS will increase in animal feeds using the GTC varieties. The magnitude of this increase has not been directly determined because although the endogenous canola EPSPS can be detected by western blots in extracts from canola seed (using antibodies raised to petunia EPSPS), no reliable and accurate assay exists to quantitate the canola EPSPS. Because CP4 EPSPS and GOX proteins are present in canola seed at only 0.02% and 0.064% of the fresh weight, lower than that of seed storage proteins, they should not be considered a macronutrient. In addition, the lack of macronutrient status of the introduced proteins has been confirmed by measurements of total seed protein (Figures 10, 11 and 12), which demonstrate that the level of total protein has not increased in GTC canola seeds compared to the control.

2. Lack of effects on carbohydrates, fats or oils.
The addition of the CP4 EPSPS and GOX proteins are not expected to alter the carbohydrate, fat, or oil composition, structure, or levels in GTC. The proximate (Figures 10 and 11) and other analyses (Figure 12) confirmed that there has been no alteration in the level of carbohydrates or level and composition of fats in oil from GT73 (section IV.A.1.b).

3. Summary of expected or intended effects due to the CP4 EPSPS and gox genes and expressed proteins in GTC line GT73.
We have concluded that there are no deleterious effects due to the insertion of the CP4 EPSPS and gox genes and the expression of their encoded proteins in GTC line GT73. This was demonstrated by: 1) the relationship between CP4 EPSPS and EPSPS enzymes found in food and microbes; 2) the lack of allergenic potential of CP4 EPSPS and GOX proteins; 3) the lack of homology of CP4 EPSPS and GOX with known protein toxins; 4) the lack of acute toxicity of CP4 EPSPS and GOX proteins as determined by a mouse gavage study; 5) the rapid digestion of CP4 EPSPS and GOX proteins in simulated gastric and intestinal fluids; 6) the lack of macroconstituent status of CP4 EPSPS and GOX proteins; and 7) the lack of expected alterations in carbohydrates, fats, or
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oils in line GT73. This data allowed us to reach the conclusion of "no concern" using the flowchart shown in Figure 21, via the pathways highlighted in bold type.

V. CONCLUSION FOR GT73 SAFETY ASSESSMENT

Glyphosate-tolerant canola (GTC) is not materially different from the parental canola line (Westar) and canola now being sold, in any meaningful way except for the ability to tolerate glyphosate. Over 1800 individual analytical measurements were compared. The results demonstrated that the levels of nutrients (protein, oil, fiber, minerals, carbohydrates, calories, amino acids, and fatty acids) in GT73 are comparable to the parental variety or are within established ranges for canola. Natural canola antinutrients (glucosinolates and erucic acid) were also measured, and comparisons again showed no material difference when compared to the parental control. Upon quantitatively and qualitatively evaluating all of the data available, we have been able to establish that, in all instances, there are no meaningful differences between GT73 and Westar in the parameters measured which include all the commercially significant properties. In all cases, data for GT73 were within established limits for canola (<30 μmol glucosinolates/g defatted meal and <2% erucic acid), and ranges of literature values for nutrients. In some instances, slight differences were noted specifically between GT73 and Westar which result from the fact that GTC was selected from Westar which is a heterogeneous genotype (personal communications with [redacted] and [redacted] Appendix C) and variation is expected. Most importantly, those values or effects are well within established ranges and limits documented and reported in the literature generally for canola.

One of the new proteins found in GTC, CP4 EPSPS, is related to EPSPSs already found in foods and feeds derived from plants, microbes, and fungi. Therefore, these proteins are considered safe for human or animal consumption. The gavage studies with these proteins support these conclusions. Further, the CP4 EPSPS and GOX proteins are inactivated by the heat processing required prior to most consumption of canola by farm animals. The CP4 EPSPS and GOX proteins are rapidly degraded in simulated digestive fluids and do not possess properties associated with allergenic proteins. Since protein is not detectable in the RBD canola oil, which is the only canola derived food consumed by humans, the introduced proteins present no risk in the human food portion of this crop.

These data lead to a conclusion of "no concern" for every criterion in the flowcharts outlined in the Food Policy. Canola modified to be tolerant to glyphosate are not materially different in composition, safety, wholesomeness or any relevant parameter from canola now grown, marketed, and consumed. Sales and consumption of these canola and all progenies derived from crosses
between GTC line GT73 and traditional canola would be fully consistent with the agency's Food Policy, the Federal Food Drug and Cosmetic Act, and current practices for the development and introduction of new canola varieties.

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68. Personal communication with seed company partners. Segregation data from backcrossing programs using GTC line GT73.


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83. Personal communication with [name redacted], Professor Emeritus, University of Saskatchewan, Department of Animal and Poultry Science, Saskatoon, Saskatchewan.


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115. Preparation and Confirmation of Doses for Acute Mouse Feeding Studies with GOX(M4-C1) and GOXv247(M4-C1). Monsanto Technical Report, MSL 12967, St. Louis.


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Appendix A. Summary of analytical methods.

A. Methods for the Analysis of Leaf, Seed, and Meal.
Following are brief descriptions of the primary assays used in the analytical studies for GTC safety assessments.

1. **CP4 EPSPS ELISA:** A double antibody indirect enzyme-linked immunosorbent assay (ELISA) was developed and validated (131) for detection of CP4 EPSPS. Levels of CP4 EPSPS are determined by extrapolation from the logistic curve fit of the purified CP4 EPSPS standard curve. The ELISA utilizes two antibodies from two different animal species raised against the native CP4 EPSPS protein. The double antibody sandwich is detected with donkey anti-rabbit AP conjugate followed by development with pNPP that yields a soluble yellow product that can be measured by optical density to quantitate analyte levels. The assay recognizes the native CP4 EPSPS, but has no interference from endogenous EPSPS. An SOP and validated computer software (132) were developed for this assay.

2. **CP4 EPSPS enzymatic assay:** The procedure utilized for determining the amount of functionally active EPSPS entailed the use of an HPLC with radioactivity detector, which has been previously described (2,19). Labelled substrate $^{14}$C-phosphoenolpyruvate ($^{14}$C-PEP) is converted to $^{14}$C-5-enolpyruvylshikimate-3-phosphate ($^{14}$C-EPSP) in the presence of shikimate-3-phosphate (S3P) by EPSPS, and the resultant $^{14}$C-EPSP is detected using HPLC and radioactive flow detection. The final reagent concentrations in the assay were 50 mM Hepes, 0.1 mM ammonium molybdate, 5 mM potassium fluoride, 1 mM $^{14}$C-PEP, and 2 mM S3P, pH 7.0. Reactions were run at approximately 25°C. The reactions were quenched after 2 to 5 minutes with an equal volume of 9:1 ethanol: 0.1 M acetic acid, pH 4.5. The samples were then centrifuged and chromatographed by HPLC anion exchange using approximately 0.25 M potassium phosphate eluent, pH 6.5, at 1 ml/min flow rate. The percent turnover of $^{14}$C-PEP to $^{14}$C-EPSP was determined by peak integration. For EPSPS, 1 unit (U) is defined to be 1 μmol EPSP produced/min at approximately 25°C, under the assay conditions described.

3. **GOX ELISA:** A direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed and validated (133) to adequately quantitate the levels of GOX proteins. This ELISA uses a purified polyclonal antibody from goat immobilized on 96-well plates to complex with GOX. The initial complex is then captured by a second antibody conjugated to alkaline phosphatase (AP). Development of the AP with pNPP, an AP substrate, yields a soluble yellow product. The optical density is monitored using a spectrophotometric plate reader. Levels of GOX in samples are ultimately determined using the four parameter logistic equation to fit the standards which were obtained from an E. coli expression system. The ELISA
was validated (133) after running experiments that addressed extraction efficiency, the overall variability of the assay, and the stability of GOX toward storage in leaf and seed tissue preparations. An SOP and validated computer software (132) were developed for this assay.

4. GOX enzymatic assay: The assay, developed for the detection the enzymatic activity of GOX proteins, measures the conversion of glyphosate or IDA (iminodiacetic acid) into glyoxylate. AMPA (aminomethylphosphonic acid) or glycine (from IDA) are also products from this reaction, but are not directly measured. Glyoxylate is detected by conversion to its 2,4-dinitrophenyl hydrazone derivative which is sequentially extracted into ethyl acetate followed by a solution of sodium carbonate. The sodium salt of the hydrazone is finally separated by reverse phase HPLC, detected at 360 nm (134). Quantitation is determined using a standard curve of known amounts of glyoxylate derivatized in the same manner. Thus, a sample containing GOX is mixed with buffer at pH 7.4 at approximately 30°C. The reaction is initiated by the addition of glyphosate or IDA and allowed to proceed for 2 to 30 min. Reagents are mixed such that the final volume is 100 µL and the final concentrations are 0.1M MOPS, 10 mM Tricine, 10 µM FAD, 10 mM magnesium chloride, and 50 mM glyphosate or IDA. The reaction is quenched by the addition of 250 µL of 0.5 mg/mL solution of 2,4-dinitrophenylhydrazine (DNPH) in water. This solution is then extracted with ethyl acetate, and the hydrazone product is re-extracted into sodium carbonate. Reverse phase HPLC involves using an isocratic mobile phase of methanol-water-acetic acid (60:38.5:1.5) with 5 mM tetrabutylammonium phosphate as an ion pairing agent at a flow rate of 1 mL/min. When increased sensitivity was needed, 5 mM 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q-0) was added to the reaction. For GOX, 1 unit (U) is defined to be 1 µmol glyoxylate produced/min. at approximately 30°C, under the assay conditions described. An SOP was developed for this assay.

