FOOD DERIVED FROM GLYPHOSATE-TOLERANT CANOLA LINE GT73

A Safety Assessment

TECHNICAL REPORT SERIES NO. 8

AUSTRALIA NEW ZEALAND FOOD AUTHORITY
November 2001

ISBN 0 642 34537 6
ISSN 1446-4977
Published November 2001

ANZFA Australia
PO Box 7186
Canberra BC ACT 2610
Australia
Tel +61 2 6271 2241
Fax +61 2 6271 2278
Email info@anzfa.gov.au

ANZFA New Zealand
PO Box 10599
Wellington
New Zealand
Tel + 64 4 473 9942
Fax +64 4 473 9855
Mail nz.reception@anzfa.gov.au

TABLE OF CONTENTS

SUMMARY

Food derived from glyphosate-tolerant canola line GT73 has been assessed to determine its suitability for human consumption. The evaluation criteria included characterisation of the transferred genes, analysis of changes at the DNA, protein and whole food levels, stability of the introduced genes, evaluation of intended and unintended changes and assessment of the potential allergenicity or toxicity of any newly expressed proteins.

Nature of the genetic modification

A genetically modified canola line (GT73) was generated by the transfer of the CP4 EPSPS and *gox* genes which both confer glyphosate tolerance to the plant. The protein products are both enzymes that have a distinct mode of action. The CP4 EPSPS enzyme is not sensitive to applications of glyphosate and the GOX protein can degrade the herbicide providing additional tolerance.

The molecular and genetic analyses indicated that the introduced genes have been stably integrated into the plant genome and were stably inherited for multiple generations.

General safety issues

The novel CP4 EPSPS and GOX proteins were detected in the seed at low levels (>0.02% fresh weight). Additionally, the only canola product considered to be a human food fraction is oil which has no DNA or protein present as they are removed during processing.

The glyphosate-tolerant canola line GT73 does not contain any antibiotic resistance genes and therefore poses no risk to the development of antibiotic resistant pathogenic bacteria.

Toxicological issues

The newly expressed CP4 EPSPS and GOX proteins in the glyphosate-tolerant canola lines have been evaluated for their potential toxicity. Neither protein possesses any characteristics of known toxins. No signs of toxicity were observed in mice exposed to doses of these proteins 1000 fold greater than likely human exposure.

In addition, exposure of the proteins to simulated mammalian digestive systems resulted in rapid digestion of the proteins. The proteins do not have chemical or physical characteristics that are typical of known food allergens and do not share significant amino acid sequence similarity with known allergens. Therefore, there is no evidence for any potential toxicity or allergenicity for either protein in humans.

Nutritional issues

The compositional analyses were comprehensive and demonstrate that there are no significant differences in the levels of major constituents, nutrients, anti-nutritional

factors or natural toxicants between glyphosate-tolerant canola line GT73 and the control canola line Westar. The components measured were proximate (protein, fat, moisture, fibre, ash, carbohydrates and calories), fatty acids and amino acids.

The major toxicants and anti-nutrient factors in canola were also assessed. Erucic acid levels in the canola oil were lower than in parental canola lines and glucosinolate levels in canola meal were higher than the control line but within an accepted industry standard.

Analysis of the refined, bleached and deodorised oil, which is the only product for human consumption, demonstrated that the composition is comparable, in all respects, to the control Westar line.

These analyses confirm that glyphosate-tolerant canola line GT73 is nutritionally and compositionally comparable to other canola lines and that no health or safety risks are posed by consuming food derived from the genetically modified canola.

Conclusion

No potential public health and safety concerns have been identified in the assessment of glyphosate-tolerant canola GT73. Based on the currently available data, food derived from glyphosate-tolerant canola line GT73 is comparable to food derived from conventional canola in terms of its safety and nutritional adequacy.

FOOD DERIVED FROM GLYPHOSATE-TOLERANT CANOLA LINE GT73

A SAFETY ASSESSMENT

INTRODUCTION

A safety assessment has been conducted on food derived from canola which has been genetically modified to be tolerant to the herbicide glyphosate. The genetically modified canola plants are referred to an glyphosate-tolerant canola line GT73.

The glyphosate-tolerant phenotype has been developed in canola through two distinct mechanisms: firstly, the introduction of an enzyme that is not sensitive to applications of glyphosate and secondly, the introduction of an enzyme that can degrade the herbicide. Canola based products produced from these plants may have been imported into Australia and New Zealand for several years.

Canola seeds are processed into two major products, oil and meal. The oil is the only product for human consumption and the only product assessed for approval in this application. Toasted meal is used as an animal feed. Canola seed oil is a premium quality oil that is used in a variety of manufactured food products including salad and cooking oil, margarine, shortening, mayonnaise, sandwich spreads, creamers and coffee whiteners. As a result of the processing steps, canola oil contains negligible protein. Canola oil may be present as an ingredient in some imported processed foods.

Canola (*Brassica napus*) is a leading oilseed crop because it has a good ratio of fatty acids comprising a very low level of saturated fatty acids, a moderate level of polyunsaturated fatty acids and a high level of the monounsaturated fatty acid, oleic acid (McDonald, 1999). It is also considered an important export crop in Australia. Over 550 000 tonnes of canola were produced in 1995-1996 with over 60% being exported. All new canola oil varieties including canola from glyphosate-tolerant canola line GT73 must meet Codex specifications for oil quality. All canola varieties that meet Codex specifications also meet specifications for canola as outlined in the Australian *Food Standards Code*.

DESCRIPTION OF THE MODIFICATION

Methods used in the genetic modification

Studies evaluated:

Kolacz, K.H. et al. 1994. Glyphosate-tolerant canola: plant transformation vectors and transformation procedure. Monsanto Company, USA 63198.

Using *Agrobacterium*-mediated transformation, the parental canola line (Westar) was transformed with the plasmid, PV-BMNGT04 which carries the *gox* and CP4 EPSPS genes. Both genes allow the selection of transformed plants under application of glyphosate.

Glyphosate-tolerant canola line GT73 was produced by the above transformation event as a result of the transfer of the following genes:

. The 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS) gene from *Agrobacterium sp*. strain CP4 EPSPS under the control of the modified figwort mosaic virus 35S promoter.

. the glyphosate oxidoreductase (*gox*) gene from *Ochromobactrum anthropii* strain LBAA [previously *Achromobacter sp*] under the control of the modified figwort mosaic virus 35S promoter. The gene encodes the GOXv247 variant protein.

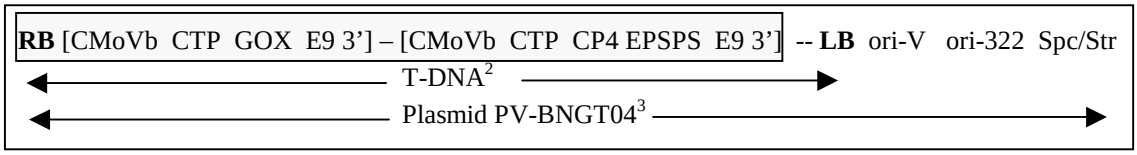
The *Agrobacterium* mediated DNA transformation system is well understood (Zambryski, 1992). The genes of interest were inserted into the plasmid between DNA sequences known as the Left and Right Borders (LB and RB). These sequences have been isolated from Ti plasmids from *Agrobacterium* and are 25 base pair repeat sequences. The Left and Right Borders delimit the DNA to be transferred (T-DNA), which includes the CP4 EPSPS and *gox* genes.

Genes outside the Left and Right Border segments are generally not transferred during the transformation. The genes in the plasmid outside the border sequences are:

. the vegetative origin of replication (ori-V) that permits plasmid replication in *Agrobacterium* (Rodgers *et al*, 1987).
. the bacterial origin of replication (ori-322) that permits plasmid replication in *Escherichia coli* (Sutcliffe, 1979)
. the spectinomycin (spc) and streptomycin (str) genes for antibiotic resistance (Fling *et al*, 1985).

The gene arrangement is shown in Figure 1.

Figure 1: Schematic diagram of PV-BNGT04[1]



[1]See text or Table 1 for an explanation of the abbreviations.
[2]The boxed region denotes the T-DNA – genes within the LB and RB which are transferred to canola.
[3]The genes in the entire plasmid. Genes outside the LB and RB are not transferred.

Function and regulation of the introduced gene(s)

Studies evaluated:

Barry, G.F. et al, 1994. Cloning and expression in *Escherichia coli* of the glyphosate-to-aminomethylphosphonic acid degrading activity from *Achromobacter sp*. strain LBAA. Monsanto Company, USA 63198.

Padgette, S.R. et al. 1994. Characterisation of glyphosate oxidoreductase. Monsanto Company, USA 63198.

Woodward, H.D. etal. 1994. Isolation and characterisation of a variant of the enzyme glyphosate oxidoreductase with improved kinetic properties. Monsanto Company, USA 63198.

Each gene transferred to canola requires regulatory sequences that allow it to be transcribed into RNA and then translated into a protein product. A promoter is the key control element that enables a gene to be transcribed into messenger RNA (mRNA) and a terminator is a DNA (polyadenylation) sequence which stops the transcription of mRNA. These sequences can be unique in each organism and thus regulatory elements that already exist in plants are often used in gene constructs to enable functioning in the plant. Regulatory regions for each of the transferred genes are summarised in the table below.

Table 1: Description of Genes transferred to Canola

Gene	Region	Name	Origin
CP4 EPSPS	Promoter Chloroplast Transit Peptide Terminator	P-CMoVb CTP 2 E9 3'	Modified figwort mosaic virus 35S promoter CTP sequence from *A. thaliana* EPSPS gene Pea rbcS E9 gene
gox	Promoter Chloroplast Transit Peptide Terminator	P-CMoVb CTP 1 E9 3'	Modified figwort mosaic virus 35S promoter CTP sequence from *A. thaliana* SSU1A gene Pea rbcS E9 gene

CP4 EPSPS

EPSPS is an essential enzyme involved in the biosynthesis of the aromatic amino acids by the shikimate metabolic pathway. This metabolic pathway is present in all plants, bacteria and fungi (Haslam, 1993). Thus plants naturally contain an EPSPS enzyme but they are inhibited by the herbicide glyphosate, whereas the bacterial EPSPS enzyme is not inhibited (Schültz *et al*, 1985). The *Agrobacterium*–derived CP4 EPSPS gene has a reduced affinity for glyphosate and has been transferred to canola to confer tolerance to glyphosate.

