FOOD DERIVED FROM GLYPHOSATE - TOLERANT COTTON LINE 1445

A Safety Assessment

TECHNICAL REPORT SERIES NO. 6

AUSTRALIA NEW ZEALAND FOOD AUTHORITY
November 2001

ISBN 0 642 34525 2
ISSN 1446-4977
Published November 2001

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SUMMARY

Food derived from glyphosate-tolerant cotton line 1445 has been evaluated 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

Glyphosate-tolerant cotton line 1445 was generated by the transfer of the CP4 EPSPS gene which confers glyphosate tolerance to the plant. The CP4 EPSPS protein is an enzyme that is not sensitive to inhibition by glyphosate because of reduced affinity for glyphosate.

Other genes transferred along with the CP4 EPSPS gene were the *nptII* gene and the *aad* gene. The *nptII* gene was used as a marker to select transformed plant cells during the cotton transformation procedure. It codes for the enzyme neomycin phosphotransferase and is derived from Transposon Tn5 from the bacterium *Escherichia coli*. It confers resistance to the aminoglycoside antibiotics neomycin, kanamycin and gentamicin. The *aad* gene is a marker used to select transformed bacteria from non-transformed bacteria during the DNA cloning and recombination steps undertaken prior to transformation of the plant cells. It codes for the enzyme aminoglycoside adenyltransferase (AAD) and confers resistance to the antibiotics spectinomycin and streptomycin.

The molecular and genetic analyses indicated that the introduced genes have been stably integrated into the genome of glyphosate-tolerant cotton line 1445 and were stably inherited for multiple generations.

General safety issues

The only human food products derived from cotton are refined oil and processed linters (>99% cellulose) and these have a long history of safe use as human food. Refined oil and linters may be used in processed foods such as frying oil, mayonnaise, salad dressing, margarine, high fibre dietary products, sausage casings and thickeners.

One of the important issues to consider in relation to genetically modified foods is the impact on human health from potential transfer of novel genetic material to cells or bacteria in the human digestive tract. Much of the concern in this regard is with the presence of antibiotic resistance genes in genetically modified foods. In the case of the glyphosate-tolerant cotton, it was concluded that the *nptII* and *aad* genes would be extremely unlikely to transfer to bacteria in the human digestive tract because of the number and complexity of the steps that would need to take place consecutively. More importantly however, in the highly unlikely event that transfer did occur, the human health impacts would be negligible because kanamycin and streptomycin resistant bacteria are already commonly found in the human gut and in the environment. Transfer of novel genetic material from the glyphosate-tolerant cotton to human cells via the digestive tract was also considered to be equally unlikely.

Processing of cottonseed to produce refined oil and cellulose from linters is expected to remove and destroy DNA, further reducing the chances of any transfer. No DNA was detected in refined cottonseed oil of line 1445.

Toxicological issues

The *aad* gene is not expressed in line 1445. CP4 EPSPS and NPT II proteins are present at very low levels in cottonseed of line 1445. Refined cottonseed oil and processed linters are considered free of protein. No CP4 EPSPS protein was detected in linters after the first processing step.

Data for the newly expressed CP4 EPSPS and NPT II proteins in glyphosate-tolerant cotton line 1445 have been evaluated for its potential toxicity to humans. Previous studies showed no signs of toxicity among mice following acute oral doses up to 572 mg/kg for CP4 EPSPS and 5000 mg/kg for NPT II. No significant similarity to the amino acid sequences of known toxins was identified for either protein.

Neither of the expressed proteins exhibits characteristics of known allergens. Both proteins have been shown to be rapidly digested in simulated mammalian digestive systems. Amino acid sequence analyses did not reveal any similarities to known allergens.

Therefore, the evidence does not indicate that there is any potential for either protein to be toxic or allergenic to humans.