5. Western blot assays for GOX and CP4 EPSPS: Detection of GOX proteins and CP4 EPSPS at low levels was accomplished by separating protein samples on polyacrylamide gels and electrophoretically blotting the proteins onto nitrocellulose paper or PVDF membrane. Specific antibodies were hybridized to the blots of GOX and CP4 EPSPS, then reacted with 125I-Protein G. Quantitation of the results was either by autoradiography or by imaging the blot directly using a PhosphorImager and quantitative image analysis. More information on the western blot assays may be found in the GOX and CP4 EPSPS ELISA reports (131,133). SOPs were developed for this assay.

6. Protein assays for extracts (Bio-Rad assay): Protein levels in extracts used for ELISA and western blot analyses, except for extracts containing SDS, were determined using the Bio-Rad protein assay in a 96-well plate format (135). An SOP was developed for this assay.
7. **Protein / amino acids in oil assays**: In order to investigate whether any appreciable amount of protein (or amino acids) is present in canola oil, a new method was developed at Monsanto. This method involves hydrolyzing one-half milliliter samples of oil with equal volumes of trifluoroacetic acid: HCl: propionic acid (50:25:25) for approximately 24 h at approximately 145°C. After cooling, the released amino acids are extracted from the oil phase with 2 x 0.5 ml of 30% methanol/0.1 N HCl. The combined extracts are dried down, reconstituted in 200 μl Na-S citrate buffer and analyzed on a Beckman 6300 amino acid analyzer. An SOP was developed for this assay.

8. **Quality assays**: The canola seed was analyzed using well-established methods at Ralston Analytical Laboratories (RAL), Agriculture and Agri-food Canada Research Station in Saskatoon, Saskatchewan, POS Pilot Plant Corporation of Saskatoon, Saskatchewan, and the Grain Research Laboratories (GRL) in Winnipeg, Manitoba. The following are brief descriptions of the RAL, Ag Canada, and GRL methods utilized in this study.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Literature Methods</th>
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<tbody>
<tr>
<td>fat (RAL)</td>
<td>ether extraction (136-138)</td>
</tr>
<tr>
<td>protein (AL)</td>
<td>Kjeldahl (139)</td>
</tr>
<tr>
<td>nitrogen solubility (RAL)</td>
<td>water extraction (140)</td>
</tr>
<tr>
<td>moisture (RAL)</td>
<td>loss on drying (141,142)</td>
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<tr>
<td>fiber (RAL)</td>
<td>crude fiber (143)</td>
</tr>
<tr>
<td>ash (RAL)</td>
<td>muffle furnace (144)</td>
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<tr>
<td>calories (RAL)</td>
<td>by calculation</td>
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<tr>
<td>amino acids (RAL)</td>
<td>AAHV (145-147)</td>
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<td>tryptophan (RAL)</td>
<td>alkaline hydrolysis (148,149)</td>
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<td>cysteine and methionine (RAL)</td>
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<td>fat (Ag Canada)</td>
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<td>protein (Ag Canada)</td>
<td>Dumas (160-162)</td>
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<tr>
<td>fatty acid profile (Ag Canada)</td>
<td>transesterification (164-168)</td>
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<td>glucosinate profile (Ag Canada and POS)</td>
<td>desulfation/HPLC (169-175)</td>
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<tr>
<td>sinapines (Ag Canada)</td>
<td>HPLC (176)</td>
</tr>
<tr>
<td>chlorophyll (GRL)</td>
<td>NIR (177)</td>
</tr>
</tbody>
</table>

**a. Fat, ether extraction (RAL)**: Ether-soluble material (primarily "free" fats and oils) was extracted from the sample with petroleum ether in a soxhlet extractor for 16 hours. The ether was volatilized, and the residue was dried, quantitated gravimetrically, and calculated as percent fat. Using a 2 g sample, the lowest confidence level of this method is 0.1% fat. The method is based on...
published procedures (136-138) and was validated for canola seed.

b. Total kjeldahl nitrogen - protein (RAL): Nitrogenous compounds in the sample were reduced, in the presence of boiling sulfuric acid, catalyzed by a potassium sulfate/titanium dioxide/cupric sulfate mixture, to form ammonium sulfate. The resultant solution was cooled, diluted, and made alkaline with a sodium hydroxide-thiosulfate solution. Ammonia was liberated and distilled into a known amount of standard acid. The distillate was titrated, and nitrogen or protein (N x 6.25) was calculated from the known amount of reacting acid. Using a 1 g sample, the lowest confidence level of this method is 0.1% protein (0.02% nitrogen). The method is based on a published procedure (139).

c. Nitrogen solubility analysis (RAL): Nitrogen solubility was assayed using an official AOCS method (140). The nitrogen solubility index was defined as [(% water soluble protein)/ (% total protein)] x 100. The lowest confidence level of this assay was 0.5%, using a 5 g sample.

d. Moisture (RAL): The sample was placed in a force-draft oven, set at 133°C, for two hours. The loss in weight was quantitated and calculated to percent moisture (141,142).

e. Crude fiber (RAL): The sample was dried, if necessary, to remove excessive moisture, ground to pass through a 1.0 mm screen and extracted in refluxing ether to remove excessive fat. It was then digested in refluxing 1.25% H₂SO₄, filtered, digested in refluxing 1.25% NaOH, and filtered. The residue was washed, dried, weighed, ignited, and reweighed. Using a 2 g sample, the lowest confidence level of this method is 0.2%. This method has been published (143).

f. Ash (RAL): The sample was charred on a hot plate, and ashed to a constant weight at 550°C in a muffle furnace. The residue was quantitated and calculated to percent ash. Using a 3 g sample, the lowest confidence level of this method is 0.2%. This method has been previously published (144).

g. Carbohydrates (RAL): Carbohydrates were calculated by difference using the fresh weight-derived data and the following equation:

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

h. Calories (RAL): Calories were calculated using the soybean Atwater factors with the fresh weight-derived data and the following equation:

\[ \text{calories (kcal/100 g)} = (3.47 \times \% \text{protein}) + (8.37 \times \% \text{fat}) + (4.07 \times \% \text{carbohydrates}) \]

i. Amino acids (AAHV method) (RAL): Samples were hydrolyzed with hydrochloric acid; after acid hydrolysis, the sample was separated on an anion exchange column, and detected by ninhydrin reaction. This assay was based
on previously published references (145-147).

j. Tryptophan-alkaline hydrolysis (RAL): For tryptophan determination, a portion of sample was mixed with sodium hydroxide solution, and precautions were taken to prevent oxidation of the amino acids as they were hydrolyzed. Samples were prepared and analyzed by HPLC, based on a modification of several methods (148,149).

k. Cysteine and methionine (CYP A method) (RAL): Cystine, cysteine, and methionine in samples were oxidized to cysteic acid and methionine sulfone by treatment with performic acid solution for 16 h at approximately 0°C. After acid hydrolysis, the sample was separated on an anion exchange column, and detected by ninhydrin reaction. This assay has been previously described (150-152).

l. Mineral screen (ICP method) (RAL): Samples were ashed to remove the organic material. The ash residue was dissolved in 3N HCl on a hot plate at medium heat for approximately 15 minutes or until the residue went into solution. After diluting the solutions 10 fold in water, the concentration of minerals was determined by comparing the emission of the unknown sample to the emissions of standard solutions, measured by inductively coupled plasma atomic emission spectroscopy (ICP) (153-155). If any sample absorbance exceeded the absorbance of the highest standard, an appropriate dilution with 0.3N HCl was made so that the sample emission fell approximately at the midpoint of the emission range of the working standards, and the sample was analyzed again. This method can measure calcium, cobalt, copper, iron, potassium, magnesium, manganese, sodium, phosphorus, and zinc.

m. Phytic acid (RAL): Phytic acid was extracted with 2.4% HCl and separated from inorganic phosphates by anion exchange chromatography. The eluant was digested with sulfuric/nitric acid, freeing phosphorus, which upon reaction with ammonium molybdate and sulfonic acid forms a blue colored complex. The absorbance of the complex was measured spectrophotometrically, and the phosphorus concentration was quantitated and compared to a set of standards of known concentration that were processed in the same manner. Absorbance values were converted based on molecular weight equivalence to quantitate the amount of phytic acid in the sample (150-158).

n. % fat (Ag Canada): The % fat was determined on a whole seed basis using an Oxford 4000 NMR Analyzer which was calibrated with a check sample of B. napus prior to analysis. Samples weighing approximately 20 grams which had been cleaned, and predried at approximately 50°C, 25 mm Hg to a moisture content of ≤3%, were measured in triplicate. This method is based on published methods (159) and has been demonstrated on a variety of seed with
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Oil contents from 1.5 to 53%.