The CP4-EPSPS gene is fused to the following regulatory sequences: the 35S promoter from a modified figwort mosaic virus (P-CMoVb) and the 3' end of the pea rbcS E9 gene (E9 3'). The bacterial EPSPS enzyme is targeted to the plastid using a chloroplast transit peptide sequence derived from the *Arabidopsis thaliana* EPSPS (CTP 2) which has been shown to deliver bacterial EPSPSs to the chloroplasts of higher plants where the aromatic amino acid biosynthetic pathway and endogenous EPSPS activity is located (della Ciopa *et al*, 1986).

gox

The *gox* (glyphosate oxidoreductase) gene is derived from *Ochromobactrum anthropii* strain LBAA [formerly *Achromobacter sp*] which is a commonly found bacteria in the soil. As in other bacteria, it degrades glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate thus effectively inactivating the herbicide (Pipke and Amrhein, 1988; Barry *et al*, 1992). AMPA is the principal metabolite of glyphosate that is degraded by several microorganisms and glyoxylate is commonly found in plant cells and is broken down by the glyoxylic pathway for lipid metabolism.

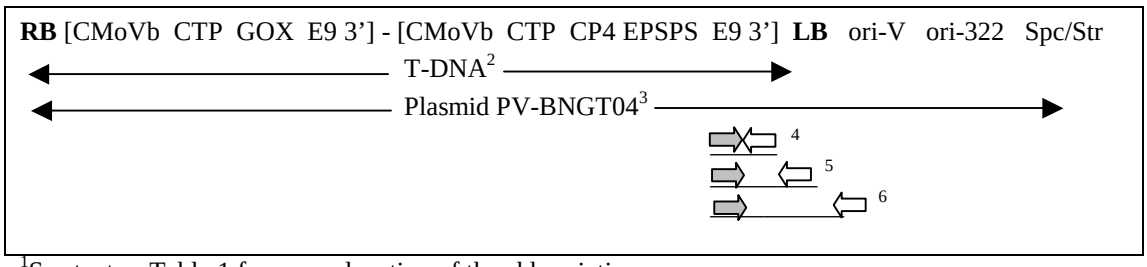
The *gox* gene is fused to the following regulatory sequences: the 35S promoter from a modified figwort mosaic virus and the 3' end of the pea rbcS E9 gene (E9 3'). The gene is targeted to the plastid by the action of the N-terminal of the small subunit 1A of the ribulose 1,5-bisphosphate carboxylase chloroplast transit peptide of *Arabidopsis thaliana* (CTP1) (Timko *et al*, 1988) which has been fused to the gene.

Characterisation of the genes in the plant

Southern blot analysis is used to detect the presence of specific DNA sequences and to determine the mode, number and stability of inserted DNA. It was used by the applicant to demonstrate that there is a single DNA insertion in line GT73 consisting of the T-DNA (ie. the DNA contained within the left and right border as shown in Figure 1). The T-DNA contains one complete copy of the CP4 EPSPS gene and a complete copy of the *gox* gene and their respective regulatory sequences.

PCR analyses using specifically designed primers for the T-DNA, the Left Border region and vector DNA also supported that only the T-DNA is inserted into the plant genome. A diagram of these primers is shown in Figure 2. PCR analysis supported that no other vector DNA including the antibiotic resistance genes was transferred to glyphosate tolerant canola line GT73.

Figure 2: Primer locations for PCR analysis of the transferred genes[1]



[1]See text or Table 1 for an explanation of the abbreviations.
[2]Denotes the T-DNA – genes within the LB and RB which are transferred to canola.
[3]The entire plasmid. Genes outside the LB and RB are not transferred.
[4]Both PCR primers are within the T-DNA (within the E9 3' element) and produce a 252 bp product in GT73
[5]One PCR primer is within the T-DNA (within the E9 3' element) and the other primer lies across the E9 3' and LB sequences and produces a 559 bp product in GT73.
[6]One PCR primer is within the T-DNA (within the E9 3' element) and the other primer is located in the vector sequence and produces a 661 bp product which was not produced using GT73 DNA.

Stability of the genetic changes

Studies evaluated:
Kolacz, K.H. et al. 1994. Determination of the stability of the GT genes in glyphosate-tolerant canola line GT73. Monsanto Company, USA 63198.

The stability of inserted DNA was demonstrated from R_3 generation and R_5 generation using Southern blot analysis. Segregation analysis for line GT73 is consistent with a stable, single dominant gene segregating according to Mendelian genetics. The glyphosate-tolerant phenotype and inheritance pattern have been consistent for multiple generations.

Conclusions regarding the genetic modification

Glyphosate-tolerant canola line GT73 contains two new genes - CP4 EPSPS and *gox* – which were transferred using an *Agrobacterium* mediated transformation system. No other genes were transferred during transformation. The DNA has transferred into the canola genome as a single and stable insert.

GENERAL SAFETY ISSUES

Canola is grown in Australia largely as an export crop but some processed foods, including imported processed foods may contain genetically modified canola. These foods include salad and cooking oil, margarine, shortening, mayonnaise, sandwich spreads, creamers and coffee whiteners.

The glyphosate-tolerant canola has been evaluated against the safety assessment guidelines developed by ANZFA (ANZFA, 1999a). As the data presented is for canola seed and processed fractions, in particular, refined, bleached and deodorised canola oil (RBDO), the safety assessment issues relate to Group D foods – food ingredients.

History of the use of canola as a food source

Rapeseed (*Brassica napus* or *Brassica campestris*) was not widely grown as a commercial crop for consumption until the late 1940's and it was previously grown largely for the production of oil to be used as an industrial lubricant. Early rapeseed varieties were very high in erucic acid and glucosinolates, which made them unsuitable for consumption. Initial endeavours in breeding programs resulted in the development of varieties with lower amounts of these natural toxicants but were found to have poor yields and high susceptibility to disease.

In the 1970's, very intensive breeding programs in several countries including Australia produced high quality varieties that were significantly lower in erucic acid and glucosinolates. These varieties are largely *Brassica napus* species and were called canola, the term denoting an industry standard that these varieties contain an erucic acid level below 2% in oil and less than 30 micromoles of total glucosinolates in toasted meal. Canola oil is the only fraction considered to be fit for human consumption and toasted meal is used in animal feeds.

The demand for canola has risen sharply, particularly in canola oil, margarine and other canola based products. Canola is the leading oilseed crop in Australia and is a growing export industry. These canola-based products are routinely used in food and have a history of safe use.

Nature of the novel protein

CP4 EPSPS Protein

Studies evaluated:

Donovan, D.E. etal. 1993. Validation of the ELISA V3.0 excel macro and template. Monsanto Company, USA 63198.

Taylor, M. 1994. Validation of an indirect ELISA to quantitate of CP4 EPSPS in genetically improved canola. Monsanto Company, USA 63198.

Harrison L.A., et al. 1993. Characterisation of microbially-expressed protein: CP4 EPSPS. Monsanto Company, USA 63198.

Harrison L.A., et al. 1994. Equivalence of plant- and microbially expressed proteins: CP4 EPSPS from glyphosate-tolerant canola and *E. coli*. Monsanto Company, USA 63198.

Heeren, R.A. et al. 1993. The purification of recombinant *Escherichia coli* CP4 5-enolpyruval-shikimate-3-phosphate synthase for equivalence studies. Monsanto Company, USA 63198.

The CP4 EPSPS gene is a 47.6 KDa protein consisting of a single polypeptide of 455 amino acids. In the genetically modified canola line, the CP4 EPSPS gene has been fused to the *A. thaliana* EPSPS CTP. *In vitro* chloroplast uptake assays have shown that the *A. thaliana* EPSPS CTP delivers CP4 EPSPS to the chloroplast and is subsequently cleaved from the pre–protein, yielding mature CP4 EPSPS with no CTP amino acids retained (della Ciopa *et al*, 1986). It has been shown that the chloroplast transit peptides are rapidly degraded after cleavage *in vivo* by cellular proteases. Thus, the only newly expressed protein present in the glyphosate-tolerant canola line would be mature CP4 EPSPS, without any additional CTP residues at the amino terminus.

GOX protein

Studies evaluated:

Harrison L.A., et al. 1994. Characterisation of microbially-expressed protein: GOX (M4-C1) and GOXv247 (M4-C1). Monsanto Company, USA 63198.

Harrison L.A., et al. 1994. Characterisation of GOX (canola) and GOXv247 (canola) and assessment of equivalence relative to E. coli GOX (M4-C1) and GOXv247 (M4-C1). Monsanto Company, USA 63198.

Nickson, T.E. 1994. Validation of an ELISA for the detection and quantification of glyphosate oxidoreductase (GOX). Monsanto Company, USA 63198.

The *gox* gene encodes a single polypeptide of 431 amino acids with a molecular mass of 46.1 KDa. The glyphosate oxidoreductase (GOX) protein breaks glyphosate down

to aminomethylphosphonic acid (AMPA) and glyoxylate. The metabolism and toxicology of AMPA is discussed further in Section 6. As the gox gene is under the control of a constitutive promoter in glyphosate-tolerant canola, the GOX gene will be present but targeted to the chloroplast using the *A. thaliana* SSU1A gene chloroplast transit peptide (CTP).

The *gox* gene has been modified to improve the affinity of the enzyme for glyphosate and is referred to as the *gox* variant (GOXv247). Nucleotide sequencing has determined that there are three amino acid substitutions in the *gox* variant protein and that the two proteins are greater than 99% identical.

Expression of the novel protein in the plant

Expression levels of the introduced proteins were measured using enzyme linked immuno-sorbent assay (ELISA) which is a highly sensitive technique that can detect the presence of a protein generally to a sensitivity of 10-100 pg. ELISA analysis was used in the analysis of leaf tissue, seed and processed fractions (toasted meal) from the glyphosate-tolerant canola line. The level of total protein present in RBDO was also determined.

Three separate field trials of glyphosate-tolerant canola were done, two in Canada and a third in Europe. In the 1992 Canadian season, the seed analysed was not treated with herbicide. In the 1993 and 1994 seasons, plants were both untreated and treated with the herbicide Roundup (active ingredient is glyphosate).

ELISA analysis of glyphosate-tolerant canola and control Westar seed from all trials as well as leaf tissue from the 1992 trial demonstrated that the introduced proteins CP4 EPSPS and GOX are expressed at very low levels in these tissues (Table 2). The level of expression constitutes less than 0.02% of the seed on a fresh weight basis. No detectable CP4 EPSPS or GOX protein was measured in Westar seed or tissue from any year.