Nutritional issues

The compositional analyses were comprehensive and indicated that there are no substantial differences in the levels of major constituents, nutrients, anti-nutritional factors or natural toxicants in cottonseed between glyphosate-tolerant cotton line 1445 and the control line Coker 312, and that there was no change due to the application of glyphosate during cultivation. The components measured were proximate (protein, fat, moisture, fibre, ash, carbohydrates and calories), fatty acids and amino acids.

Analysis of the refined oil demonstrated that the composition is comparable in all respects to the control line C312.

The levels of natural toxicants of cotton, gossypol and the cyclopropenoid fatty acids, were also assessed. Cottonseed of line 1445 was found to have a slightly higher gossypol content compared to Coker 312 but well within the range for commercial cultivars. No gossypol was detected in refined cottonseed oil of either line 1445 or the control line C312. There was no difference in the levels of cyclopropenoid fatty acids in either cottonseed or refined oil from line 1445 and C312 indicating that the genetic modification process has not altered the levels of naturally-occurring toxins.

These analyses confirm that insect protected cotton line 1445 is nutritionally and compositionally comparable to other cotton lines and that no health or safety risks are posed by consuming food derived from the genetically modified cotton.

Conclusion

No public health and safety concerns have been identified in the assessment of glyphosate-tolerant cotton line 1445. Based on currently available data, refined oil and processed linters derived from glyphosate-tolerant cotton line 1445 are comparable to refined oil and linters derived from conventional cotton in terms of their safety and nutritional adequacy.

FOOD DERIVED FROM GLYPHOSATE-TOLERANT COTTON LINE 1445

A SAFETY ASSESSMENT

INTRODUCTION

A safety assessment has been conducted on foods derived from cotton that has been genetically modified to be tolerant to glyphosate. This cotton is referred to as glyphosate-tolerant cotton line 1445.

Glyphosate is a herbicide which is used widely as a non-selective agent for controlling weeds in primary crops. The mode of action of glyphosate is to specifically bind to and block the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an essential enzyme involved in the biosynthesis of aromatic amino acids in all plants, bacteria and fungi. The glyphosate-tolerant phenotype of RR cotton was conferred by the introduction of a bacterial gene which produces an EPSPS enzyme with a reduced affinity for glyphosate. The resultant level of enzyme activity is sufficient to sustain the plant in the presence of the herbicide.

The only human food products obtained from the cotton (*Gossypium hirsutum*) are cottonseed oil and linters. Cottonseed oil is a premium quality oil that may be used in a variety of foods including frying oil, mayonnaise, salad dressing, shortening, margarine and packing oil. Linters are short fibres removed from the cottonseed during processing (delinting). After extensive processing at alkaline pH and high temperatures, the linters may be used as high fibre dietary products, sausage casings and thickeners in ice cream and salad dressings. The linters consist primarily of cellulose (>99%).

The cotton was developed for cultivation in the United States and was approved for commercial release in the USA in September 1995. Cottonseed harvested from these plants or processed products containing cottonseed oil or linters may have been imported into Australia and New Zealand since 1996.

Glyphosate-tolerant cotton has been the subject of research and field trials in Australia under GMAC planned release guidelines, including proposal PR-83X(2). Approval for commercial release of glyphosate-tolerant cotton is currently being sought.

The data regarding the generation and characterisation of glyphosate-tolerant cotton line 1445 have been published in the scientific literature (Nida *et al* 1996a, 1996b, Sims *et al* 1996).

DESCRIPTION OF THE GENETIC MODIFICATION

Studies evaluated:

Barry, G.F., Taylor, M.L., Padgette, S.R., Kolacz, K.H., Hallas, L.E., della-Cioppa, G. and Kishore, G.M. 1994. Cloning and expression in *Escherichia coli* of the glyphosate-to-aminomethylphosphonic acid degrading activity from *Achromobacter* sp. strain LBAA. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-13245

Padgette, S.R., Barry, G.F., Re, D.B., Eichholtz, D.A., Weldon, M., Kolacz, K., Kishore, G.M. 1993 Purification, cloning and characterisation of a highly glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-12738

Shaw, M. 1997 Safety, compositional and nutritional aspects of cotton with the Roundup Ready gene, line 1445: conclusion based on studies and information evaluated according to FDA's policy on foods from new plant varieties. from Kolacz, K., Nida, D.L. and Serdy, F.S. 1995 Monsanto #95-106.