o. % Protein (Ag Canada): The % protein value was obtained from a sample of defatted meal, also referred to as oil free meal, using a LECO FP-428 "Nitrogen Determinator". The analysis was run in triplicate on one sample per plot. Means, standard deviations, and % CV's for each sample were calculated and are in the raw data. This method was based on that of Dumas (160), and measured nitrogen gas evolution following combustion of a sample. The sample was combusted giving principally N₂, NOₓ, CO₂, H₂O, and O₂. The O₂ is converted to CuO, CO₂ was absorbed in Ascarite II, H₂O was absorbed by Mg(ClO₄)₂, and NOₓ was converted to N₂. Nitrogen gas was measured as it flowed through a thermal conductivity cell. As with the Kjeldahl method, protein is computed by the following: N₂ x 6.25 = % protein. This procedure has been published (161,162). It is an accepted method by the AOAC for crude protein in animal feed.

p. Fatty acid profile (Ag Canada): This method used sodium methoxide mediated transesterification in methanol of a sample of oil extracted from seed. The Raney grinder (163) was used with petroleum ether (Skellysolve B) to obtain the oil in solution. Petroleum ether samples were concentrated, and the solvent free oil was then mixed with sodium methoxide in methanol and petroleum ether for 30 min. Water was added to separate layers, and the top (organic) layer was analyzed via flame ionization detection gas chromatography. The percents of individual fatty acid methyl esters were calculated relative to the total amount of fatty acid methyl esters present. This assay has been previously described (164-168).

q. Glucosinolate profile (Ag Canada and POS): The analysis required extraction of accurately weighed oil-free meal (163) with methanol containing lead-barium acetate. The solution was transferred for partial purification on an anion exchange column and elution via enzymatic cleavage of the sulfate moiety with sulfatase from Helix pomatia. The desulfated eluent was then derivatized with bistrimethylsilyl trifluoroacetamide or bistrimethylsilylacacetamide premixed in acetone with 1-methylimidazole and trimethylchlorosilane, followed by gas chromatographic analysis. This procedure is a modification of a Canadian Grain Commission (169) method, and is well suited for samples containing ≥10 μmoles/g defatted meal (170-175).

r. Sinapine analysis (Ag Canada): The method used detected total choline esters. Defatted meal was extracted with 70% methanol at approximately 75°C for 20 minutes. Debris was removed by centrifugation, and the supernatant was applied to a cation exchange column (CM-Sephadex C25). Purified material was eluted from the column with 2M acetic acid. The absorbance of the eluate containing the choline esters was measured at 330 nm. From a linear regression of the plot of absorbance versus sinapine
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bisulphate concentration (standard curve), sinapine concentration of the meal was calculated:

\[
\text{Sinapine (mg/g)} = \frac{(\text{ABS}-a)}{b} \times \frac{V}{1.0} \times 10.0 \times \frac{0.050}{1000}
\]

ABS = absorbance at 330 nm, \(a = y\) intercept of standard curve (-0.0027),
b = slope of standard curve (0.0439 mL/µg), V = total volume (mL) of extract (10 mL),
1.0 = volume (mL) of extract applied to column, 0.050 = weight of meal sample analyzed,
10.0 = total volume (mL) eluted from column, 1000 = conversion factor (µg/mg)

Sinapine (choline esters) is a constituent not typically measured in rapeseed. The method used is extracted from a masters thesis submitted to Ag Canada, and is based on published methods (87,176).

s. Chlorophyll content (GRL): Seed samples were analyzed using a Dickey-john Instalab NIR product analyzer. This method has been used to analyze approximately 20 gram samples of whole seed, and the values are reported on an as is moisture basis. The instrument was calibrated using an ISO method (177).

B. Methods for the Analysis of RBD Oil.
The following are brief descriptions of the RAL standard methods used to analyze RBD oil from canola.

Food Chemical Codex Methods (88)
a. Fatty acid profile (RAL): This method used sodium methoxide mediated transesterification of a sample of oil extracted from seed. Methylation was complete when cloudiness disappeared upon gently refluxing. A solution of 0.5% HCl saturated with NaCl was added and allowed to thoroughly mix. The esters were extracted into hexane, filtered through reagent grade sodium sulfate, and the hexane was evaporated with dry nitrogen. A small volume of sample was injected onto the chromatograph. The percents of individual fatty acid methyl esters were calculated relative to the total amount of fatty acid methyl esters present. The method did not measure free fatty acids.

b. Acid value (RAL): The acid value was defined as the number of mg of potassium hydroxide required to neutralize the fatty acids in 1 g of the test substance. Sample was completely dissolved in hot alcohol, previously boiled and neutralized with sodium hydroxide. Phenolphthalein TS was added and sodium hydroxide titrated until a pink color persisted for at least 30 seconds. Calculation for the acid value was then made.

c. Arsenic (RAL): The sample, mixed with magnesium oxide/nitrate, was ashed in a muffle furnace to remove organic material. The ash residue was dissolved in hydrochloric acid and treated with potassium iodide and zinc chloride to reduce any pentavalent arsenic. Trivalent arsenic reacts with metallic zinc, liberating arsine gas, which was passed through lead acetate-
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treated cotton to precipitate hydrogen sulfide and other contaminating hydrides. The arsine gas was bubbled into potassium permanganate solution, converting arsenic to the pentavalent state, which was treated with ammonium molybdate in the presence of hydrazine sulfate. The resulting blue-colored solution was measured spectrophotometrically, and arsenic content was quantitated from a set of standards of known concentrations taken through the color reaction. Using a 10 g sample, the lowest confidence level was 0.2 ppm arsenic. This method measures total arsenic, both inorganic and organic arsenicals.

d. Cold test (RAL): A 200-300 ml sample was filtered and transferred to completely fill a cork stoppered bottle. The sealed bottle was equilibrated at 25°C in a water bath completely covering the bottle. The bottle was then completely immersed in an ice and water bath for 5.5 hrs taking care to replenish ice as needed to keep the temperature at 0°C. This was a pass/fail test, and all of the sample had to be clear such that all fat crystals or cloudiness was absent.

e. Free fatty acids (RAL): A portion of fat was dissolved in hot, neutralized ethanol, with a phenolphthalein indicator. The solution was quickly titrated with standardized sodium hydroxide solution to the pink phenolphthalein end point. Percent free fatty acids was calculated from the number of equivalents of standardized base it took to neutralize the sample, and the molecular weight of the particular acid in which terms the results were expressed. Free fatty acids in most types of fats and oils were calculated as oleic acid.

f. Heavy metals (RAL): The sample was ashed in a muffle furnace to remove organic material. The ash residue was dissolved in dilute acid. The sample solution was pH adjusted and treated with sulfide solution, precipitating insoluble sulfides. The resulting colored precipitate in solution was evaluated against a white background and compared to a lead standard of known concentration that had been taken though the precipitation step. This method measured total metals that form precipitates with sulfide ions, including silver, arsenic, bismuth, cadmium, mercury, lead, antimony, and tin. Results were qualitative: less than or greater than a stated level of heavy metals, calculated as lead sulfide.

g. Iodine number (RAL): This method measured unsaturation, expressed as centigrams iodine adsorbed per gram of sample, in fats and oils that do not have conjugated systems. A portion of filtered sample was dissolved in carbon tetrachloride. WLS iodine solution was added and the solution was allowed to set, during which time the iodine attached to any unsaturated carbon bonds. The excess free iodine was titrated with standardized sodium thiosulfate solution, using a starch indicator. The centigrams of iodine adsorbed by the sample were calculated from the difference between the total centigrams of
iodine added and the centigrams iodine left after the reaction, as detected by the thiosulfate titration. Results were reported as centigrams iodine adsorbed per gram of sample.

h. Lead analysis (RAL): Concentration of lead (ppm) was determined by comparing the absorbance of an unknown sample to the absorbances of standard solutions, using atomic absorption spectroscopy. The sample was ashed in a muffle furnace to remove organic material. The ash residue was dissolved in dilute acid. Standards and samples were complexed with ammonium pyrrolidone dithiocarbamate (APPC), in an acidic solution, and extracted into an organic solvent. A known control sample was included in every analytical run and was evaluated and recorded.

i. Peroxide value (RAL): This method measured the milliequivalents of peroxide per kilogram of fat in fats and oils. The sample was dissolved in an acetic acid-chloroform solution. Potassium iodide was added and oxidized by any peroxide present, liberating iodine. The liberated iodine was titrated with standardized sodium thiosulfate, using a starch indicator solution end point. The amount of peroxide was calculated from the known amount of titer of sodium thiosulfate consumed.

j. Refractive index (RAL): An Abbe refractometer was used to measure refractive index of a sample. Enough sample was applied to a clean measurement prism to completely fill the space taking care to eliminate bubbles. The illuminator was then adjusted for the best contrast in the reflection borderline. The hand wheel was used to set the borderline on the cross hair intersection. The contact switch was depressed and the sample value in index of refraction is read.

k. Saponifiable value (RAL): This method measured the amount of alkali necessary to saponify a definite quantity of the sample. It was expressed as the number of milligrams of potassium hydroxide required to saponify 1 g of the sample. Clarity and homogeneity of the test solution were partial indicators of complete saponification which usually takes approximately 1 hr. A blank determination was made along with the sample. After saponification was complete, 1 ml of phenolphthalein indicator was added and 0.5N HCl was titrated until the pink color disappeared.