Table 2: Protein expression levels in canola as determined by ELISA[1]

	Expression levels in seed (µg/mg fresh weight)			
	Mean	**Range**	**Mean**	**Range**
	1992 leaf[2]		**1992 seed[2]**	
GT73				
CP4 EPSPS	0.034	0.028-0.037	0.049	0.044-0.051
GOX	0.108	0.071-0.161	0.154	0.109-0.203
Westar[6]				
CP4 EPSPS	nd	-	nd	-
GOX	nd	-	nd	-
GT73	**1993 un-treated[3]**		**1993 treated[3,5]**	
CP4 EPSPS	0.028	0.018-0.047	0.030	0.014-0.042
GOX	0.193	0.108-0.334	0.206	0.125-0.379
Westar[6]				
CP4 EPSPS	nd	-	nd	-
GOX	nd	-	nd	-

Table 2 continued: Protein expression levels in canola as determined by ELISA[1]

	1994 un-treated[4]		1994 treated[4,5]	
GT73				
CP4 EPSPS	0.018	0.016-0.022	0.018	0.012-0.022
GOX	0.160	0.126-0.240	0.186	0.119-0.232
Westar[6]				
CP4 EPSPS	nd	-	nd	-
GOX	nd	-	nd	-

[1]Means of all samples taken from all locations except for 1992 where samples were taken from 3 of the 7 sites.
[2]CP4 EPSPS & GOX Leaf n=4: Seed CP4 EPSPS n=3, GOX n= 6; Westar n=7. No treated values for 1992
[3]Untreated and Treated CP4 EPSPS n=8, GOX n= 16; Westar n=4.
[4]Untreated CP4 EPSPS n=7, GOX n= 7; Treated EPSPS n=9, GOX n= 9; Westar n=2.
[5]Early post application plot of Roundup at 0.45 kg a.i./ha and 2 L/ha in 1993 and 1994 respectively.
[6]Expression of the novel proteins in Westar was not detected.

In line GT73, expression of both CP4 EPSPS and GOX proteins in the seed was comparable for all trials (Table 2). The expression of the novel proteins in the seed was also comparable for plants treated with the herbicide glyphosate.

Processed Fractions

Analyses of the processed fractions of canola, refined, bleached and deodorised oil (RBDO) and toasted meal were also done (Table 3). It is widely accepted that many refined oils, do not contain any protein or only negligible amounts (Tattrie and Yaguchi, 1973; Klurfeld and Kritchevski, 1987). In the 1992 trial, the level of total protein present in canola oil was determined for both glyphosate-tolerant canola line GT73 and Westar. The total protein in both canola lines was present only in trace amounts (0.290 ppm in GT73 and 0.327 ppm in Westar) which was not considerably different to the level determined for an acid blank control sample (0.217 ppm).

Table 3: Total protein present in refine oil produced from the 1992 field trial

SAMPLE	Total protein present in refined oil (ppm)
GT73	0.290
Westar	0.327
Acid blank control	0.217

The trace protein in the oil represents less than 0.0001% protein and is at the limit of detection. This amount of protein is considered to be negligible. Given that the novel protein was present in unprocessed seed at very low levels and that all protein is virtually removed upon processing canola seed, the refined oil is not considered to contain any novel protein.

The amount of the novel proteins in toasted meal was found to be considerably reduced upon processing. In the 1992 and 1993 trials, the CP4 EPSPS protein was reduced by over 40% and the GOX protein was reduced by more than 20%. Additionally, the proteins were not found to have any enzymatic activity, as expected, since processing denatures the proteins and therefore its activity.

Impact on human health from potential transfer of novel genetic material to cells in the human digestive tract

The human health considerations in this regard depend on the nature of the novel genes and must be assessed on a case-by case basis.

In 1991, the World Health Organization (WHO) issued a report of a Joint FAO[1]/WHO Expert Consultation which looked at strategies for assessing the safety of foods produced by biotechnology (WHO 1991). It was concluded by that consultation that as DNA from all living organisms is structurally similar, the presence of transferred DNA in food products, in itself, poses no health risk to consumers.

The major concern in relation to the transfer of novel genetic material to gut microorganisms is with antibiotic resistance genes. It is generally accepted that there are no safety concerns with regard to the presence in the food of antibiotic resistance gene DNA *per se* (WHO 1993). There are concerns, however, that there could be horizontal gene transfer of the antibiotic resistance gene from ingested food to gut microorganisms and that if the microorganisms are able to express the transferred resistance gene this could lead to an increase, in the gastrointestinal tract, of microorganisms resistant to a specific antibiotic. This, in turn, might lead to an increased potential for the transfer of the antibiotic resistance gene to pathogenic microorganisms, thus compromising the therapeutic use of such antibiotics. There are further concerns that, if the antibiotic resistance gene is expressed in the plant, the expressed protein, when ingested, could inactivate oral doses of the antibiotic to which it confers resistance.

The glyphosate-tolerant canola line assessed in this application does not contain any antibiotic resistance genes as indicated by the Southern blot and specific PCR experiments. Only DNA contained within the Left and Right Borders of the *Agrobacterium*–based plasmid is transferred. This refers only to the genes conferring glyphosate tolerance which are not considered to pose any health risk.

Additionally, refined oil is the only product for human consumption derived from glyphosate-tolerant canola and there is virtually no protein present since it is removed during processing of the oil.

As discussed above, it is extremely unlikely that novel genetic material will transfer from GM foods to bacteria in the human digestive tract because of the number of complex and unlikely steps that would need to take place consecutively.

It is equally unlikely that novel genetic material will transfer from GM foods to human cells via the digestive tract. In considering the potential impact on human health, it is important to note that humans have always consumed large amounts of DNA as a normal component of food and there is no evidence that this consumption has had any adverse effect on human health. Furthermore, current scientific knowledge has not revealed any DNA sequences from ingested foods that have been incorporated into human DNA. Novel DNA sequences in GM foods comprise only a minute fraction of the total DNA in the food (generally less than 0.01%) and are

[1] Food and Agriculture Organization.

therefore unlikely to pose any special additional risks compared with the large amount of DNA naturally present in all foods.

Given the information above, the horizontal gene transfer of any genetic material from the glyphosate tolerant canola, whether novel DNA or not, is not considered to pose any risk to public health and safety, particularly in relation to the development of antibiotic resistant pathogenic bacteria.

Conclusions regarding general safety issues

CP4 EPSPS and GOX are both expressed at relatively low levels in the seed. The only canola product intended for human consumption is the refined oil, which does not contain any detectable CP4 EPSPS or GOX protein. The CP4 EPSPS gene and protein have been well characterised and are considered similar to plant EPSPS genes which are readily consumed. The *gox* gene has been sourced from a common soil bacterium, which has no history of pathogenicity.

The risk of transfer of the novel genetic material to gut bacteria is considered negligible and additionally, there are no antibiotic resistance genes present in glyphosate-tolerant canola.

TOXICOLOGICAL ISSUES

Levels of naturally-occurring toxins

Rapeseed varieties naturally have very high levels of the toxic components erucic acid and glucosinolates both of which have dietary concerns. Erucic acid has cardiopathogenic potential and glucosinolates have goitrogenic properties, which makes rapeseed unsuitable for human consumption (McDonald, 1999). Canola refers to those varieties of rapeseed that must meet specific standards on the levels of erucic acid and glucosinolates.

Although refined oil is the only human food fraction derived from canola, data has also been presented for toasted meal. Canola meal is not considered to be a human food fraction and has been evaluated in this assessment to compare levels of major components to determine any potentially unintended effects. Canola meal, whether genetically modified or not, is not regarded as a food fraction due to the presence of natural toxicants, erucic acid and glucosinolates and the genetic modification does not change this pattern of consumption.

Erucic acid analysis

Erucic acid is a mono-unsaturated fatty acid (22:1), which is a natural constituent of rapeseed. High erucic acid rapeseed (HEAR) oil has been shown to have cardiopathic potential in laboratory animals (reviewed in ANZFA, 1999b). Canola has been developed from rapeseed and canola oil must conform to a standard defined as less than 2 percent erucic acid in oil and less than 30 micromoles of total glucosinolates in toasted meal to conform to CODEX standards (CODEX, 1993). Conformance to these standards ensures that canola oil is essentially free of cardiopathogenic

potential. All canola varieties that meet CODEX specifications also meet specifications for canola oil as outlined in the Australian *Food Standards Code*.

Data for erucic acid in line GT73 has been statistically analysed to ensure that it does not exceed the 2% maximum level permitted in oil. The mean values for erucic acid in GT73 are well below the maximum limit allowed for canola and are also below the values determined for the control line Westar (Table 4). Breeding in canola continues to reduce the erucic acid levels and the fact that the glyphosate tolerant canola line has a low content is considered beneficial.

Table 4: Erucic acid levels in oil from glyphosate tolerant canola line GT73 and Westar[1]

	1992 seed	1993 untreated	1993 treated[4]	1994 untreated	1994 treated[4]
GT73[2]	0.12	0.04	0.00	0.10	0.12
Westar[3]	0.3-0.6	0.15-0.57	0.15-0.57	0.29-0.36	0.29-0.36

[1]Means of all samples taken from all locations except for 1992 where samples were taken from 3 of the 7 sites. Values for Westar samples from all trials were below the calculated limit of detection.
[2]1992 n=7; 1993 Untreated n=4, Treated n=5; 1994 Untreated n=2, Treated n=4.
[3]1992 n=7; 1993 n=7; 1994 n=2.
[4]Early post application plot of Roundup at 0.45 kg a.i./ha and 2 L/ha in 1993 and 1994 respectively.

Glucosinolate analysis

There are over 100 known structural types of glucosinolates, nine of which are closely monitored in canola because they are reported as having toxic properties. Five compounds referred to as the alkyl glucosinolates are thought to have the anti-nutritional properties. The sum of four of these five alkyl glucosinolates (gluconapin, progoitrin, glucobrassicanapin and napoleiferin) must be less than a total of 30 μmoles/gram oil free meal for the seed to be classified as canola quality (the value is likely to be decreased 20 μmoles/gram). Of similar concentration but of less concern are the indol glucosinolates, two of which are monitored. Two types from a third group of glucosinolates, the thioalkyl glucosinolates are measured but are typically present in very low concentrations. Benzylglucosinolates are glucosinolates derived from phenylalanine and are also monitored in canola meal.

Glucosinolates are goitre-inducing when they are hydrolysed by myrosinase, an enzyme localised within cells of *Brassica* seeds. When the seed is crushed, the enzyme acts upon the glucosinolate to produce isothiocyanates, thiocyanates and possibly nitriles depending on temperature and moisture conditions. However, during processing, a cooking step inactivates myrosinase leaving glucosinolates intact. Some destruction and reduction of glucosinolates may occur in further processing steps. Breeders are encouraged to work towards the elimination of glucosinolates in canola.

During processing of canola seed to produce oil, the seed is flaked rupturing the oil cells and cooked at 75-85C. The cooking ruptures any remaining intact cells and compresses the flakes into cake fragments. These cake fragments are then solvent extracted to remove most of the remaining oil. Heal treatment of the processed fractions is important for removing volatile components which often are toxicants. The solvent is removed from the oil fraction which then undergoes a degumming process producing a semi-refined oil. These processing steps as well as the final refinement effectively remove glucosinolates from the refined bleached deodorised oil

(Genser and Eskin, 1979).

The applicant provided data for the analysis of glucosinolates in canola seeds and meal. Defatted meal from genetically modified canola line GT73 and the control Westar from the 1992 and 1993 field trials were analysed for glucosinolates by Agriculture Canada using standard methods of the Co-Op Test (Table 5). These analyses by Agriculture Canada (the Co-Op Test) allow a comparison of seed from GT73 to a much larger data set of values for Westar seed enabling an estimation of the considerable variation observed in the heterogeneous Westar genotype.