Methods used in the genetic modification

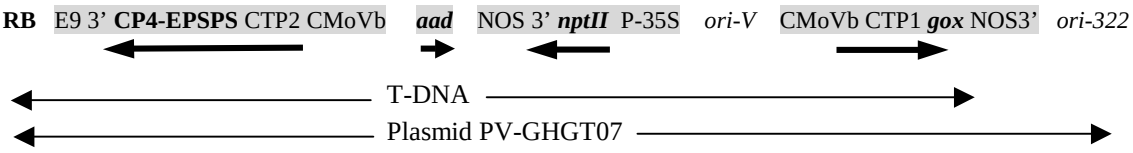
The glyphosate-tolerant (Roundup Ready®) cotton line 1445 was produced by *Agrobacterium*-mediated transformation of the parental cotton line, *Gossypium hirsutum* L. cv Coker C312, with plasmid PV-GHGT07. The *Agrobacterium*-mediated DNA transformation system is well understood (Zambryski, 1992).

The genes of interest were inserted into the plasmid adjacent to a DNA sequence known as the right border (RB). There is no *Agrobacterium* left border (LB) sequence present in PV-GHGT07 (Nida *et al* 1996a). The LB and RB have been isolated from Ti plasmids from *Agrobacterium* and are 25 base pair repeat sequences that delimit the DNA to transferred (T-DNA) in the transformation event. However it has been shown that the left border is not essential for integration of DNA into the plant genome (Jen and Chilton, 1986).

The plasmid PV-GHGT07 carries the genes encoding EPSPS from *Agrobacterium* strain CP4 (CP4-PESPS, Padgette *et al* 1996a), which is resistant to inhibition by glyphosate and the *nptII* gene which confers resistance to kanamycin (Beck *et al* 1982). Transformed plants were selected on the basis of their ability to grow in the presence of the antibiotic kanamycin conferred by the transfer of the *nptII* gene.

In addition, plasmid PV-GHGT07 also contains: (i) bacterial origin of replication (*ori*) sequences for replication in *Escherichia coli* and *Agrobacterium tumefaciens*; (ii) the bacterial *aad* gene which encodes aminoglycoside adenyltransferase (AAD), which confers resistance to spectinomycin and streptomycin allowing for selection of bacteria containing the plasmid; and (iii) the *gox* gene from the bacterium *Ochromobactrum anthropii* strain LBAA (formerly *Achromobacter* sp.), encoding the glyphosate metabolising enzyme glyphosate oxidoreductase (GOX). The arrangement of the genes in PV-GHGT07 is shown in Figure 1 and their origin and function are listed in Table 1.

Figure 1. Schematic diagram of PV-GHGT07[1]



[1] See Table 1 for an explanation of the abbreviations.

Function and regulation of the introduced gene(s)

5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene

The CP4 EPSPS gene introduced into cotton line 1445 was derived from *Agrobacterium* strain CP4 and encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase. The CP4 EPSPS protein is resistant to inhibition by glyphosate and has previously been transferred to soybeans (Padgette *et al* 1996b) to confer tolerance to glyphosate.
The native CP4 EPSPS gene from *Agrobacterium* CP4 contains sequences that might impair its expression in plants. A plant-preferred version of the CP4 EPSPS gene was synthesised in order to substitute plant preferred codons while producing an identical CP4 EPSPS protein (Harrison *et al* 1996). The activity of the resultant CP4 EPSPS enzyme was unaltered and was used for transformation of cotton.

Expression of the CP4-EPSPS gene in plant cells is under the control of the CMoVb promoter from Modified Figwort virus and the 3' non-translated region of the pea *rbcS* (small subunit ribulose bisphosphate carboxylase oxygenase) E9 gene (E9 3').