The saponification value= \((B - S) \times (\frac{N}{W}) \times 56.1\), where

\[ B = \text{ml of 0.5N HCl required to titrate blank} \]
\[ S = \text{ml of 0.5N HCl required to titrate sample} \]
\[ N = \text{normality of HCl solution} \]
\[ W = \text{weight of sample in grams} \]
Safety Assessment of Glyphosate-tolerant Canola

1. Fat stability (RAL): This method measured the time required to attain a specified peroxide value in fats and oils. A sample was held at 97.8°C and aerated with a stream of purified air for a specified amount of time. The aerated sample was sampled at least two times, estimated to give values bracketing 100 milliequivalents of peroxide. A sample (5 g) was dissolved in acetic acid/chloroform solution and treated with saturated potassium iodide. Peroxide oxidized iodide to free iodine which was titrated with standardized sodium thiosulfate solution and starch indicator. Milliequivalents of peroxide per 1000 grams of sample were calculated from the mls of titrant.

\[
\text{Milliequivalents/kg} = \frac{(\text{mls standardized thiosulfate solution}) \times (\text{Sample weight, g})}{1000}
\]

Peroxide values were plotted against time, and the number of hours at which 100 milliequivalents of peroxide were formed, was read off the curve. The length of time required to 100 milliequivalents/kg was assumed to be an index of resistance to rancidity. The relationship between the peroxide value and qualities such as shelf-life, actual rancidity and oxidative stability, has not been established.

m. Sulfur (RAL): The levels of sulfur in the oil are determined based on comparison to a standard curve of known amounts of sulfur (1-20 ppm). Recovery of sulfur through the method was also determined using standard additions. The sulfur content of all samples was determined by aspiration into an inductively coupled plasma atomic emission spectrometer operating at a wavelength of 180.732 nm.

n. Unsaponifiable matter (RAL): Unsaponifiable matter includes substances frequently found in fats and oils that cannot be saponified by caustic alkali but are soluble in ordinary fat solvents. This includes such components as higher aliphatic alcohols, sterols, pigments, and hydrocarbons. A portion of the sample was saponified with hot ethanolic potassium hydroxide. The digested sample was diluted with alcohol and water, and shaken with portions of petroleum ether, extracting unsaponified matter that was ether soluble. The ether extract was rinsed free of caustic, taken to dryness, and weighed. The residue was dissolved in warm, neutralized, ethanol (with phenolphthalein indicator) and titrated with dilute sodium hydroxide to the phenolphthalein endpoint. The weight of the residue, corrected for weight of fatty acids present (mls of 0.02N NaOH it took to achieve the endpoint x 0.0056, which is the conversion from fatty acid to fat), was calculated as percent unsaponifiable matter.

o. Moisture (RAL): The sample was extracted in a chloroform-methanol solution and an aliquot titrated with Karl Fischer reagent. Water in the sample
promoted oxidation of the sulfur dioxide by iodine both present in the reagent to produce sulfuric acid. When no more water remained in the reaction mixture, the excess of free iodine triggered the titration endpoint. This known titration was then calculated to percent moisture, using a known factor of the amount of water consumed per milliliter of titer.

p. Lovibond color analysis (Medallion Labs): Diatomaceous earth was added to a sample and agitated at room temperature and then filtered. The temperature was adjusted to 25°C-35°C, and the color tube was filled to a predetermined level. The tube was placed in the tintometer and matched as close as possible to one of a set of standard glasses.
Safety Assessment of Glyphosate-tolerant Canola

Appendix B. Food Directorate, Health Protection Branch, Canada
letter of no objection to food use of oil derived from GT73 and progeny
Dear [Name],

This will refer to your Novel Food Submission dated June, 1994 concerning a transgenic, glyphosate-tolerant canola (Brassica napus) variety developed through the use of recombinant DNA techniques.

The procedure of note consists of the following:

The introduction, into canola, of the genes encoding for the expression of two proteins: (i) the enzyme, 5-enolpyruvylshikimate-3-phosphate synthetase (EPSPS) from Agrobacterium sp. strain CP4 (CP4-EPSPS); and (ii) the enzyme, glyphosate oxidoreductase (GOX) from Achromobacter sp. strain LBAA. The two proteins together confer tolerance to glyphosate, the active ingredient in Roundup™ herbicide.

It is noted that this glyphosate tolerant canola does not contain the kanamycin resistant marker gene NPTII (derived from Escherichia coli).

Officers of the Health Protection Branch have reviewed the information you provided to characterize the transgenic canola variety. They have also noted that neither the canola wholeseed nor the meal is expected to be used for human consumption. On the basis of the data submitted, we have no objection to food uses of the refined oil from the glyphosate tolerant canola varieties developed through the use of the above-noted transgenic system.
Concerning the acceptability of the meal for use in animal feed, we would suggest that you continue to keep in direct contact with the Department of Agriculture and Agri-food as the evaluation of animal feed falls under the purview of that Department.

By copy of this letter, we are informing our colleagues in the Department of Agriculture and Agri-food of our decision.

Yours truly,

[Blank]

Director General
Food Directorate

cc: [Blank]
Appendix C. Letters from [Blank] and [Blank] of Agriculture and Agri-Food Canada.
To: Monsanto
700 Chesterfield Parkway N.
Mail Zone AA31
St-Louis, Missouri
USA 63198

From: Agriculture Canada Research Centre
430 Blvd. Gouin
St-Jean-sur-Richelieu, Quebec
CANADA J3B 3E6

Subject: Heterozygosity of canola cultivar Westar

Dear [name],

Following your request we compiled our data on heterozygosity of cultivar Westar and estimated that the minimum value for the percentage of heterozygosity in the seed lot we are using is 12.7%.

Our calculation is based on the percentage of heterozygosity detected from one F2 population and two doubled haploid populations derived from three F1 plants produced by a cross between individual plants of Topas and Westar. From the analysis of 126 RFLP markers, we identified the markers that were heterozygous in Westar as those that were polymorphic between the parents Westar and Topas but were not segregating in the F2 due to the absence of the Westar allele. We also did the same calculation with the same markers in the two doubled haploid populations. In addition, we identified the markers that were monomorphic between the parental lines and not segregating in the F2 population but were polymorphic in either one of the two doubled haploid populations. This again indicated heterozygosity at this locus. The number of different markers was added to give the estimate. It is interesting to note that for a given marker, it was scored as being heterozygous in more than one population as expected.

Considering that Westar is heterozygous, you must also realize that this estimate applies only to our seed lot. Probably the estimate in other seed lot will be similar, but it is as likely that more or less heterozygosity will be present in other seed lots as a direct consequence of heterozygosity (heterozygosity generates heterogeneity).

I hope this answer you question about Westar.

Sincerely yours,

[signature]

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Appendix D. Summary of Wholesomeness Studies in Rats, Trout, and Quail.

(1) 4 week rat feeding study with unprocessed and processed canola (184)

PURPOSE
This study, conducted with rats, was undertaken to compare the wholesomeness of unprocessed and processed (defatted, toasted) meal from glyphosate-tolerant canola to the parental (Westar) line. The diets formulated with canola meal were fed ad libitum to rats for approximately one month.

METHODS
Processed and unprocessed canola meal were administered separately in the diet to rats fed ad libitum for approximately one month. Incorporation rates for canola meal in rodent diets were selected based on results of a pilot 4 week rat feeding study conducted earlier with non-transformed processed or unprocessed Westar canola meal (ML-92-331, ML-93-332). Canola seed from parental and glyphosate-tolerant lines (GT73, GT200) used in the rat feeding studies were grown in test plots in Canada in 1992 (Monsanto Study # 92-02-30-01; Experiment #s 92-213-701,2,4,5,6,7,8) (77).

Samples of each line of canola seed were to be pooled into a composite prior to processing into meal (solvent-extracted, toasted). Processing was carried out at the Food Protein Research and Development Center (Texas A&M University, College Station, Texas) (Monsanto Study #92-02-30-01; Experiment # 92-447-702). Processed and unprocessed meal was shipped to Test Diets ( ) for formulation into rodent diets. A proximate analysis (fat, protein, fiber, ash, and moisture) was completed (Ralston) on each line of canola meal prior to formulation of the diets. Based on the proximate analysis, the rodent diets were formulated to be isonitrogenous to and as similar as possible to the nutrient profile for this commercial rodent diet. Processed and unprocessed canola meal from each line was incorporated into the rodent diets at levels of 5 and 15% w/w (50,000 and 150,000 ppm). The unprocessed and processed canola meal supplemented rodent diets were shipped to for conduct of the rat feeding studies.

Male and female rats (approximately 6 weeks of age) were fed rodent chow supplemented with processed or unprocessed canola meal ad libitum. Rat chow consumption was measured for each rat on a weekly basis. During the course of the study, test animals were observed twice daily for mortality and adverse clinical signs. Body weights were recorded twice daily for randomization and weekly for each animal. The intent of the study was to test discrete samples of canola.
April 16, 1994

Monsanto Agriculture Co.,
700 Chesterfield Village Parkway
Chesterfield, Missouri 63198

FAX: [Redacted]

Dear [Redacted],

RE: Glucosinolate values of the TR Canola

I have reviewed your data with [Redacted] and [Redacted], and we note that pretty well all the quality parameters for the transgenic lines RT 200 and RT 73 fall within the range of values recorded for untransformed Westar. However, there appeared to be a tendency for RT 200 and RT 73 to have slightly higher values for the alkyl glucosinolates (averaging 2 to 2.5 µ moles per gram of oil free meal higher when averaged over all trials.) In our opinion, this is a very minor deviation and one which would be expected when single plants are selected from the heterozygous plant population that constitutes the cultivar Westar.