In the 1994 field trial, Cargill used an alternative technique to determine the glucosinolates content, which makes a direct comparison to previous years' values invalid.

The levels of glucosinolates in all samples from GT73 are well below the 30 µmole limit for defatted meal (Table 5). A comparison of mean levels of the alkyl glucosinolates in the genetically modified canola shows that all values except the 1992 GT73 value (16.8 µmol/g) are within the range of the Co-Op Test values (7.0-12.5 µmol/g). The level of glucosinolates in the genetically modified line is higher than in the control line but it is well below the accepted industry maximum limit (30 µmol/g).

Table 5. Glucosinolate composition in meal from Westar and glyphosate tolerant canola line GT73[1]

1992[2]	Westar		Westar Co-op		GT73	
	Mean	Range	Mean	Range	Mean	Range
Alkyl	8.75	6.11-11.4	9.66	7.0-12.5	16.8	13.8-19.8
Thioalkyl	0.26	0.18-0.40	0.36	0.2-0.8	0.46	0.38-0.55
Indolyl	11.4	9.8-13.4	11.0	7.0-13.7	11.6	11.55-11.63

1993[3]	Westar		Westar Co-op		GT73 Untreated		GT73 Treated[4]	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Alkyl	8.93	6.7-11.1	7.56	5.3-9.4	10.56	7.97-12.9	10.8	5.57-13.2
Thioalkyl	0.28	0.2-0.37	0.30	0.2-0.4	0.28	0.23-0.33	0.28	0.13-0.37
Indolyl	11.5	11.0-12.5	11.5	10.7-12.5	11.4	10.9-12.0	11.4	10.5-12.5

1994[5]	Westar	GT73 Untreated	GT73 Treated[4]
Alkyl	10.6	11.6	10.8
Indolyl	3.92	4.06	4.67

[1]Values are in µmoles/gram of defatted meal.
[2]1992 Westar n=7, GT73 n=2 Co-op Westar n=13.
[3]1993 Westar n=5, Untreated GT73 n=5, Treated GT73 n=5, Co-op n=9.
[4]Early post application plot of Roundup at 0.45 kg a.i./ha and 2 L/ha in 1993 and 1994 respectively.
[5]1994 Westar n=2, Untreated n=2, Treated n=2. Cargill used a different method of analysis.

Processed fractions – toasted meal

Independent laboratories at POS Pilot Plant Corporation of Saskatoon, Saskatchewan (POS) determined the glucosinolate content of the meal samples in 1992 and 1993

and at Cargill, Centre de Bolssay, Cedex in 1994. The content in GT73 in the 1992 trial was 9.9 μmol/g oil free meal and 4.4 μmol/g in Westar oil free meal. In the 1993 trial, glucosinolate content in untreated GT73 meal was also more than double that of Westar (10.5 and 4.7 μmol/g respectively). These values, although higher in the genetically modified line than in the control line are well below the 30 μmol/g defatted meal limit set by the industry in their definition of canola (McDonald, 1999).

Although the level of glucosinolates in line GT73 seed and meal appear to be consistently higher than the average determined for Westar, it is consistent with the variability known to occur in the heterozygous canola variety (Downey, 1994). It is also important to note that canola meal is not considered a food fraction fit for human consumption.

Potential toxicity of novel proteins

The safety of the EPSPS protein used in this application has been addressed in previous assessments (A338 Roundup Ready Soybeans). This data has also been published in the scientific literature as cited in the text.

Studies evaluated:

Bishop, B.R. and M.E. Gustafson. 1993. Production of glyphosate oxidoreductase (GOX) in recombinant *E. coli*. Monsanto Company, USA 63198.

Kolacz, K.H. et al. 1994. *E. coli* vectors for the expression of plant-processed form of CP1-GOX and CTP1-GOXv247. Monsanto Company, USA 63198

Naylor, M.W. 1994. Acute oral toxicity study of GOX (M4-C1) protein in albino mice. Monsanto Company, USA 63198.

Naylor, M.W. 1994. Acute oral toxicity study of GOXv247 (M4-C1) protein in albino mice. Monsanto Company, USA 63198.

Nickson, T.E. et al. 1994. Preparation and confirmation of doses for acute oral toxicity studies in mice with glyphosate oxidoreductase GOX (M4-C1) and GOXv247 (M4-C1). Monsanto Company, USA 63198.

Harrison L.A., et al. 1993. Characterisation of microbially-expressed protein: CP4 EPSPS. Monsanto Company, USA 63198.

Harrison L.A., et al. 1994. Equivalence of plant- and microbially expressed proteins: CP4 EPSPS from glyphosate-tolerant canola and *E. coli*. Monsanto Company, USA 63198.

Bishop, B.R. 1992. Production of CP4 EPSP synthase in a 100 litre recombinant *Escherichia coli* fermentation. Monsanto Company, USA 63198

The potential for toxicity of the newly expressed proteins, CP4 EPSPS and GOX, were evaluated based on:

. the amino acid sequence similarity with known toxins
. acute toxicity testing in mice.
. the resistance to digestion by proteases and acids in the model digestive/gastric system
. their presence as a major protein component in a specified food.

The amino acid sequences of both the CP4 EPSPS and GOX proteins were compared to the amino acid sequences of 1935 known protein toxins. No significant similarity was found other than would be expected given that certain functional domains are generally conserved between proteins.

The acute oral toxicity of bacterially produced CP4 EPSPS, lacking the CTP (Harrison et al, 1996), GOX and GOXv247 proteins, was studied in groups of ten CD-1 mice/sex in order to directly assess the potential for toxicity associated with this protein. Physical and chemical integrity and identity between the bacterially-produced and plant-plant produced proteins was demonstrated using Western blot analysis, N-terminal amino acid sequencing and enzymatic activity. Thus the novel proteins that were produced by fermentation that were used in acute toxicity tests are equivalent to the novel proteins produced in the plant.

There were no adverse effects or mortalities noted in mice administered CP4 EPSPS protein by gavage at doses up to 572 mg/kg (Harrison et al, 1996). This data from application A338 Roundup Ready Soybean has been previously assessed by ANZFA (ANZFA, 1999c). The GOX protein used in the acute toxicity test included four amino acids of the CTP since evidence supports that processing of the mature protein includes these four amino acids. There were no adverse effects observed in mice administered the GOX protein by gavage at doses up to 100 and 104 mg/kg for GOX and GOXv247 respectively.

These doses are well above the level of expression of the proteins found in glyphosate-tolerant canola plants (refer to Table 2) and represent a test using an estimated 1300-fold and 5000-fold increase in exposure to CP4 EPSPS and GOX proteins respectively, that would be expected by consuming the genetically modified canola.

Clinical observations were performed and body weights and food consumption were determined. All surviving animals were necropsied at study termination (8-9 days). Mice were observed up to 9 days after dosing and no signs of toxicity were observed (ie no adverse effects for either protein on body weight, food consumption, survival, or gross pathology).

Levels of naturally occurring allergenic proteins

Canola oil has been shown in this application to contain negligible levels of protein (discussed in 3.3) and given that most allergens are proteins, its consumption is unlikely to cause an allergic reaction. Many refined oils have been shown not to be allergenic even if the source can be allergenic (Taylor *et al*, 1981; Tattrie and Yaguchi, 1973).

In all cases of documented allergies to foods including both common and unusual allergies, there is only a single entry for rapeseed and this is considered a very uncommon allergy (Bush and Hefle, 1996).

Potential allergenicity of novel proteins

Studies evaluated:

Astwood, J. 1995. Glyphosate oxidoreductase (GOX) shares no significant sequence similarity with proteins associated with allergy or Coeliac disease. Monsanto Company, USA 63198.

Ream, J.E., Bailey, M.R., Leach, J.N. and Padgette, S.R. 1993 Assessment of the *in vitro* digestive fate of CP4 EPSP synthase Monsanto Company, USA 63198. MSL-12949

Ream, J.E. et al. 1994. Assessment of the in vitro digestive fate of glyphosate oxidoreductase GOX and GOXv247 variant. Monsanto Company, USA 63198.

Although there are no predictive assays available to assess the allergic potential of proteins, a number of characteristics are common among many of the allergens that have been characterised. For instance, amino acid sequence similarity with known allergens may be a useful gauge of allergenic potential. A string of 8-12 consecutive amino acid residues in common with known allergens could be an indicator for allergenicity given that many T-cell epitopes of allergenic proteins are that length (Taylor and Lehrer, 1996). In terms of the chemical and physical nature of proteins, known allergens tend to be glycosylated proteins with a molecular weight of 10–70 KDa (Lehrer et al, 1996).

Allergens also tend to be heat stable as well as resistant to peptic and tryptic digestion and the acidic conditions of the stomach. Consequently, many allergenic factors tend to be resistant to proteolytic digestion. The CP4 EPSPS and GOX proteins were evaluated for potential allergenicity against these criteria.

On the basis that amino acid sequence similarity with known allergens is a useful indicator of allergenic potential, the amino acid sequence of the CP4 EPSPS and GOX proteins were compared to the amino acid sequences of 219 known allergens present in public domain databases (eg GenBank, EMBL, Swissprot, PIR). No significant similarity (i.e. a sequence of more than 8 consecutive amino acids) was found with any of these known allergens.

The CP4 EPSPS protein is one of many EPSPS proteins that occur in plants, fungi and bacteria. The EPSPS proteins are naturally present in foods derived from plants and microbes and have no history of being allergenic. The bacterially sourced CP4 EPSPS protein is 47.6 KDa.

The GOX and GOXv247 proteins are both 46.7 KDa (there is a 17 Da difference). Thus each protein fits the molecular mass criteria recognised for many allergens of 10–70 KDa. The GOX protein is a single polypeptide that has a narrow substrate specificity for glyphosate.

Protein allergens must be stable to the peptic and acidic conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergenic response. A study of the digestibility of both proteins in model digestion systems was done using *in vitro* using simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) as mammalian digestion models. The method of preparation of the simulated mammalian gastric and intestinal digestive solutions used is described in the United States Pharmacopoeia (1989). The exposure of CP4 EPSPS and GOX proteins to SGF and SIF was conducted over a series of timed incubations at 37°C.

The products of the digestion were analysed using gel electrophoresis, Western blot analysis and enzymatic activity assays.

Both the CP4 EPSPS and GOX proteins are digested by proteases present in the mammalian digestive system, suggesting that they would not survive peptic and tryptic digestion or the acidic conditions of the human digestive system. From the simulated digestion experiments and Western blot analyses, the CP4 EPSPS protein had a half–life of less than 15 seconds in the gastric system and 10 minutes in the intestinal system. The GOX protein had a half–life of less than 30 seconds in the intestinal system as determined by Western blot analyses.