The endogenous EPSPS enzyme of plants is located within chloroplasts, the site of aromatic amino acid biosynthesis in plant cells. Many proteins with subcellular locations are synthesised as pre-proteins and directed to a particular organelle by a transit peptide at the end of the mature protein. Following delivery to the organelle, the short transit peptide is cleaved from the mature protein and is rapidly degraded. The CP4 EPSPS enzyme is targeted to the plastid by a chloroplast transit peptide sequence derived from the *Arabidopsis thaliana* EPSPS (CTP 2, Klee *et al* 1987). The CTP2 gene sequence was fused to the 5' end of the CP4 EPSPS gene. The CTP2 peptide sequence 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).

Neomycin phosphotransferase (nptII) gene

The *nptII* gene is derived from the bacterial Transposon Tn5 and encodes neomycin phosphotransferase II which confers resistance to the aminoglycoside antibiotics kanamycin, neomycin and geneticin, and is widely used as a marker in the initial selection of transformed plant cells. The *nptII* gene is transferred along with the CP4 EPSPS gene, enabling those plant cells successfully transformed with the CP4 EPSPS gene to grow in the presence of kanamycin. Those cells which lack the *nptII* gene, and hence the CP4 EPSPS gene, will not grow and divide in the presence of kanamycin.

Table 1. Genetic Elements contained in PV-GHGT07

Genetic element	Region	Name	Function	Source
Right Border		**RB**	**initiates T-DNA transfer**	*Agrobacterium tumefaciens*
gox	**Promoter** **Chloroplast Transit Peptide** ***gox*** **Terminator**	**P-CMoVb** **CTP 2** ***gox*** **NOS 3'**	**drives expression in plant cells** **directs protein to the chloroplast** **metabolises glyphosate** **signals termination of transcription**	Modified Figwort Virus 35S promoter CTP sequence from *Arabidopsis thaliana* EPSPS gene *Ochromobactrum anthropii* NOS 3' gene
CP4 EPSPS	**Promoter** **Chloroplast Transit Peptide** **CP4 EPSPS** **Terminator**	**P-CmoVb** **CTP 2** **CP4 EPSPS** **E9 3'**	**drives expression in plant cells** **directs protein to the chloroplast** **CP4 EPSPS protein** **signals termination of transcription**	Modified Figwort Virus 35S promoter CTP sequence from *Arabidopsis thaliana* EPSPS gene from *Agrobacterium* strain CP4 Pea *rbcS* E9 gene
aad		*aad*	Spectinomycin, Streptomycin resistance in bacterial cells	Transposon Tn7
nptII	Promoter *nptII* Terminator	P-35S *nptII* NOS 3'	drives expression in plant cells Kanamycin resistance in plant cells signals termination of transcription	Cauliflower Mosaic Virus Transposon Tn5 *A tumefaciens* strain T37 pTiT37
oriV		***oriV***	**origin of plasmid replication in *Agrobacterium***	*Agrobacterium tumefaciens*
Ori322		*ori322*	origin of plasmid replication in *Escherichia coli*	plasmid pBR322

The expression of the *nptII* gene in plant tissues requires the presence of a promoter that functions in a plant background. The expression of the *nptII* gene from PV-GHGT07 in cotton is effected by the 35S promoter from cauliflower mosaic virus and the 3' non-translated region of the nopaline synthase gene from the pTiT37 plasmid of *Agrobacterium tumefaciens* strain T37 (NOS 3').

Aminoglycoside adenyltransferase (aad) gene

The *aad* gene is derived from bacterial Transposon Tn7 and encodes aminoglycoside adenyltransferase (AAD) which confers resistance to the antibiotics spectinomycin and streptomycin. The *aad* gene is under the control of a bacterial promoter and was included in the construct as a marker to allow for selection of bacteria containing PV-GHGT07 prior to transformation of the plant cells. The *aad* gene has no plant regulatory sequences and would not be predicted to be expressed in plant tissues.