In the late 1980's, [Redacted] and [Redacted] made single plant selections to try and select out of Westar, lines that had the genetic make up to produce no more than 1 or 2 µ moles/g alkyl glucosinolates. It has taken some time to locate these data, but they indicate that in the low alkyl year (unselected Westar 10.8 µ moles) of 1991, they were able to identify single plant lines from Westar that were genetically stable at 6.7 µ moles. Since all the high glucosinolate plants were discarded in the selection process we can not say for certain what the upper level of the glucosinolate range within single plants of Westar would be. However, given our knowledge of biological systems, we can confidently predict that there would be plants within Westar that would produce at least 4 µ moles/g more than the Westar average. We feel very confident in this conclusion, since the evaluation test with low glucosinolate lines was a six rep field test with glucosinolate values determined for each line in all six reps.

It is unfortunate that Monsanto happened to choose plants for transformation that had genotypes capable of producing very slightly more alkyl glucosinolates than the average of the genotypes that make up Westar.

[Redacted]
There is also agreement among canola researchers here, that an increase of 1 to 2 μ moles in the range of about 6 to 16 μ moles is insignificant as to the quality of the product from such strains, particularly since in no test did the lines approach the maximum acceptable value of 20 μ moles per gram oil free meal.

I hope you find this information useful.

Yours truly,

[Name]
Research Scientist

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Diets containing processed or unprocessed meal from each of the canola lines were fed to 10 male and 10 female rats randomly assigned to each group. Control animals (10 rats/sex) were fed that was not substituted with processed or unprocessed canola meal. At the end of the study, all test animals were sacrificed and necropsied. Liver, testes and kidneys were weighed and approximately 40 tissues were collected and saved for each animal.

RESULTS

(1) Diet preparation
Since the test substance consisted of processed or unprocessed canola meal, no practical analytical method was available to measure the levels of canola meal mixed into test diets. Therefore, no analytical tests were performed to determine concentration, uniformity and stability of the test substances incorporated into rodent diets. However, the correct amount of test substances were added to test diets based on gravimetric measurements. The mixers used to prepare test diets for this study have been verified to mix diets homogeneously.

The study was designed to compare the wholesomeness of meal from each GTC line to meal from the parental line. Subsequent to the completion of the study, it was discovered that composites of canola seed for the two GTC lines were inadvertently commingled (77). For example, line GT73 was actually 83% GT73 and 17% GT200 and line GT200 was actually 86% GT200 and 14% GT73. Processed GT73 and GT200 diets contained approximately 50/50 mixtures of seed from both GTC lines, e.g. both processed GTC diets were similar composition (Diet GT73 was actually 53% GT200/47% GT73; diet GT200 was 53% GT73/47% GT200). The Westar parental control seed was not commingled with GTC seed (77).

(2) In-life study results
There was no mortality in the study. No treatment related adverse clinical signs were observed and all animals appeared healthy. Occasional differences in body weights or body weight gains were observed between GTC and parental lines as discussed below.

There were no statistically significant differences in absolute body weights for males or females fed either line of unprocessed or processed GTC meal when compared to parental controls with one exception. A slight, but statistically significant, reduction in absolute body weight was observed for males fed 15% unprocessed GT200/GT73 (86/14) meal at week 4.

Slight, but statistically significant, reductions in body weight gains were observed at some study intervals (Table 10). Body weight gains were reduced.
Safety Assessment of Glyphosate-tolerant Canola

slightly for males fed 15% unprocessed GT73/GT200 (83/17) at weeks 1 and 2. Body weight gains were also reduced for males fed 15% unprocessed GT200/GT73 (86/14) at weeks 2 and 4.

For the processed canola meal feeding studies, body weight gains were slightly reduced for males fed 5% diet (47% GT200/53% GT73) at weeks 1, 2 and 4 and at week 4 for males fed the 15% diet (47% GT200/53% GT73). Females fed the 5% processed diet (47% GT200/53% GT73) exhibited slight, but statistically significant, reductions in body weight gain at weeks 3 and 4. There were no differences in body weight gains for females fed the same 15% diet (47% GT200/53% GT73). There were no differences in body weight gains for males and females fed the other processed GTC diet (53% GT200/47% GT73). A possible explanation for the differences in body weight gain responses for both groups of rats fed similar diet compositions of processed GTC seed is the processing of canola meal prior to formulation of rodent diets.

Canola meal is not fed unprocessed to farm animals as it contains natural toxicants called glucosinolates which are converted by an enzyme in the meal called myrosinase into products that are goitrogens and hepatotoxins. Myrosinase is inactivated during processing of the meal. For our feeding studies, processing of canola seed was carried out on pilot scale (40-100 lbs seed) to provide sufficient material for testing. Each parental and GTC line of seed was processed as a separate lot.

The nitrogen solubility index of the processed canola used in the 47% GT200/53% GT73 diet was lower (14.7) than the other GTC diet (20.0) and the Westar (19.8) diet (77). This means that the lot of canola meal with the lower nitrogen solubility index received more heat treatment than the other canola meal lots. The canola meal with the most heat treatment was used in the 47% GT200/53% GT73 diet which, when fed to rats, resulted in lower body weight gains than the other diets tested.

There were no differences in food consumption between any of the groups.

There were differences in absolute and relative organ weights (liver, kidney, testes) between rats fed the negative control diets and rats fed unprocessed or processed canola meal (GTC and parental lines). However, there were no differences in absolute or relative organ weights between rats fed GTC meal and parental line canola meal (both unprocessed and processed). Therefore, these organ weight changes were attributed to feeding canola meal to rats and not to insertion of genes imparting glyphosate tolerance into canola.

There were no gross pathology findings observed at necropsy that were considered treatment related. The gross findings in this study are commonly observed in control animals at the testing laboratory and the incidences were
CONCLUSIONS
Variable decreased weight gain was observed in some groups fed unprocessed and processed GTC. Most of the decreases were either not dose related or were not consistently observed between groups of rats fed diets of comparable processed canola composition (due to commingling of GTC seed). The differences in weight gain observed between both groups of rats fed similar processed canola meal diets may be due to differences in conditions used to process canola meal (based on differences in the nitrogen solubility index for both lots of processed canola meal tested). Insertion of glyphosate-tolerant genes into canola was not considered to have induced any unintended effects on the wholesomeness of canola meal.

(2) 4 week rat feeding study with processed canola (185)

PURPOSE
As a consequence of commingling of GTC seed in the previous rat feeding study, a second study was undertaken. The purpose of the study was the same as the first, to compare the wholesomeness of GTC and parental line canola meal.

METHODS
This feeding study was carried out with one GTC line (GT73) as commercial development of the other GTC line was discontinued. The feeding of unprocessed ground canola seed to rats was not repeated as this was considered to have little practical relevance to commercial practices since unprocessed ground canola seed is not fed to farm animals. There was no diet control group in this study. The rest of the experimental design was the same as the first study. Processed and unprocessed canola meal were administered separately in the diet to rats fed ad libitum for approximately one month. Incorporation rates for canola meal in rodent diets were selected based on results of a pilot 4 week rat feeding study conducted earlier with non-transformed processed or unprocessed Westar canola meal (ML-92-331, ML-93-332). Canola seed from parental and glyphosate-tolerant line GT73 used in the rat feeding study were grown in test plots in Canada in 1993 (Monsanto Study # 93-63-R-3) (79).

Samples of each line of canola seed were to be pooled into a composite prior to processing into meal (solvent-extracted, toasted). Processing was carried out at the Test Diets (fat, protein, fiber, ash, and moisture) was completed (Ralston) on each line of canola meal prior to formulation of the diets (79). Based on the proximate
Safety Assessment of Glyphosate-tolerant Canola

analysis, the rodent diets were formulated to be isonitrogenous to Purina Rodent Laboratory Chow and as similar as possible to the nutrient profile for this commercial rodent diet. Processed canola meal from each line was incorporated into the rodent diets at levels of 5 and 15% w/w (50,000 and 150,000 ppm). The processed canola meal supplemented rodent diets were shipped to Monsanto's Environmental Health Laboratory for conduct of the rat feeding studies.

Male and female Charles River CD® rats (approximately 6 weeks of age) were fed rodent chow supplemented with processed canola meal *ad libitum*. Rat chow consumption was measured for each rat on a weekly basis. During the course of the study, test animals were observed twice daily for mortality and adverse clinical signs. Body weights were recorded prior to randomization and weekly for each animal. The intent of the study was to test discrete samples of canola.

Diets containing processed meal from each of the canola lines were fed to 10 male and 10 female rats randomly assigned to each group. At the end of the study, all test animals were sacrificed and necropsied. Liver, testes and kidneys were weighed and approximately 40 tissues were collected and saved for each animal.

RESULTS

(1) Diet preparation

Since the test substance consisted of processed canola meal, no practical analytical method was available to measure the levels of canola meal mixed into test diets. Therefore, no analytical tests were performed to determine concentration, uniformity and stability of the test substances incorporated into rodent diets. However, the correct amount of test substances were added to test diets based on gravimetric measurements. The mixers used to prepare test diets for this study have been verified to mix diets homogeneously.

(2) In-life study results

There was no mortality in the study. No treatment related adverse clinical signs were observed and all animals appeared healthy. No differences in body weights or body weight gains were observed between GTC and parental lines with the exception of an increased body weight gain observed at week 3 for male rats fed 15% GT73.