Conclusions regarding toxicological issues

There is no evidence to indicate that there is any potential for the EPSPS or the GOX proteins to be either toxic or allergenic to humans. Proteins from the EPSPS family of proteins are naturally present in our food source. Although the GOX protein is not present in foods naturally, it does not possess characteristics or sequence homology common to many allergens or toxins. Furthermore, the proteins are expressed at relatively low levels in the canola and are rapidly digested in conditions that mimic human digestion. Additionally, neither protein had toxic effects on mice given acute doses of the equivalent bacterially produced proteins.

Finally, there is no protein present in refined oil as it is removed during processing.

NUTRITIONAL ISSUES

Studies evaluated:

Nickson, T.E., and M.L. Taylor. 1994. Evaluation of seed from glyphosate-tolerant canola lines from the 1993 Canadian field trials. Monsanto Company, USA 63198.

Nickson, et al T.E., D.B. Re, B.G. Hammond, R.L. Fuchs and S.G. Rogers. 1994. Evaluation of glyphosate-tolerant canola lines from the 1992 Canadian field trials. Monsanto Company, USA 63198

Nickson, T.E., D.B. Re, B.G. Hammond, R.L. Fuchs and S.G. Rogers. 1995. Safety, compositional and nutritional aspects of glyphosate-tolerant canola: conclusion based on studies and information evaluated according to FDA's consultation process. Monsanto Company, USA 63198.

Taylor, M.L. 1995. The evaluation of seed from glyphosate-tolerant canola 1994 European field trials. Monsanto Company, USA 63198.

Taylor, M. and T.E. Nickson. 1995. The evaluation of refined, bleached, deodorised oil from glyphosate-tolerant canola. Monsanto Company, USA 63198.

Compositional analysis

Compositional analyses were done on the glyphosate-tolerant canola line GT73 and the control/parental line Westar. Comparisons were made to the database maintained by Agriculture Canada and Agrifood Canada (the Canadian Rapeseed Co-Op Tests). Three rounds of field trials of line GT73 were conducted according to Good Laboratory Practice (GLP) guidelines: 1992 Canadian trials grown in 7 field

locations; 1993 Canadian trials grown in 4 field locations; and 1994 European trials grown in 3 field locations (France, Belgium and the UK). Seed grown from each of the sites were analysed and statistical analyses of the data were done. The seed, leaf and processed fractions were analysed by independent laboratories for compositional quality characteristics according to GLP using standardised analytical methods by either the Ralston Analytical Laboratories (RAL), St Louis, Missouri, the Grains Research Laboratory (GRL) and at the Agriculture Canada Research Station (Agriculture Canada) in Saskatoon.

Processed fractions: Analysis of refined, bleached, deodorised oil (RBDO)

All new varieties of canola oil must be analysed to ensure they meet CODEX specifications for canola. This includes 18 quality analyses that define canola oil and includes a fatty acid analysis and 17 other food chemical tests. The results for all analyses of glyphosate-tolerant canola line GT73 were within CODEX specifications except for the values for four minor fatty acids: arachidic acid (C20:0), behenic acid (C22:0), lignoceric acid (C24:0) and nervonic acid (C24:1) (Table 6). However, the value for these four fatty acids exceeded the CODEX specifications in both the control line Westar and GT73.

Table 6: Fatty acid profile for refined, bleached and deodorised oil.

Fatty Acid	Westar	GT73	Codex
Arachidic 20:0	1.02	1.06	<1.00
Behenic 22:0	0.51	0.52	<0.50
Lignoceric 24:0	0.24	0.23	<0.20
Nervonic 24:1	0.30	0.31	<0.20

This result is considered to reflect the natural variation within canola rather than any effect of the genetic modification on the canola line. Furthermore, there is no anti-nutritional or toxicological significance associated with these fatty acids. With the exception of four slightly elevated minor fatty acids, the oil derived from glyphosate-tolerant canola line GT73 is comparable to oil derived from Westar.

Processed fractions: Analysis of toasted meal

Samples of toasted meal from glyphosate-tolerant canola line GT73 were sent to RAL for proximate analysis, amino acid composition, nitrogen solubility indexes and a mineral screen. The results for all analyses of toasted meal from glyphosate-tolerant canola were comparable to the samples derived from the Westar line and consistent with published values.

Proximate analysis for major constituents

Proximate analysis was done on genetically modified and control canola seeds at both RAL and the protein and oil components were also done at the Agriculture Canada in the 1992 and 1993 field trials. Components measured were protein, fat, moisture, fibre, ash and carbohydrates as well as calories and are all reported on a dry weight basis except for moisture (Table 7).

The proximate analyses were done on GT73 canola from all years including analyses on seeds from herbicide treated and untreated plants in 1993 and 1994. In all of the component analyses of line GT73, there were no significant differences between the glyphosate-tolerant canola and the control line Westar, nor for the seeds from plants treated with herbicide (p=0.05).

Table 7: Mean values and ranges for the Proximate Analyses of Canola from three field trials

1992	Westar[2]		GT73[2]			
	Mean	Range	Mean	Range		
Protein[1]	23.4	21.0-26.1	25.4	25.4-25.7		
Fat[1,2]	46.5	42.3-49.9	45.8	44.6-47.1		
Fibre[1]	8.21	7.16-9.90	7.37	6.26-8.19		
Moisture[3]	4.39	3.69-4.86	4.85	4.32-5.38		
Calories Kcal/100g[1]	551	536-567	546	539-554		
Ash[1]	3.68	3.44-3.91	3.59	3.39-3.79		
Carbohydrate[1]	26.4	23.6-28.0	25.2	23.4-26.9		
1993	Westar[2]		Untreated[2]		Treated[2,4]	
	Mean	Range	Mean	Range	Mean	Range
Protein	23.8	22.8-26.7	23.4	22.3-26.2	23.5	22.7-25.5
Fat	45.7	43.3-47.2	46.4	42.7-48.8	46.2	44.3-47.4
fibre	8.62	8.07-9.59	8.36	7.98-8.77	8.38	8.1-8.94
Moisture	10.4	8.44-11.6	9.22	8.49-9.49	9.67	9.20-10.1
Calories Kcal/100g	513	495-533	523	501-534	520	507-528
Ash	4.07	3.58-4.26	4.00	3.72-4.47	3.93	3.49-4.30
Carbohydrate	26.4	25.8-27.9	26.1	24.9-27.1	26.4	25.7-27.2
1994	Westar[2]		Untreated[2]		Treated[2,4]	
	Mean	Range	Mean	Range	Mean	Range
Protein	27.5	26.3-28.6	25.6	23.9-27.2	25.6	24.5-27.1
Fat	39.3	39.0-39.6	42.4	42.1-42.8	43.2	42.3-44.2
fibre	10.9	10.5-11.2	10.7	10.5-11.0	10.1	9.7-10.6
Moisture	8.30	8.18-8.43	8.43	8.34-8.52	8.63	7.68-9.31
Calories Kcal/100g	495	494-496	510	507-513	512	505-517
Ash	4.83	4.76-4.90	4.26	4.22-4.31	4.25	4.18-4.40
Carbohydrate	28.4	27.6-29.2	27.8	26.4-29.1	24.6	23.9-25.4

[1]Data as a percentage of dry weight
[2]1992: Westar n=7, GT73 n=2, Westar fat n=6; 1993 All n=4; 1994 Westar n=2, Untreated GT73 n=2, Treated GT73 n=3.
[3]Equilibrium moisture value
[4]Early post application plot of Roundup at 0.45 kg a.i./ha and 2 L/ha in 1993 and 1994 respectively.

% Fat and % Protein

Additional analyses (protein and oil) by Agriculture Canada (the Co-Op Test) allowed a comparison of seed from GT73 to a much larger data set of values for Westar seed. This enabled an estimation of the considerable variation observed in the heterogeneous Westar genotype. Statistical analyses on the fat content (whole seed, dry weight basis) and on protein content (defatted meal) noted one significant difference in line GT73 compared to Westar (p=0.05) (Table 8). The mean fat values

in 1993 (Untreated GT73: 45.8% and Treated GT73: 45.5%) were significantly higher than Westar. These findings were not consistent year to year and nor were they consistently noted in the proximate analyses and could be attributed to the natural range of variation that occurs in canola. The fat values, even though different to those for the control, were within the range reported for Westar grown during the field trial (fat: 42.4-47.3% and protein: 38.5-44.9%) and were also within the range reported for canola varieties from the Co-op Test Database (fat: 37.9-51.1% and protein: 34.0-50.8%).

Table 8: Mean values for % protein and % fat in canola seed

	Westar		Co-op Westar		GT73			
1992[1]	Mean	Range	Mean	Range	Mean	Range		
% Protein[1,2]	41.1	38.4-42.9	43.3	34.8-48.0	44.8	42.9-46.6		
% Fat[1,3]	44.8	41.9-47.7	42.8	37.7-47.6	44.8	44.1-45.4		
1993[4]	Westar		Co-op Westar		Untreated		Treated[5]	
% Protein[1,2]	41.2	38.3-45.0	42.3	34.0-50.8	41.8	39.6-44.8	42.2	40.2-44.7
% Fat[1,3]	45.1	42.4-47.3	44.8	37.9-51.1	45.8	43.7-47.1	45.5	42.8-48.5
1994[6]	Westar		Co-op Westar		Untreated		Treated[5]	
% Protein[1,2]	39.4	37.8-41.0	-		38.2	36.0-40.5	38.6	37.1-40.2
% Fat[1,3]	39.3	39.0-39.6	-		42.4	42.1-42.8	43.2	42.3-44.2

[1]Westar n=7; Co-op Westar n=52; GT73 n=2. Analyses done at Ag Canada.
[2]% Protein in defatted meal on samples ≤3% moisture
[3]% Fat on a whole seed basis dried to constant moisture (≤3%)
[4]Westar n=5; Co-op Westar n=87; Untreated GT73 n=4; Treated GT73 n=5. Analyses done at Ag Canada.
[5]Early post application plot of Roundup at 0.45 kg a.i./ha and 2 L/ha in 1993 and 1994 respectively.
[6]Westar n=2; Untreated GT73 n=2; Treated GT73 n=2. Analyses done at RAL.

Fatty acid analysis

Canola has a high content of long-chain unsaturated fatty acids. Refined canola oil is about 90% unsaturated C18 fatty acids which make it ideal for human consumption. Erucic acid (C22:1) content is monitored to ensure the canola maintains its GRAS (generally regarded as safe) status. Canola oil has considerable natural variation in fatty acid composition and thus some variation in the composition of commercial canola oil is acceptable.

Two methods of comparison of canola oil from GT73 and Westar seed using standard methods of the Co-Op Test were done. The first method was based on profile: total saturated (eg. 16:0, 18:0, 20:0 and 22:0), mono-unsaturated, di-unsaturated and tri-unsaturated fatty acid esters. There were no differences in fatty acid profiles between mean values for the treated or untreated GT73 and Westar seed.

Individual fatty acid esters were also monitored and compared (Tables 9.1 and 9.2). The components measured were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1 cis), linoleic (C18:2), and linolenic (C18:3), arachidic acid (C20:0), eicosenoic acid (C20:1), eicosadienoic acid (C20:2), behenic acid (C22:0), and erucic acid (C22:1).