Glyphosate oxidoreductase gene

The glyphosate oxidoreductase *(gox)* gene is derived from the bacterium *O. anthropii* and encodes the enzyme glyphosate oxidoreductase (GOX). GOX catalyses the conversion of glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate thus effectively inactivating the herbicide (Pipke and Amrhein 1988, Jacob *et al* 1988). To ensure expression of the *gox* gene in plant tissues it was fused to the CMoVb promoter from Modified Figwort virus and the 3' non-translated region of the nopaline synthase gene from the pTiT37 plasmid of *A. tumefaciens* strain T37 (NOS 3'). Introduction of both the CP4 EPSPS and *gox* genes would provide an increased likelihood of tolerance to glyphosate, however molecular analysis revealed that the *gox* gene was not integrated into line 1445 (see 2.3 below).

Characterisation of the genes in the plant

Molecular characterisation of the integrated DNA present in glyphosate-tolerant cotton line 1445 was performed using untransformed cotton DNA and plasmid PV-GHGT07 and plasmid PV-GHGT06 as reference material (Nida *et al* 1996a). Plasmid PV-GHGT06 is identical to PV-GHGT07 except that it does not contain the *gox* gene sequence.

To determine the number of sites of insertion and the integrity of the genetic elements contained within the inserted DNA, genomic DNA from the transformed line 1445 was subjected to Southern blot analysis.

The analyses revealed that the modification in line 1445 is a single insertion event resulting from the introduction of a segment of DNA of approximately 6.1 Kb, comprised of the region of PV-GHGT07 from the right border to *oriV*, including CP4 EPSPS, *aad* and *nptII*. Table 2 lists the genes integrated into line 1445. Further analysis demonstrated that that none of the *gox* gene sequence and only a truncated segment of *oriV* was integrated into the genome of line 1445. The Southern blot data support the conclusion that all of the DNA required for expression of CP4 EPSPS and

nptII has been integrated into line 1445. Western blot data (see below) verify that these proteins are expressed.

Table 2. Genes transferred to glyphosate-tolerant Cotton line 1445

Gene	Function	Source
CP4 EPSPS	gene encoding CP4 EPSPS protein	*Agrobacterium* strain CP4
aad	Spectinomycin, Streptomycin resistance	Transposon Tn7
nptII	Kanamycin resistance	Transposon Tn5
oriV (incomplete)	origin of replication	*Agrobacterium tumefaciens*

Stability of the genetic changes

Progeny from three successive homozygous generations (R_3 through R_5) of the transgenic line 1445 were tested by Southern blot analysis to determine the stability of the integration of the DNA. The data show that the inserted DNA was present in each generation and is stably integrated into the cotton genome.

Conclusions regarding the genetic modification

The CP4 EPSPS, *npt II* and *aad* genes were transferred to cotton via an *Agrobacterium*-mediated transformation system resulting in the generation of the glyphosate-tolerant cotton line 1445. No other genes were transferred as a result of the transformation. The DNA has integrated into the genome of cotton line 1445 as a single and stable insert.

GENERAL SAFETY ISSUES

Glyphosate-tolerant cotton is grown in the USA for both domestic use and for export. Glyphosate tolerant cotton was approved for environmental release in the USA in 1995 (USDA/APHIS 1995). Refined oil and processed linters from glyphosate-tolerant cotton were approved for use in human food in the USA in 1995 (US FDA 1999) and in Canada in 1996 (Health Canada 1996). Processed foods, including imported processed foods may contain genetically modified cottonseed oil or cellulose from processed linters.

Glyphosate-tolerant cotton has been evaluated against the safety assessment guidelines developed by ANZFA. The data presented has been derived from whole cottonseed, cottonseed meal and flour (used only in animal feed) and refined cottonseed oil and processed linters, the only human food products derived from cottonseed. The safety assessment issues relate to Group B foods – food ingredients - as indicated in the guidelines for safety assessment of food produced using gene technology (ANZFA 1999a).

History of use of cottonseed products as foods

Only processed elements of cottonseed, ie oil and linters, are used as food in humans and these have a history of safe use. Neither whole cottonseed nor cottonseed meal is used in human food.