There were no differences in food consumption between any of the groups.

There were no differences in absolute or relative organ weights between rats fed GT73 meal and parental line (Westar) meal with the following exceptions. Absolute liver weight was increased 8% for males and 13% for females; relative liver weights were increased 9% (males) and 16% (females) for rats fed the
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GT73 diet compared to parental line controls. This increase was not observed in the previous rat feeding study with the processed diets (~50/50 GT200/GT73) or with the unprocessed GTC diets (83/17% GT73/GT200; 86/17% GT200/GT73).

The occurrence of increased liver weights in the second rat feeding study but not the first study is attributed to two factors.

(1) Seed from GTC lines contained, in comparison to the parental line, a higher level of a family of natural toxicants called glucosinolates. While the level of glucosinolates was higher in the GTC line fed to rats in the 2nd study (4 gm/kg compared to 1.8 gm/kg for parental line), they were well within limits (>12 gm/kg seed) established for commercial cultivars that are considered to be low glucosinolate varieties (79).

In the 2nd rat feeding study, the level of total glucosinolates in the processed GTC meal rodent diet (15% incorporation rate of GTC meal) was approximately 0.6 gm/kg compared to 0.3 gm/kg for the parental line. Various animal species fed defatted canola meal have been observed to have increased liver weights which is attributed to the presence of glucosinolates and their degradation products in the meal (74). When rodent diets (no canola meal added) are spiked with individual glucosinolates at concentrations of 1 gm/kg and fed to rats for 29 days, relative liver weights may increase up to 7% relative to diet controls (not spiked with glucosinolates) (182). Relative liver weights are increased more (15%) at higher dietary levels (3-7 gm/kg) of glucosinolates (182, 183). The degradation products of glucosinolates are more potent inducers of liver enlargement than the parent molecules. An enzyme called myrosinase which is present in canola catalyzes the hydrolysis of glucose from glucosinolates yielding a variety of degradation products such as alkyl, alkenyl and aromatic nitriles, isothiocyanates etc. When myrosinase was added to rat diets spiked with glucosinolates, liver enlargement was exacerbated over that observed without the addition of the enzyme (74). This is why canola meal is processed in commercial practice to inactivate the enzyme myrosinase and reduce the generation of more toxic glucosinolate degradation products.

(2) Processing of canola meal is another variable which can affect measured responses in rat wholesomeness studies. Processing of canola meal involves heating to inactivate myrosinase and solvent extraction to remove the oil. For our feeding studies, processing of canola seed was carried out on pilot scale (40-100 lbs seed) to provide sufficient material for testing. Each parental and GTC line of seed was processed as a separate lot. In the first rat feeding study, canola meal was processed in the United States and one GTC lot (GT200) had a lower nitrogen solubility index (14.7) than the other GTC (20.0) and parental line (19.8) lot indicating it had received more heat treatment (77). This was
corrected in the second study (canola meal processed in Canada) where both the GTC line and the parental line meal were processed very similarly (based on equivalent nitrogen solubilities of 28.3 and 29.1 respectively) (79). However, the nitrogen solubility for these meal samples was higher than the first feeding study indicating these processed canola meal samples received less heat treatment. If the milder heat processing in the second study resulted in less inactivation of myrosinase, then the higher level of glucosinolates could have resulted in higher levels of degradation products in the GTC line. This could have caused the higher relative liver weights (16%) in the GTC line group compared to parental line controls. As indicated earlier, glucosinolate degradation products are more potent than parent molecules for increasing liver weight.

There were no gross pathology findings observed at necropsy that were considered treatment related. The gross findings in this study are commonly observed in control animals at the testing laboratory and the incidences were similar across all groups.

CONCLUSIONS
The only finding observed in rats fed up to 15% w/w (150,000 ppm) processed glyphosate-tolerant canola meal was increased liver weight. This effect has been reported in various animal species fed canola meal and has been attributed to the presence of glucosinolates and their degradation products (naturally occurring toxicants) in canola. The level of glucosinolates in GTC canola meal was two fold higher than in the parental line which could have accounted for the differences in liver weight. The level of glucosinolates in the GTC line, although higher than the parental line, were still within the limits established for "low" glucosinolate varieties of canola. Cultivars derived from the Westar line from which the GTC line was derived have been reported to have variable glucosinolate levels. There was no evidence that insertion of glyphosate-tolerance genes into canola had any unintended effects on wholesomeness.

PURPOSE
(3) 10 week feeding study with processed canola in trout (186)

METHODS
Processed canola meal was administered in the diet to trout fed for approximately 10 weeks. Incorporation rates for canola meal in trout diets were selected based on commercial aquaculture practices. Canola seed from parental and GTC lines were grown at the same time and in the same seven
Safety Assessment of Glyphosate-tolerant Canola

Field test plots (Monsanto Study # 92-02-30-01; Experiment #s 92-213-701,2,4,5,6,7,8) (78).

The intent of the study was to test 3 lines of processed canola; Westar - (parental line)
glyphosate tolerant line GT73
glyphosate tolerant line GT200

Samples of each line of canola seed were processed into meal at the Food Protein Research and Development Center (Texas A&M University, College Station, Texas) (Monsanto Study #92-02-30-01; Experiment # 92-447-702). The processed meal was the same lot used in the rat feeding study and was shipped to the for formulation into trout diets. Processed canola meal from each line was incorporated into the trout diets at substitution levels of 5, 10, 15 and 20% of the dry matter in place of fish meal on a nitrogen basis and maintaining recommended levels of essential amino acids. Additionally, one diet contained no canola meal (reference diet). The diets were formulated so that all nutritional requirements of the trout would be met.

Trout were obtained from the and were acclimated to laboratory conditions prior to initiation of the study. At study initiation, trout were approximately 10 grams in weight. Trout were grown in 120-L aquaria, 15 fish to a tank. There were 3 replicates per dietary treatment or 45 trout per treatment group. Water in the aquaria was recirculated and filtered; flow rates were adjusted to approximately 1 Liter/minute. Water temperature, dissolved oxygen, ammonia-N and nitrite-N were monitored on a regular basis to maintain acceptable levels for growing trout.

Trout were fed their respective diets at a rate of 3% of their body weight per day, with the daily allotment divided into two equal meals offered in the morning and the afternoon. Each tank was observed for uneaten food (pellets). Each tank with all of the fish was weighed as a group every two weeks to adjust feed allotment. This rate of feeding is near satiation for trout of this size.

At the end of 10 weeks, all fish were removed from the tank and weighed as a group (15 fish/tank) and euthanized by cold shock. Total weight gain/tank over the 10 weeks was divided by the total food offered/tank to calculate feed efficiency. In those tanks in which mortality occurred, average weights of fish at the beginning and end of the study were used to calculate weight gain as a percent increase and average feed offered was used to calculate efficiency. From each tank, 3 average sized fish were used for proximate analysis.
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determinations. Each fish was analyzed separately for moisture, crude protein, fat and ash according to AOAC methods. These values were used to calculate protein efficiency ratios (PER) and protein retention (PR). PER was calculated as wet weight gain divided by protein intake. PR was calculated as final body protein minus initial body protein divided by protein intake times 100.

RESULTS

(1) Diet preparation
Since the test substance consisted of processed canola meal, no practical analytical method was available to measure the levels of canola meal mixed into test diets. Therefore, no analytical tests were performed to determine concentration, uniformity and stability of the test substances incorporated into trout diets. However, the correct amount of test substances were added to test diets based on gravimetric measurements.

The study was designed to compare the wholesomeness of meal from each GTC line to meal from the parental line. Subsequent to the completion of the study, it was discovered that composites of canola seed for the two GTC lines were inadvertently commingled.

Test Group Composition
Westar 100% Westar (control)
GT73 53% GT200/47% GT73
GT200 47% GT200/53% GT73

(2) In-life study results
Survival of fish was very good in the study with only a few deaths occurring randomly in some groups fed either parental or GTC meal. Fish fed diets containing canola meal (both transgenic and parental lines) exhibited slightly lower weight gain and feed efficiencies than fish fed the reference diet (Table 11). There was no statistically significant difference in body weight gain or feed efficiency between the canola fed groups although trout fed diet 53% GT73/47% GT200 tended to have a lower weight gain than the other groups fed 15 and 20% canola meal.

There were no statistically significant differences in feed efficiency between any of the groups although the group fed 20% GTC diet (53% GT73/47% GT200) had a numerically lower feed efficiency.

In regard to proximate analysis of the fish, there were no statistically significant differences in moisture content and ash levels between any group (Table 12). A few statistically significant differences in protein and fat were observed between groups. These differences were not concentration related.
and occurred sporadically in all groups. They were considered to be due to variation inherent in the analysis. There was a trend for protein levels to be lower and fat levels to be higher for fish fed canola meal compared to fish fed the reference diet. Protein efficiency ratios (PER) were not statistically different between groups and followed a similar trend as feed efficiency since they are calculated similarly. Protein retention (PR) values were statistically different for the 5 and 10% diets (47% GTC 200/53% GT73). However, there was no dose relationship to these changes as no differences were apparent at higher (15 and 20%) dietary rates. Therefore, these statistical differences were attributed to random variation. PER and PR values tended to be lower for all groups fed canola meal compared to fish fed the reference diet.

CONCLUSIONS
There were no adverse effects observed in trout that were attributed to the insertion of glyphosate tolerance trait into canola.