Table 9.1 Fatty acid ester profiles for GT73 and Westar canola for the 1992 and 1993 trials

Fatty	1992[2]			1993[3]			
Acid	Westar	Co-op	GT73	Westar	Co-op	Not treated	Treated[4]
16:0	3.9-4.2	3.7-4.8	3.98	3.8-4.3	4.0-4.3	4.1	4.1
16:1	0.3-0.4	0.0-0.6	0.32	0.2[5]	0.2-0.3	0.2	0.2
18:0	1.4-2.0	1.2-2.1	1.72	1.4-1.9	1.7-1.9	1.7	1.8
18:1	58.8-62.5	57.4-63.4	61.4	60.1-62.8	61.9-63.1	62.9	62.8
18:2	18.9-20.2	18.3-22.1	18.9	18.8-20.6	18.4-19.8	18.7	18.7
18:3	8.1-12.1	8.2-13.0	10.8	8.6-10.13	8.5-9.8	9.65	9.73
20:0	0.6-0.8	0.4-0.9	0.72	0.6-0.7	0.6-0.7	0.65	0.68
20:1	1.7-2.0	1.3-2.3	1.58	1.57-2.0	1.4-1.9	1.49	1.51
20:2	0.1[5]	0.1-0.2	0.17	0.1[5]	0.1[5]	0.09	0.1
22:0	0.3-0.4	0.3-0.4	0.40	0.4-0.5	0.4[5]	0.4	0.43
22:1[6]	0.3-0.6	0.1-1.4	0.12	0.15-0.57	0.1-0.5	0.04	0.0

[1]Values are % of fatty acid ester profile. Analysis by Ag Canada.
[2]Westar n=7; Co-op Westar n=13; GT73 n=2;.
[3]Westar n=15; Co-op Westar n=8; Untreated GT73 n=12; Treated GT73 n=15.
[4]Treated: Early post application plot of Roundup at 0.45 kg a.i./ha
[5]Single value obtained for all samples.
[6]Erucic Acid

In 1994, the fatty acid analysis also included docosadienoic acid (C22:2), lignoceric acid (C24:0) and nervonic acid (C24:1) (Table 9.2). In all years, the values for fatty acid esters from GT73 were within the range for Westar from the Co-Op Test except erucic acid which was below that for Westar (Tables 9.1 and 9.2) in 1993. Since canola continues to be bred for lower erucic acid content because of its adverse cardiopathic potential, this difference is considered to be a positive attribute. Erucic acid is discussed in greater detail in Section 4.1 Naturally Occurring Toxins.

Table 9.2 Fatty acid ester profiles for GT73 and Westar canola from seed from the 1994 trial[1]

1994			
Fatty Acid	Westar	Not treated	Treated[2]
16:0	4.52	4.51	4.50
16:1	0.24	0.24	0.24
18:0	1.90	1.5	1.89
18:1	62.6	64.8	64.4
18:2	20.2	19.0	19.1
18:3	7.11	6.94	7.00
20:0	0.77	0.78	0.74
20:1	1.46	1.16	1.17
20:2	0.1	0.1	0.1
22:0	0.36	0.36	0.34
22:1	0.32	0.1	0.12
22:2	0.1	0.1	0.1
24:0	0.20	0.18	0.18
24:1[6]	0.18	0.14	0.15

[1]Westar n=2; Untreated GT73 n=2; Treated GT73 n=3.
[2]Treated: Early post application plot of Roundup at 2 L/ha

Amino acid analysis

Amino acid analyses were done on glyphosate-tolerant canola seeds from line GT73 in 1992 and from untreated plants and plants treated with glyphosate in 1993 and 1994. The results are reported as a dry weight and per protein basis (i.e. the amino acid value divided by the percent protein as determined from proximate analyses).

Of the 18 amino acids analysed, the values for each year were comparable for treated or untreated glyphosate-tolerant canola plants and the control line Westar with few exceptions. Table 10 lists the amino acids that were found to be slightly lower in the genetically modified canola plants. In 1992, the only exception was the mean value for proline on a per unit protein basis in GT73 (6.61%), which exceeds the range for Westar (mean value of 6.24% and a range of 6.09-6.36%). However this difference between the genetically modified and control line is consistent with previously reported values (up to 7.79%, Baidoo and Aherne, 1985).

In the 1993 trials, amino acid mean values (g/100g seed dry weight) for line GT73 were within the ranges determined for Westar except the means were higher for cysteine (0.43 versus 0.33 and a range of 0.20-0.42 for Westar) and methionine (0.35 versus 0.26 and a range of 0.16-0.32 for Westar) in untreated plants and proline in treated plants (1.46 versus 1.38 and a range of 1.28-1.45 for Westar). Upon statistical analysis, the mean tryptophan value was significantly different (p=0.05) in untreated GT73 (0.24 versus 0.26) to that for Westar. All values however, were within the range for canola (0.24-0.29) and the differences are considered within the natural variation range known for canola.

Table 10. Amino Acid values that were different between GT73 and Westar.

	Westar	Westar range	GT73	GT73 range
1992				
Proline[1]	6.24	6.09-6.36	6.61	6.46-6.70
1993				
cysteine[3]	0.33	0.20-0.42	0.43	0.29-0.57
methionine[3]	0.26	0.16-0.32	0.35	0.23-0.51
proline[4]	1.38	1.28-1.45	1.46	1.31-1.64
tryptophan[5]		0.24-0.29	0.24	0.23-0.28
1994[1]				
glutamic acid	17.7	17.3-18.1	16.5	16.0-16.9
histidine	2.36	2.32-2.40	2.26	2.24-2.29
proline	5.69	5.60-5.78	5.46	5.39-5.54

[1]Value is mean value on a per unit protein basis
[2]Value is g/100g seed dry weight
[3]untreated plants
[4]Treated plants. Early post application plot of Roundup at 0.45 kg a.i./ha and 2 L/ha in 1993 and 1994 respectively.
[5]Significantly different p=0.05.

In the 1994 trials, the values for glutamic acid, histidine and proline were all lower than those found for Westar. However, all values were within the range found for Westar. The values for glutamic acid (16.5 versus 17.7 and a range of 17.3-18.1 for Westar), histidine (2.26 versus 2.36 and a range of 2.32-2.40 for Westar) and proline (5.46 versus 5.69 and a range of 5.60-5.78 for Westar) in treated and untreated GT73 seeds were all lower than the mean value found for Westar but were within the range found for Westar.

Levels of anti-nutrients

Canola has been through extensive breeding programs to become one of the most widely used oils for human consumption. Canola has been bred from rapeseed for reduced anti-nutritional factors.

Sinapine analysis

Sinapines are a family of choline esters that naturally occur in canola and can be found in canola meal. Sinapines are known to render an off-odour to chicken eggs if the chickens are fed canola meal and have some significance to the poultry feed industry. The analysis for sinapines was done by Agriculture Canada using published methods. The mean value for sinapine content in line GT73 (12.7) was determined in the 1992 and 1993 trials and was the same as that for Westar (12.7).

Mineral/phytic acid analysis in processed fractions

Canola meal is rich in many essential minerals but their content in meal can be influenced by environmental factors. As phytic acid can adversely affect the uptake of phosphorous, calcium, magnesium and zinc, all of these constituents were assessed in untreated canola line GT73 and the control Westar. The values for all minerals and phytic acid were determined in the 1992 and 1993 trials and were comparable to those found in canola.

Conclusion regarding compositional data

Analysis of the compositional data of the canola seed and processed fractions indicates that there were no meaningful differences in the levels of major constituents, nutrients, anti–nutritional factors or natural toxicants between glyphosate-tolerant canola line GT73 and the control canola line Westar. Since new varieties of canola must undergo assessment to ensure that it meets the compositional standards required for canola (eg CODEX standards), a valuable resource is available for comparison. The glyphosate-tolerant canola line GT73 assessed in this application has been analysed by Agriculture Canada and the results compared to the database (the Canadian Rapeseed Co-Op). In terms of the anti-nutrients erucic acid and glucosinolates, GT73 seeds were found to be well below the maximum acceptable limit for both of these compounds and comparable to Westar.

Genetically modified canola plants that were treated with the herbicide Roundup during growing were also analysed and found to be comparable to Westar canola.

Additionally, an analysis of oil derived from GT73 and Westar seeds found a negligible amount of protein in the refined canola oil, which was at the limit of detection for both lines. There was no meaningful difference between oil derived from the genetically modified and control lines. Proximate analyses and some compositional studies of the toasted meal were also done and no meaningful differences to toasted meal from Westar were found.

Ability to support typical growth and well-being

Studies evaluated:

Brown, P.B. 1994. Evaluation of glyphosate-tolerant canola as a feed for rainbow trout. Monsanto Company, USA 63198.

Cambell, S.M. et al. 1993. Glyphosate-tolerant canola seed meal, a dietary toxicity study with the Northern bobwhite, Wildlife International Ltd. Monsanto Company, USA 63198.

Cambell, S.M. and J.B. Beavers. 1994. A dietary toxicity study with glyphosate-tolerant canola seed meal in the bobwhite. Monsanto Company, USA 63198.

Naylor, M.W. 1994. One month feeding study with processed and unprocessed glyphosate-tolerant canola meal in Sprague Dawley rats. Monsanto Company, USA 63198.

Naylor, M.W. 1995. One month feeding study with processed canola (line GT73) in Sprague Dawley rats. Monsanto Company, USA 63198.

Naylor, MW and RM Folk. 1996. One month feeding study in Sprague Dawley Rats with processed meal from canola or oilseed rape. Monsanto Company, USA. ML-96-153

In assessing the safety of a genetically modified food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well-being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences together with an extensive compositional analysis of the food. Where, on the basis of available data, there is still concern or doubt in this regard, carefully designed feeding studies in animals may provide further re-assurance that the food is nutritionally adequate. Such studies may be considered necessary where the compositional analysis indicates significant differences in a number of important components or nutrients or where there is concern that the bioavailability of key nutrients may be compromised by the nature of the genetic changes to the food.

In the case of glyphosate-tolerant canola, the applicant submitted data from several feeding study trials in order to demonstrate wholesomeness of the canola meal. Although canola meal is not a human food fraction, the studies have been assessed as supporting data for the wholesomeness of the genetically modified canola. These include two four-week rat studies on processed and unprocessed meal, a ten-week trout study on processed meal and a five-day quail (Northern Bobwhite) study on unprocessed meal. A third one-month study on rats fed meal was repeated to test for the effect of glyphosate tolerant canola meal on liver and kidney weights.

Rat feeding studies

Six-week-old Sprague-Dawley rats (10/sex/group) were fed either 0, 5 or 15% w/w ground (unprocessed) and processed (toasted and defatted) glyphosate-tolerant canola (which was a composite of two genetically modified lines GT73 and GT200) and Westar canola meal and a diet control (commercial rodent chow with no added canola meal). The canola seed was incorporated into a balanced diet for four weeks. All test diets were formulated as Purina test diets to be as similar as possible in composition to commercial Purina rodent chow.