Cottonseed contains gossypol which is a biologically active terpenoid aldehyde. Gossypol is toxic *per se* and it also has adverse effects on the protein nutritive value of food by rendering lysine metabolically unavailable (Yannai and Bensai, 1983). The presence of gossypol limits the use of cottonseed as a protein source for humans or in animal feed, except for ruminants where bacteria in the rumen detoxify it (Randel *et al* 1992, Poore and Rogers 1998, Nikokyris *et al* 1991). However, the removal or inactivation of gossypol during processing enables the use of some cottonseed meal in feed for fish, poultry and pigs.

Refined cottonseed oil is free of gossypol (Cherry 1983, Gunstone *et al* 1994). The gossypol that partitions into the oil is eliminated during subsequent refining of the oil through inactivation by heat and alkali treatment. The reduction of free gossypol in oil is a measure of the food quality and processing efficiency. The refining process also removes protein from the oil.

Cottonseed oil is used as frying oil, and in mayonnaise, salad dressing, shortening, margarine and packing oil. Linters are processed to produce cellulose (99%) using alkaline pH and high temperature and used as high fibre dietary products, sausage casings and thickeners in ice cream and salad dressings.

CP4 EPSPS has previously been introduced in soybeans (Padgette *et al* 1996b) and corn. Products of these transgenic commodities, and of glyphosate-tolerant cotton, are permitted for sale in the USA, Canada and Japan.

Nature of novel proteins

Two novel proteins are expressed in line 1445 as the result of the transformation event. These are CP4 EPSPS and neomycin phosphotransferase (NPT II).

CP4 EPSPS

CP4 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. The EPSPS enzyme of plants is inhibited by glyphosate (Steinrucken and Amrhein 1980), however bacterial EPSPSs, such as CP4 EPSPS, have reduced affinity for glyphosate. The CP4 EPSPS protein exhibits approximately 50% amino acid homology with native plant EPSPS proteins.

Plant EPSPSs are localised in the chloroplast. *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,

only mature CP4 EPSPS, without any additional CTP residues at the amino terminus, would be predicted to be expressed in glyphosate resistant cotton.

NPT II

Neomycin phosphotransferase II (also known as aminoglycoside 3'-phosphotransferase II) is an enzyme with a molecular weight of 29 kD that catalyses the transfer of a phosphate group from adenosine 5'-triphosphate (ATP) to a hydroxyl group of aminoglycoside antibiotics, including neomycin, kanamycin and gentamicin A and B, thereby inactivating the antibiotics (Wright and Thompson 1999). The enzyme is encoded by the *nptII* gene, which is derived from transposon Tn5 from the bacterium *E. coli* (Beck *et al* 1982).

Expression of novel proteins in the plant

Studies evaluated:

Berberich, S.A. 1993 Evaluation of protein content in refined cottonseed oil produced from the 1992 insect resistant cotton field trials. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. Study Number 92-01-36-07

Burnette, B.L., Taylor, M.L., Lakemeyer, L.L., Smith, C.E., Bailey, M. and Nida, D.L. 1993 Characterisation of 5-enol-pyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) produced by glyphosate-tolerant cotton and assessment of equivalence relative to CP4 EPSPS produced by *E. coli*. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-13166

Heeren, R.A., Padgette, S.R. and Gustafson, M.E. 1993 The purification of recombinant *Escherichia coli* CP4 5-enolpyruval-shikimate-3-phosphate synthase for equivalence studies. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-12574

Nida, D.L., Halsey, M., Jackson, T., Taylor, M., Ebert, C., Taylor, N. and Sims, S. 1994 Evaluation of cotton with Roundup Ready™ genes generated in 1993 US field test locations. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-13613

Nida, D.L., Rogan, G.J. and Taylor, M.L. 1995 Evaluation of cotton with Roundup Ready™ genes generated in 1994 US field test locations. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-14046