(4) 5 Day Quail Feeding Studies with Unprocessed Canola Meal

(1) 1st quail feeding study (187)

PURPOSE
Since birds such as quail may feed on canola seeds in the field, a test of the wholesomeness of glyphosate tolerant varieties of canola seed was undertaken.

METHODS
Northern bobwhite chicks were obtained from the . All test birds were from the same hatch. At study initiation, birds were 10 days of age. Thirty bobwhite chicks of mixed sex were assigned by indiscriminate draw to each treatment and housed in groups of ten birds/pen. Each treatment group was fed nominal dietary concentrations of 20% w/w (200,000 ppm) unprocessed canola meal that was added directly to the game bird ration and mixed together in a Hobart mixer. This dietary level was selected based on the results of a pilot quail feeding study performed with unprocessed Westar variety canola meal (Study # ML-92-281) There were 2 lines of GTC seed meal tested; GT73 and GT200 and control (parental line - Westar) was also tested. An additional basal diet control group (no added canola seed meal) of thirty birds was also included in the study.

Canola seed from the parental line (Westar) and GTC lines GT73 and GT200 were grown at the same time in the same field test plots (Study #92-02-30-01, Experiment #s 92-213-701, 2, and 4) (77) for the bobwhite dietary toxicity study. Seed from these field test plots were also used for the first 4 week rat feeding study. The raw canola seed was ground into meal by Monsanto personnel in St. Louis and shipped to for testing. Canola seed was ground into meal to facilitate homogeneity of mixing which was considered
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easier to achieve with meal than with intact seeds.

The original intent of the study was to test each experimental line independently. Subsequent to the completion of the study, it was discovered that composites of canola seed from the two GTC lines were inadvertently commingled. Thus, line GT73 was actually 83% GT73 and 17% GT200 and line GT200 was actually 86% GT200 and 14% GT73. The Westar control seed was not commingled with GTC seed.

Each control and treatment group was fed the test diet for 5 days and then switched to basal (unsupplemented) diets for the last 3 days of the study. Food and water were provided ad libitum. Food consumption was recorded daily for each pen (days 1-5) and average food consumption/pen was recorded for days 6 to 8. Food consumption is an estimate due to the unavoidable wastage by the birds. Individual body weights were recorded at study initiation, on study day 5 and at study termination. The average temperature in the brooding compartment of the pens was 39°C ± 2°C. Average ambient room temperature was 24.1°C ± 1.0°C with an average relative humidity of 29% ± 10%. The photoperiod was 16 hrs. light/day during acclimation and throughout the test. Birds were observed twice daily during the study for mortality or signs of toxicity.

RESULTS

There were no mortalities observed during the study in any of the groups tested. One bird in the parental control group was observed to have a head injury on day 8. All other birds in this study were normal in appearance. Body weight gain and estimated food consumption were comparable for quail fed canola seed meal from the GTC lines and those fed the control (parental line) and basal diet.

Consumption of a diet containing 200,000 ppm (20% of the diet w/w) of raw canola seed meal is equivalent to eating the following number of canola seeds:

20% X 6 gm food/bird/day = 1.2 gm canola flour/bird/day.

Body weight for a young quail is approximately 25 gm, therefore, 1.2 gm/day/25 gm/bird = 48 gm flour/kg body weight bird.

The average weight of canola seed of the westar variety is approximately 0.005 gm (personal communication from [redacted], project coordinator for glyphosate-tolerant canola, Monsanto Agricultural Group).

Approximate consumption of canola seeds was therefore 9600 seeds/kg body weight bird per day. In a commercial field, there will be about 200-300 seed produced per canola plant and about 200 plants per square meter. Thus, these
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bird consumed the equivalent of about 32 plants/kg bw per day or about 0.16 m² of a field.

CONCLUSIONS
There was no evidence of any differences in the wholesomeness of glyphosate tolerant canola compared to parental line canola when fed to quail.

(2) 2nd quail feeding study (188)

METHODS
Northern bobwhite chicks were obtained from the [redacted]. All test birds were from the same hatch. At study initiation, birds were 10 days of age. Thirty bobwhite chicks of mixed sex were assigned by indiscriminate draw to each treatment and housed in groups of ten birds/pen. Each treatment group was fed dietary concentrations of 20% w/w (nominal 200,000 ppm) unprocessed canola meal that was added directly to the game bird ration and mixed together in a Hobart mixer. This dietary level was selected based on the results of a pilot quail feeding study performed with unprocessed Westar canola meal (Study # [redacted] volume). GTC cottonseed meal (line GT73) and parental line (Westar) were tested. An additional basal diet control group (no added canola seed) of thirty birds (3 groups, 10 birds/group) was also included in the study.

Canola seed from the parental line (Westar) and GTC line GT73 were grown at the same time in the same field test plots (Study # 93-63-R-3) (79) for the bobwhite dietary toxicity study. The raw canola seed was ground into meal by Monsanto personnel in St. Louis and shipped to [redacted] for testing. Canola seed was ground into meal to facilitate homogeneity of mixing which was considered easier to achieve with meal than with intact seeds.

Each control and treatment group was fed the test diet for 5 days and then switched to basal (unsupplemented) diets for the last 3 days of the study. Food and water were provided ad libitum. Food consumption was recorded daily for each pen (days 1-5) and average food consumption/pen was recorded for days 6 to 8. Food consumption is an estimate due to the unavoidable wastage by the birds. Individual body weights were recorded at study initiation, on study day 5 and at study termination. The average temperature in the brooding compartment of the pens was 37°C ± 2°C. Average ambient room temperature was 28°C ± 0.6 °C with an average relative humidity of 70% ± 9%. The photoperiod was 16 hrs. light/day during acclimation and throughout the test. Birds were observed twice daily during the study for mortality or signs of toxicity.
**RESULTS**

There were no mortalities observed during the study in any of the groups tested. All birds in this study were reported to be normal in appearance. When compared to the parental control group, quail in the GTC group exhibited a slight reduction in body weight during the exposure period (day 0 - day 5). However, there was no apparent reduction in body weight for the entire test duration (day 0 - day 8) for GTC quail. Feed consumption was comparable for all groups.

**CONCLUSIONS**

The wholesomeness of glyphosate tolerant canola was considered to be comparable to parental line canola when fed to quail.
Appendix E. Additional information on sequence searches for homology comparisons to known allergens and toxins

In order to gain insight on the potential allergenicity or toxicity of CP4 EPSPS and GOX proteins, which have no known allergenic or toxic properties, 121 allergenic proteins and 1,935 toxin proteins were extracted from the Pir protein, Swissprot, and Genpept protein databases to compare amino acid sequence homology to these introduced proteins. These sequences were compiled by searching the databases for proteins with keywords matching the text pattern “allergen” or “toxin”.

The database of allergens included proteins and peptides that ranged from pollen allergens to allergens from insect venoms. The database of allergenic proteins covers all available allergens in the database of protein sequences. The toxin database is more complex. Not all of the toxins in the toxin database are toxic to humans. There are several proteins in the database which are derived from *R. thuringiensis* cry genes, known only to be toxic to certain insects. Still other proteins are not even toxic proteins, but are involved in the response to a toxin protein, such as a receptor, or a precursor protein which may not be toxic until the protein is processed into its active form. The database of toxin proteins and peptides includes known sequences of ribosome inactivating proteins (RIPs), neurotoxins from *Clostridium botulinum*, scorpions and spiders, diphtheria toxins, and snake venoms. Because there are several different protein databases available, including Pir, Swissprot, and Genpept, duplications of proteins exist within the allergen and toxin databases.

Methods
All of the protein comparisons were assisted by the Genetics Computer Group (GCG) of Madison, WI., sequence analysis software package (version 7.1 March 1992) (178). The peptide sequence used for comparisons between the database of allergen and toxin proteins was translated from a given plasmid region encoding the protein of interest. For CP4 EPSPS, pMON17190 was used as the source of the gene sequence (101). The amino acid sequence of the CP4 EPSPS mature protein encoded by pMON17190.pep is identical to the amino acid sequence of the CP4 EPSPS protein encoded by pMON21104 which was utilized to provide CP4 EPSPS from *E. coli* for safety studies (2). For GOXv247, pMON17237 which is the identical sequence in the plant transformation vector was used as the source of the gene sequence for this comparison. The amino acid sequence of GOXv247 encoded by pMON17237 differed from the GOXv247 amino acid sequence encoded by pMON21115, the sequence used in the acute toxicity study in mice, by five amino acids at the N-terminus (see Section on GOX in IV.B.1.d.ii for discussion). Comparisons of the CTPs used to target CP4 EPSPS and GOXv247 were run separately using sequences encoded in pMON17237. Files containing lists of known allergen and toxin proteins were developed using the GCG command “STRINGSEARCH”.