Mild but significant decreased weight gains were observed in male rats given the 15% dose level of unprocessed seed or processed meal from glyphosate-tolerant canola compared to those fed Westar meal. There were no differences in food consumption between any of the groups that would account for the variable weight gain. These results may be attributable to higher level of glucosinolates in the glyphosate-tolerant canola line compared to the level in the parental line.

For groups fed both glyphosate-tolerant and parental line canola meal, the absolute and/or relative liver and kidney weights were increased approximately 5-20% when compared to diet controls. However there were no differences in absolute or relative organ weights between the glyphosate-tolerant canola and parental line groups.

The experiment was repeated for the processed (toasted and defatted) GT73 and Westar canola meal. Six-week-old Sprague-Dawley rats (10/sex/group) were fed either 0, 5 or 15% w/w processed (toasted and defatted) glyphosate-tolerant canola (line GT73 only) and Westar canola meal and a diet control (commercial rodent chow with no added canola meal).

No meaningful differences were observed in body weights and body weight gains in the second rat study between groups fed processed glyphosate-tolerant canola meal and parental line canola meal. Liver weights were however increased approximately 12-16% for both sexes fed 15% GT73 meal. Livers appeared normal at gross necroscopy. This increase in liver weight has been attributed to a higher level of alkyl glucosinolate toxicants in the glyphosate-tolerant canola line GT73 which was 4 g/kg compared to 1.8 g/kg for the parental line. As glucosinolate levels can vary widely in canola, it is most likely that the plant chosen for development naturally contained a slightly higher levels of glucosinolates than the average canola plant.

Liver weights can vary and this can be an adaptive change that is indicative of a higher level of metabolic activity. Increased liver weight is commonly observed in toxicity studies, when it is often considered a physiological adaptation (if dose related), that reaches a steady state with continued dosing and is reversible after cessation of treatment. It is not necessarily harmless in itself (Glaister, 1986).

Glucosinolates have been linked to enlargement of the thyroid, adrenal gland, kidney and liver in feeding studies using rapeseed (Verkerk et al, 1998). There is an industry limit of 30 µmol glucosinolates per gram of defatted canola meal (which is equivalent to 12 g/kg) to which this canola line meets. As canola meal is not considered a human food fraction, there are no standards for canola meal in the Australian *Food Standards Code*.

Additional rat feeding study

A third study on Sprague-Dawley rats was repeated specifically to determine whether the liver and kidney weights of rats fed processed meal from line RU3 fall within the range of liver and kidney weights of rats fed processed meal from non-genetically modified commercial varieties of canola in Canada and Europe. This study was conducted as per previously described studies. The meal used in this study is from glyphosate-tolerant canola line RU3, which is derived from GT73.

Two separately processed replicates of processed canola meal from line RU3 were administered in the diet to groups of rats (10/sex/group). The animals were fed 10% processed glyphosate-tolerant canola meal for approximately one month. Comparisons were made to the Alliance variety of canola, a non-genetically modified canola line that was grown at the same time and in the same field as RU3. Additional comparisons were made to nine commercial varieties of processed Canadian canola or European oilseed rape meal that were administered in the diet at target levels of 10% (two replicates each of variety). An additional negative diet control group of ten males and ten females received a commercial rodent chow.

Clinical observations were performed weekly. Mortality and behaviour were checked daily. Body weights and food consumption were determined weekly. All animals were sacrificed at study termination and their kidneys and liver were weighed.

There were no mortalities in any of the groups and no adverse clinical signs that were considered treatment related. Body weights were generally lower and liver/body and kidney/body weights were generally higher for rates fed canola/oilseed rape meal when compared to the diet control group, which is consistent with published reports (Verkerk et al, 1998). The increases seen in relative organ weights of rats fed diets containing canola or oilseed rape meal were due to the reductions in body weight (3.39 - 6.28% less than the terminal body weights of the negative controls) rather than a direct effect on absolute organ weight, since overall average absolute organ weights of the test animals were within 1.01% of the control animal's average organ weights.

There were no significant differences in body weight, cumulative weight gain, terminal body weights or food consumption for animals fed the genetically modified canola variety, when compared to the Alliance variety or other commercial varieties of canola. There were also no significant differences in absolute or relative liver or kidney weights between animals fed the RU3 variety when compared to the Alliance variety or the population of canola varieties.

It can be concluded from this study that the RU3/GT73 variety is equivalent to the Alliance canola variety in terms of its nutritional profile. In addition, a diet containing meal from RU3/GT73 has no significant difference on liver and kidney weight compared to diets containing meal from other commercially available canola varieties.

Quail feeding study

Thirty northern bobwhite (*Colinus virginianus*) chicks (three groups of ten each) were fed glyphosate-tolerant canola meal for five days and observed for a further three days. Treatment groups were fed a basal diet, Westar or glyphosate-tolerant canola (both line GT73 and GT200) incorporated at a rate of 20% of the total weight of the diet.

There were no effects on body weight or feed consumption between birds in the control or treatment groups. There were no mortalities or overt signs of toxicity in either treatment or control groups.

A second similar study was also done on the Northern Bobwhite. No treatment related mortality or differences in food consumption, body weight or behaviour occurred between birds fed 20% weight/weight glyphosate-tolerant canola or control canola meal.

Trout feeding study

Triplicate groups of 15 fish rainbow trout (*Oncorhynchus mykiss*) were fed canola meal at 0, 5, 10, 15 or 20% weight of the dry diet for 10 weeks (ie 45 fish/treatment). There was statistical overlap in weight gain of fish fed each dietary treatment and no differences were detected between glyphosate-tolerant canola (both line GT73 and GT200) diets and control diets at individual level of incorporation. Fish fed the glyphosate-tolerant canola did not exhibit any adverse effects of the sample as the level of inclusion increased. These results support the safety of meal from glyphosate-tolerant canola as a component in fish diets.

Conclusions from the feeding studies

All of the feeding studies examined the wholesomeness of glyphosate-tolerant canola meal for animal feeds. Although these studies are limited in terms of the information they provide about the human food fraction (oil), they provide support for the wholesomeness of the genetically modified canola meal.

The glyphosate-tolerant canola meal contains a higher level of glucosinolates than in the control line. The observed increase in liver weights in rats was attributed to this naturally occurring higher level of glucosinolates. The higher level of glucosinolates present in glyphosate-tolerant canola was not attributed to the genetic modification.

An important factor in the assessment of glyphosate tolerant canola is that only highly refined, bleached and deodorised oil is for human consumption. The feeding studies establish the nutritional adequacy of canola meal for animal feeds and represent a worse case scenario in terms of canola consumption by humans. In the processing of canola seed to oil, the erucic acid content is reduced to a very low level that meets Australian regulations and glucosinolates are removed. Consequently, the refined oil constitutes an even lower risk than processed and unprocessed meal.

Conclusions regarding nutritional issues

Nutritional qualities for the glyphosate-tolerant canola line GT73 were determined by compositional analyses of the major components of the seed and processed fractions and were found to be comparable in all respects to the conventional control line Westar.

Changes at the whole food level (canola meal only) have been assessed by the wholesomeness studies and these studies support that the glyphosate-tolerant canola meal is nutritionally comparable to meal from the parent line.

There is a long history of safe use of canola oil. Based on the data submitted in the present application, canola oil derived from glyphosate-tolerant canola line GT73 is considered to be equivalent in terms of its safety and nutritional adequacy to parent

varieties.

OTHER ISSUES

The significance and metabolism of AMPA in plants and animals

The GOX protein, encoded by the transferred *gox* gene, confers glyphosate tolerance by breaking down glyphosate to glyoxylate and aminomethylphosphonic acid (AMPA), which effectively reduces cellular levels of glyphosate. AMPA is the primary plant metabolite of glyphosate and does not have herbicidal activity. The applicant has provided additional data for the evaluation of the metabolism and toxicology of AMPA. This data addresses the issue that residues would be expected to be higher in some GM crops such as canola line GT73 that can withstand over-the-top application of herbicide as opposed to conventional methods of herbicide application.

AMPA metabolism in the plant

The metabolism of glyphosate is the same in tolerant or non-tolerant plants: glyphosate is metabolised to AMPA. The only difference between glyphosate metabolism in tolerant and non-tolerant plants is that the relative distribution of metabolites depends on the speed and extent to which glyphosate is converted to AMPA. AMPA has one of three fates in a plant: it is either non-selectively bound to natural plant constituents, further degraded to one-carbon fragments that are incorporated into natural products or conjugated with naturally occurring organic acids to give trace level metabolites.

AMPA residues in the plant

The metabolism of glyphosate (and AMPA) metabolism in canola line GT73 was investigated using two sequential applications of ^{14}C-glyphosate, each applied at a rate of approximately 0.90 kg a.e./ha at 14 and 22 days after planting. The treatments used, simulate expected commercial treatments but the total application rate of 1.80 kg a.e./ha exceeds the maximum proposed application rate of 0.90 kg a.e./ha. Canola seed was harvested 79 days after the last application. Maximum AMPA residues found in canola seed were 0.97 mg/kg. The amount of radioactivity was determined in processed fractions that had undergone processing that simulated commercial oil extraction (i.e. hexane-extracted oil, aqueous extract, extracted meal) as well as in the initial seed.

The radioactivity in the oil was due to the presence of fatty acids that had incorporated one-carbon fragments that were breakdown products of the labelled glyphosate. No glyphosate or glyphosate related metabolites were present in the oil derived from canola seed that had been treated as described above. Up to 70-80% of the total radioactivity in the unextracted seed remained in the extracted meal, with the remainder present in the aqueous extract. Further investigations characterised the non-extractable bound radioactive residues and the fate of glyphosate in the plant.

Overall, glyphosate metabolism in canola occurs as follows: as a result of the action

of the GOX enzyme, glyphosate is rapidly degraded to AMPA which is conjugated to secondary metabolites (N-glyceryl-AMPA and N-acetyl-AMPA). The results suggest that AMPA accounts for at least 15% of bound radioactivity due to unspecific adsorption and binding. In addition, AMPA is further degraded to one-carbon fragments that become broadly incorporated in a wide variety of natural products and plant constituents. Simulated digestive and gastric system studies showed that less than 8% of the bound ^{14}C-activity was released and that therefore only a very small fraction of the bound components would be biologically available if ingested by animals.

Because the level of AMPA was expected to be higher than in other canola lines, an evaluation of the animal metabolism and toxicology of AMPA has also been assessed.

AMPA metabolism in animals

AMPA metabolism studies were conducted by administering rats intravenously with ^{14}C-AMPA at a rate of 6.7 mg/kg body weight. These studies demonstrated that AMPA is not metabolised in animals and that greater than 90% of the administered dose is rapidly eliminated (i.e. within 48 hours) in faeces and urine.