Sims, S.R., Nida, D.L., Segalini, L.L., Leach, J.N., Ebert, C.C., Fuchs, R.L. and Berberich, S.A. 1995 Analysis of expressed proteins in fiber fractions from insect-protected and glyphosate-tolerant cotton varieties. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-14289

CP4 EPSPS

Cotton plants of line 1445 are tolerant to the normally lethal effects of glyphosate application as a result of CP4 EPSPS expression. Expression of CP4 EPSPS protein in line 1445 was verified by probing Western blots of protein extracts from cottonseed with a CP4 EPSPS-specific antibody. No expression was detected in the untransformed parental line Coker 312. The reactive protein observed in line 1445 exhibited the same apparent molecular weight (48 kD) as purified CP4 EPSPS protein isolated from *Escherichia coli* expressing the CP4 EPSPS gene (47.5 kD), also verifying that the CTP2 sequence (8.2 kD) is cleaved upon transport into the chloroplast.

The level of expression of CP4 EPSPS was measured in cottonseed and leaf from line 1445 by an enzyme linked immunosorbent assay (ELISA, Nida *et al* 1996a). The results of these studies are shown in Table 3. The data presented were obtained from two separate field trial experiments conducted at six separate locations in the USA in 1993 and 1994. The level of CP4 EPSPS protein in cotton line 1445 is low, ranging from 0.02% to 0.028% of total protein in cottonseed (calculated on the basis of protein content determined in the proximate analysis described in Tables 6 and 7).

The effect of application of glyphosate during plant growth on the expression levels of CP4 EPSPS in cottonseed was also investigated in field trials in 1994. There was no significant difference in expression of CP4 EPSPS in cottonseed between plants treated with glyphosate and those that were not treated (Table 3).
Refined cottonseed oil was not tested for the presence of CP4 EPSPS or NPT II protein. However the refining process removes protein, and refined cottonseed oil is considered free of protein (Rogers, 1990). Data submitted as part of an application from Monsanto for Ingard Cotton (A341, cotton expressing the Bt toxin of *Bacillus thuringiensis*) confirmed that no protein could not be detected in refined oil from either untransformed line C312 or Ingard cotton line 531 at a sensitivity of 1.3 ppm.

Combed lint and brown linter stock (ie linters after the first processing step) were tested for the presence of CP4 EPSPS protein by Western blotting. CP4 EPSPS was detected in combed lint but not in brown linter stock, the first product in the sequence of processing linters for cellulose (Sims *et al* 1996).

NPT II

The expression of the NPT II protein was determined by ELISA and the results are shown in Table 3. NPT II was detected at low levels in cottonseed and leaf (Nida *et al* 1996a). The NPT II protein content of cottonseed is 0.0022% of total protein (calculated as described for CP4 EPSPS above). The effect of application of glyphosate during plant growth was also investigated. There was no significant difference in expression of NPT II in cottonseed between plants treated with glyphosate and those that were not treated.

AAD

AAD expression was investigated by ELISA. No AAD expression was detected in line 1445. This result was expected as the *aad* gene is under the control of a bacterial promoter and would not be expected to be expressed in plant tissues.

Table 3. Protein expression levels in glyphosate-tolerant cotton line 1445 treated/untreated with glyphosate

Enzyme	Expression levels (µg/mg fresh weight)	
	- glyphosate	**+ glyphosate**
cottonseed		
CP4 EPSPS[1]	0.082 ± 0.017	NA
CP4 EPSPS[2]	0.06 ± 0.012	0.071 ± 0.015
NPT II[1]	0.0067 ± 0.001	NA
NPT II[2]	0.007 ± 0.0023	0.007 ± 0.003
AAD[1]	n.d.	NA
leaf		
CP4 EPSPS[1]	0.052 ± 0.016	NA
NPT II[1]	0.045 ± 0.014	NA

n=6, 1: 1993 field trial data, 2: 1994 field trial data, NA: not applicable, n.d.: not detected, +/- standard deviation

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

Studies evaluated:

Jennings, J.C., Doherty, S.C., Hamilton, K.A. and Lirette, R.P. 2000 Assessment of processed oil from Roundup Ready^{R} and Bollgard^{R} cottonseed for the presence of transgenic DNA. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-16554

In 1991, the World Health Organization (WHO) issued a report of a Joint FAO[1]/WHO 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 cells in the human digestive tract is with antibiotic resistance genes. Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes to select transformed cells. 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 have been concerns expressed, however, that there could be horizontal gene transfer of antibiotic resistance genes from ingested food to microorganisms present in the human digestive tract and that this could compromise the therapeutic use of antibiotics.