123
"STRINGSEARCH" searches the user defined databases for matches of the user defined "text pattern". Each of the available protein databases were analyzed separately using the following logicals:

"Allergen"

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<th>LOGICAL</th>
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<th>OUTPUT_FILE</th>
</tr>
</thead>
<tbody>
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<td>Swissprot:*</td>
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<td>Release 25 (Apr '93)</td>
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<tr>
<td>Genpept:*</td>
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</tr>
<tr>
<td>Pir1:*</td>
<td>Pir Protein</td>
<td>Complete Records</td>
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<td>Pir1all.strings</td>
</tr>
<tr>
<td>Pir2:*</td>
<td>Pir Protein</td>
<td>Complete Records</td>
<td>Release 36 (Apr '93)</td>
<td>Pir2all.strings</td>
</tr>
<tr>
<td>Pir3:*</td>
<td>Pir Protein</td>
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<td>Pir3all.strings</td>
</tr>
</tbody>
</table>

"Toxin"

<table>
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</tr>
</thead>
<tbody>
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<tr>
<td>Pir3:*</td>
<td>Pir Protein</td>
<td>Complete Records</td>
<td>Release 36 (Apr '93)</td>
<td>Pir3alltox.strings</td>
</tr>
</tbody>
</table>

For example, at the prompt:

STRINGSEARCH through what sequence(s) (* GenEMBL:* *) ?

The logical "Swissprot:*" was entered to replace the default "GenEMBL:*" logical to identify peptide sequences only from the Swissprot database. Each of the remaining protein databases were searched through complete definitions. The results of the "STRINGSEARCH" generated five files containing proteins matching the text pattern "allergen" or "toxin". The five output files or filenames for "allergen" matches are: Pir1all.strings, Pir2all.strings, Pir3all.strings, Swissprotall.strings, and Genpeptall.strings, and matches for "toxin" are: Pir1alltox.strings, Pir2alltox.strings, Pir3alltox.strings, Swissprotalltox.strings, and Genpeptalltox.strings. All "allergen" and "toxin" proteins were retrieved from the respective databases using the GCG command "FETCH@filename.STRINGS", until all of the protein sequences from each database were retrieved into a VAX directory.

FASTa is the standard method (102-105) for rapid comparison of a query sequence, or defined sequence of interest, to a entire nucleotide or protein database. CP4 EPSPS (pMON17190.pep), GOX (pMON17237.pep), CTP1.pep (GOX CTP, Figure 4), or CTP2.pep (CP4 EPSPS CTP, Figure 4) was the query sequence used in all FASTa comparisons. Instead of comparing sequences to each entire protein database, the FASTa comparison was divided into two separate databases limited to proteins recognized from "STRINGSEARCHII" using the text pattern "allergen" and text pattern "toxin". FASTa uses the algorithm developed by Pearson and Lipman (102) to search
for similarities between the query and any group of sequences. Basically, the FASTa algorithm uses four steps to calculate three scores (\textit{init1}, \textit{initn}, and \textit{opt}) that characterize sequence similarity. The first step uses a rapid technique for identifying shared identities or similarities between the two sequences. This method is similar to the technique which has been described by Wilbur and Lipman (103). This rapid technique is based on a lookup table to locate identities between the query and the database of allergens and toxins. The \textit{ktup}, or word size, indicate how many consecutive identities are required for a match. By searching protein databases, the amino acid's chemical similarity or mutational similarity is also considered when a match is assigned. A \textit{ktup} value of 2 is the standard or default value for protein database searches (178). A \textit{ktup} value of 1, may be applied for a more sensitive database search, but this value also increases false positive matching between the query and database sequences. The first step in the calculation of the scoring matrix in FASTa is to identify the highest pairs (\textit{ktup}=2) of identities shared between the two sequences. Next, the ten highest regions, without gaps in the sequence, that contribute to the highest scores, are rescanned using the PAM250 matrix (PAM = percent accepted mutation). The PAM250 (MDM78) matrix (179,180) was derived from the amino acid replacement analysis among related proteins. It specifies a range of positive scores for common or likely mutations and a range of negative scores for unlikely substitutions or mutations. Specifically, the values are the log of the probability that the amino acid residue arose from the mutation of a common ancestor, divided by the probability that the sequences are related by chance. Positive values indicate that the amino acid residues are more likely than chance to have come from a common ancestor and negative numbers indicate that an evolutionary relationship is less likely than chance. The PAM250 scores of the initial alignment are summed and reported as the \textit{init1}. In general, the higher the PAM250 score, the longer the stretches of homology between the two sequences. Next the \textit{initn} score is calculated by joining the initial alignments (\textit{init1}) minus a gap penalty (usually 20 for each gap). The \textit{initn} scores are used to rank the homologies generated between the query and the database, from the highest homology to the lowest homology. Finally an \textit{opt} (optimized) score is calculated considering only those residues that lie in a band 32 amino acid residues wide centered on the highest scoring region (\textit{init1}). The results of the FASTa comparison with CP4 EPSPS to the allergen and toxin protein database are described in the next section.

\textbf{Results}

All four sequences (CP4 EPSPS, GOXv247, CTP1, and CTP2) were compared to the generated database of known allergens and toxins using the FASTa algorithm for rapid database comparison. The results of the FASTa comparison used \textit{ktup}=2 for each of the protein database. The highest (most homologous) PAM250 scores used for ranking the sequences (\textit{initn}) for native proteins were: 38 for CP4 EPSPS, 37 for GOXv247, 29 for CTP1, and 31 CTP
2 for the allergen database. The highest PAM250 scores (initn) noted in searches of the native sequences against the toxin database were: 54 for CP4 EPSPS, 69 for GOXv247, 50 for CTP1, and 46 for CTP2. These scores were then compared to those obtained using randomized amino acid sequences of the same four proteins. The highest scores (initn) against allergens were: 41 for CP4 EPSPS, 72 for GOXv247, 29 for CTP1, and 25 for CTP2. Lastly, the highest scores (initn) determined against toxins for the randomized sequences were: 79 for CP4 EPSPS, 72 for GOXv247, 43 for CTP1, and 39 for CTP2.

A low PAM250 score or initn score indicates that the length of homology or similarity between the two sequences is short. A low initn score does not necessarily indicate that significant homology does not exist. For example, the length of the allergenic proteins or allergenic protein fragments ranged between 10 amino acids and 398 amino acids. Since the generated database of allergens consisted of several short allergenic peptides, 100% identity between the pMON17190.pep and the short allergenic peptide may generate a low initn score. For example, a peptide of ten amino acid identical to the first ten N-terminal amino acids of CP4 EPSPS was compared using the FASTa algorithm. An initn score of only 43 was calculated, even though there was 100% identity between the two sequences. Also another test of the FASTa algorithm was performed by comparing CP4 EPSPS to the entire database of protein sequences. Initn scores of up to 805 were generated from the protein database to the CP4 EPSPS, and the highest ranking initn scores were from Aro genes (encoding EPSPS proteins), in which considerable homology is expected. Therefore, it is the task of the biologist to independently examine each of the alignments for potential biological significance. If the proteins in the database are of comparable length to the query, which was the case in each of the four searches conducted, then a low initn score indicates little or no biological homology, unless the homology can be assigned to an active site in the protein or a site known to be important for its function or activity.

The data generated compares our protein sequences to the allergenic proteins and toxins extracted from each of the respective protein databases. The low initn numbers indicate that there are no long stretches of homology between CP4 EPSPS, GOXv247, or the CTPs and any of the allergens or toxins within the databases. The low initn scores from the top ten FASTa scores did not contain any small peptides from which a 100% identity may generate a "low" initn score. Each alignment or FASTa output within the allergen database and the toxin database was examined and in all cases, there was no indication of significant homology between CP4 EPSPS, GOXv247, CTP1, and CTP2 and the database of allergenic or toxic proteins.

The significance of the alignments was tested using the GCG command "SHUFFLE" to randomize the native sequence, but keeping the amino acid composition identical. This type of analysis using a randomized protein to
determine the statistical significance of the alignments is known as Monte Carlo analysis (181). The resulting randomized queries were named pMON17190.shuf1 for CP4 EPSPS, pMON17237.shuf1 for GOXv247, ctp1.shuf1 for CTP1, and ctp2.shuf1 for CTP2. The amino acid alignment between the native and randomized sequences was constructed using the GCG program- "GAP". The gap comparison using the default conditions of the GAP program determined that the two proteins shared only 19.570% identity and 44.869% similarity. The shuffled protein (pMON17190.shufl) was compared to the identical database of allergenic proteins and toxin proteins using the same word size (k=2) as pMON17190.pep. The range of initn scores for the randomized peptide are given above. Most significantly, the initn scores from the allergen database and the toxin database, generated with each of the randomized proteins, resemble the initn scores generated with each of the unrandomized proteins. This indicates that the generated alignments between the database of allergen proteins and toxin proteins was determined by random distribution of amino acids in a peptide sequence.

The 10 highest scores from each database were compared between each of the native proteins and each of their respective scrambled sequences. The results from one randomization of the CP4 EPSPS, GOXv247, CTP1, and CTP2 peptide sequences clearly indicates that these proteins have no relationship, other than a random distribution of its amino acids, to the database of allergens and toxins.

**Conclusion**

The evidence presented indicates, using the best methods available today, that CP4 EPSPS and GOX do not share any sequence similarity between the database of known sequenced allergens and toxins. We make this conclusion based on the fact that a randomized protein containing the identical amino acid contents as CP4 EPSPS and GOX, compared to all of the sequenced allergens and toxins, produced a similar range and homology result as found for the native sequences. This analysis indicates that the alignments were based entirely on the random occurrence that the two protein sequences shared any similarities. It is clear from the work reported that CP4 EPSPS and GOX proteins do not share extensive amino acid homology to known protein allergen sequences or toxin sequences that have been deposited in the searched protein databases.