Glyphosate metabolism studies were conducted by administering rats orally or intravenously with ^{14}C-glyphosate at a rate of 10 or 1000 mg/kg body weight. These studies demonstrated that glyphosate is absorbed to the extent of 30-36% and that its recovery in the excretia accounts for 98-99% of the administered ^{14}C-glyphosate. The metabolism of glyphosate was very minor regardless of whether it was administered orally or intravenously.

In all cases described above, after 120 hours post-administration, less than 0.7% of the administered dose remained in the tissues and organs, demonstrating that AMPA does not bio-accumulate in these tissues.

Toxicology of AMPA

Structure analysis shows AMPA to be very similar to the parent molecule glyphosate which has been extensively tested by the applicant and found not to be oncogenic and has a low order of chronic toxicity. Both compounds are poorly absorbed orally and if absorbed, is rapidly excreted unmetabolised via the urine. The toxicity profile is similar between the two compounds and neither compound bioaccumulates.

The major toxicology endpoints have been investigated for AMPA and the results demonstrate a very low order of toxicity. The acute toxicity of AMPA is low, with an oral LD_{50} of 8300 mg/kg.

Subchronic toxicity of AMPA is also low in studies using rats and dogs. AMPA was administered orally to dogs (5 per sex per group) for three months at concentrations of 0, 10, 30, 100 and 300 mg/kg/day. No treatment related effects were observed at doses up to and including the highest dose tested (analytically determined to be 263 mg/kg/day).

Several subchronic exposure in rats were conducted: a 14 day study on groups of rats

(5 per sex per group) using doses of 0, 1000, 2000 and 4000 mg/kg/day and a 90 day study on groups of rats (20 per sex per group) using doses of 0, 400, 1200 and 4800 mg/kg/day.

In the first rat study, reduced body weight gain and food consumption were observed at the highest dose tested. No other effects were observed. The NOEL (no observed effect level) was determined to be 2000 mg/kg/day.

In the second study, body weights were reduced in mid and high dose animals. There was no effect on food consumption or haematology at any dose level. There were differences in some blood chemistry parameters (i.e. increase in mean lactic dehydrogenase and SGOT levels) at the high dose level. Hyperplasia of the urinary bladder epithelium was observed at the mid (low incidence) and high dose level. Thus exposure to very high dose levels results in kidney toxicity. However the NOEL in this study was set quite high at 400 mg/kg/day.

Additional chronic and reproductive studies have been conducted to determine the toxicity of glyphosate where the presence of AMPA, as a metabolite of glyphosate, can be deduced. These include a two-generation rat reproduction study and a rat teratology study. The two-generation rat reproduction study found a decrease in pup weight at the high dose, which also produced toxicity to the parents. At the NOEL in this study, animals were exposed to approximately 3 and 740 mg/kg/day of AMPA and glyphosate, respectively. In the rat teratology study, AMPA did not produce birth defects even at levels which produced maternal toxicity.

Livestock feeding studies

Livestock feeding studies were conducted with swine, poultry and lactating cows. Test groups of animals were fed a daily ration containing a nine to one mixture of glyphosate and AMPA at total combined daily dietary levels that represent 1X, 3X and 10X the maximum expected residue levels of both compounds in the diet (i.e. 40, 120 and 400 ppm glyphosate and 4, 12 and 40 ppm AMPA respectively).

For all three species, AMPA residues were less than 0.05 ppm (non-detectable) in all fat and muscles samples from all treatment levels. At the 1X dose level, AMPA residues were less than 0.05 ppm in all liver samples and did not exceed 0.07 ppm in all kidney samples. Small residues levels were detected in liver and kidney at the 3X and 10X dose levels. AMPA residue levels in the kidney at the 10X dose level were 0.96, 0.33 and 0.94 ppm in swine, poultry and cows respectively. AMPA residues in liver at the 10X level were 0.39, 0.38 and 0.17 ppm in swine, poultry and cows respectively. Analysis of tissues following the 28 day depuration (i.e. cleansing) period demonstrated that AMPA is rapidly eliminated, with residues less than 0.05 ppm in all samples from all species.

AMPA residues were less than 0.025 ppm (non-detectable) in all egg samples collected from hens dosed at the 1X and 3X levels. With the exception of two eggs which had less than 0.035 ppm AMPA, residues in eggs were less than 0.025 ppm in all egg samples from hens dosed at the 10X level. Analysis of eggs following the depuration period demonstrated that AMPA is rapidly eliminated , with residues less than 0.05 ppm in all egg samples.

AMPA residues were less than 0.025 ppm (non-detectable) in all milk samples collected from cows dosed at the 10X levels. Since AMPA was not detected in the 10X milk samples, the 1X and 3X samples were not analysed.

Metabolism and distribution in livestock

Lactating goats exposed orally to glyphosate and AMPA (in a combined nine to one ration dose level, contained only low residue levels in the edible tissues. The highest ^{14}C residues among all edible tissues was found in the kidneys (representing 0.13% of the total administered dose) and milk contained less than 0.01% of the total administered dose. Similarly in laying hens exposed orally to glyphosate and AMPA, almost all radioactivity was found in the excretia. The total radioactivity in eggs and tissues accounted for less than 0.02 and 0.1% respectively of the administered dose.

Thus the results from both the feeding and metabolism studies show that AMPA residues will not be present in meat, milk or eggs of animals that consume feed containing expected or exaggerated residues.

Conclusions

Oil derived from glyphosate-tolerant canola has been shown not to contain any residues of AMPA (or glyphosate).

AMPA has only minimal toxicity in acute and subchronic toxicity studies. Animal metabolism and feeding studies demonstrated that AMPA is rapidly eliminated and does not bio-accumulate in edible tissues, milk or eggs.

ACKNOWLEDGEMENTS

ANZFA gratefully acknowledges the expert comments of Professor Geoff Fincher in the Department of Plant Science, Waite Institute, University of Adelaide in the preparation of this safety assessment report.

REFERENCES

Australia New Zealand Food Authority (ANZFA). 1999a. Guidelines for the safety assessment of foods to be included in Standard A18 – Food Produced Using Gene Technology.

Australia New Zealand Food Authority (ANZFA). 1999b. Erucic acid in food: a toxicological review and risk assessment.

Australia New Zealand Food Authority (ANZFA). 1999c. Full Assessment Report for A338 Roundup Ready Soybeans.

Baidoo S.K. and F.X. Aherne. 1985. Canola meal for livestock and poultry. Agric. Forest. Bull. 8:21-26.

Barry G., et al. 1992. Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants. In *Biosynthesis and Molecular Regulation of Amino Acids in Plants.* Singh (Ed). American Society of Plant Physiologists.

Bush, R.K. and Hefle, S.L. 1996. "Food allergens" *Critical Reviews in Food Science and Nutrition* 36(Suppl): S119-S150.

CODEX Standard 24 – 1981. 1993. CODEX Standard for edible rapeseed oil. CODEX Alimentarius Commission. Volume 8. Food and Agriculture Organisation, World Health Organisation. Rome.

della Ciopa et al, 1986. Translocation of the precursor of 5-enolpyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants in vitro. Proc Natl. Acad. Sci USA 83:6873-6877.

Downey, R.K. 1994. An evaluation from Agriculture Canada on the level of glucosinolates in glyphosate tolerant canola line GT73.

Fling, M.E., J. Kopf and C. Richards. 1985. Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3'(9)-*O*-nucleotidyltransferase. Nucl. Acids Res. 13:7095-7106.

Genser, M.V. and N.A.M. Eskin. 1979. Canola oil: properties, processes and food quality. Rapeseed Association of Canada, Winnipeg, Man.

Glaister, J.R. 1986. Chapter 3: Target-organ Pathology. In *Principles of Toxicological Pathology*. Taylor and Francis Ltd, London

Harrison, L.A., Bailey, M.R., Naylor, M.W., Ream, J.E., Hammond, B.G., Nida, D.L., Burnette, B.L., Nickson, T.E., Mitsky, T.A., Taylor, M.L., Fuchs, R.L., and Padgette, S.R. 1996 The expressed protein in glyphosate-tolerant soybean, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, is rapidly digested *in vitro* and is not toxic to acutely gavaged mice. *J Nutr* 126: 728-740.

Haslam, E. 1993. Shimikic Acid: Metabolism and Metabolites. Haslam (Ed). John Wiley and Sons, Inc. Chichester, UK.

Klurfield, D.M. and D. Kritchevski. 1987. Isolation and quantitation of lectins from vegetable oils. Lipids 22:667-668.

McDonald, B.E. 1999. Canola Oil, nutritional properties. Canola Connection, Canola Council of Canada. http://www/canola-council.org/pubs/oilprop.htm

Pipke, R. and N. Amrhein. 1988. Degradation of the phosphonate herbicide glyphosate by *Arthrobacter atrocyaneus* ATCC 13752. Appl. Environ.Microbiol. 54:1293-1296.

Rodgers, S.G., H. Klee, R. Horsch and R.T. Fraley. 1987. Improved vectors for plant transformation: Expression cassette vectors and new selectable markers. Meth. Enzymol. 153:253-277.

Schültz et al 1985. Differential sensitivity of bacterial 5-enolpyruvylshikimate-3-phosphate synthases to the herbicide glyphosate. FEMS Microbiology Letters 28:297-301.

Sutcliffe, J.G. 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symposium 43:77-90.

Tattrie, N. H. and M. Yaguchi. 1973. Protein content of various processed edible oils. J. Inst. Can. Sci. Technol. Ailment 6: 289-290.

Taylor, S.L., W.W. Busse, M.I. Sachs, J.L. Parker and J.W. Yunginger. 1981. Peanut oil is not allergenic to peanut-sensitive individuals. J. Allergy Clin. Immunol. 68(5):372-375.

Timko, M.P., L. Herdies, E. de Alameida, A.R. Cahsmore, J. Leemans and E. Krebbers. 1988. Genetic engineering of nuclear-encoded components of the photosynthetic apparatus of Arabidopsis. In *The impact of chemistry on biotechnology – a multidisciplinary discussion*. M. Phillips, S.P. Shoemaker, R.Middlekauff and R.M. Ottenbrite (Eds). ACS Books, Washington DC. 279-295

The United States Pharmacopeia, Vol. XXII. (1990). United States Pharmacopeial Convention, Inc., Rockville, MD, pp 1788-1789.

Verkerk R., M. Dekker and W.M.F. Jongen. 1998. Glucosinolates. In *Natural Toxicants in Food*. D. Wason (Ed). Sheffield Academic Press, Sheffield, England.

WHO (1991). Strategies for assessing the safety of foods produced by biotechnology. Report of a joint FAO/WHO Consultation. World Health Organization, Geneva, 59 pp.

WHO. Health aspects of marker genes in genetically modified plants. Report of a WHO workshop. 1993

Zambryski P. 1992. Chronicles from the Agrobacterium-plant cell DNA transfer story. Annu. Rev. Plant. Physiol. Plant Mol. Biol. 43: 465-490.