[1] Food and Agriculture Organization.

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.

This section of the report will evaluate the potential for therapy with antibiotics to be compromised through the presence of antibiotic resistance genes in the glyphosate-tolerant cotton line 1445.

Two antibiotic resistant genes have been transferred to the glyphosate-tolerant cotton. line 1445 contains the *nptII* gene, under the control of the 35S promoter, meaning it will be expressed in plant cells. line 1445 also contains a copy of the *aad* gene, which is under the control of a bacterial promoter, meaning it will not be expressed in plant cells (see 3.3 above).

Potential inactivation of an oral dose of kanamycin

A special concern with respect to antibiotic resistance genes is whether inactivation of an oral dose of antibiotics could occur during the treatment of humans. This may happen if the enzyme that confers antibiotic resistance is expressed in the plant and if it remains functionally active in the gastrointestinal tract once ingested.

The *nptII* gene is one of the most widely used selectable marker genes (Kärenlampi 1996). Of the antibiotics that are inactivated by NPT II, both neomycin and kanamycin still have clinical use, although neomycin tends to be used topically due to its oral toxicity (Davis *et al* 1980).

As the *nptII* gene is expressed in the cotton line 1445 the potential for ingested NPT II protein to inactivate an oral dose of kanamycin has been considered.

The potential for ingested NPT II to inactivate oral doses of kanamycin has been addressed in relation to the presence of the *nptII* gene in a delayed ripening tomato (Redenbaugh *et al* 1992, Redenbaugh *et al* 1994). The use of the *nptII* gene has also been reviewed by a WHO Workshop (WHO 1993), the Nordic Council of Ministers (Karenlampi 1996) and the United States Food and Drug Administration (US FDA 1998). It has been concluded that ingested NPT II, at the low levels likely to be present in transgenic plants, would not interfere with orally administered kanamycin therapy because firstly, NPT II is rapidly degraded in simulated gastric conditions (Fuchs *et al* 1993), secondly, even were NPT II not to be degraded, the enzyme requires ATP as a cofactor in order to inactivate kanamycin and ATP is limiting in the gastrointestinal tract, and finally, NPT II is unlikely to be active in the acidic conditions of the stomach.

The *nptII* gene used in the line 1445 cotton is identical to the gene used in the delayed ripening tomatoes. Therefore, the same conclusion regarding the use of *nptII* in tomatoes can also be applied to the cotton in this application. Furthermore, the only human food products derived from cotton, refined oil and processed linters, do not contain detectable levels of protein so the potential for inactivation of oral doses of kanamycin is negligible.

Potential for horizontal gene transfer

This section of the report will concentrate on evaluating the human health impact of the potential transfer of antibiotic resistance genes from glyphosate-tolerant cotton to microorganisms present in the human digestive tract.

The first issue that must be considered in relation to the presence of the *nptII* and *aad* genes present in glyphosate-tolerant cotton is the probability that either of these genes would be successfully transferred to, and expressed in, microorganisms present in the human digestive tract. The following steps are necessary for this to occur:

- excision of DNA fragments containing the *aad* gene and its bacterial promoter;
- survival of DNA fragments containing the *aad* gene in the digestive tract;
- natural transformation of bacteria inhabiting the digestive tract;
- survival of the bacterial restriction system by the DNA fragment containing the *aad* gene;
- stable integration of the DNA fragment containing the *aad* gene into the bacterial chromosome or plasmid;
- maintenance and expression of the *aad* gene by the bacteria.

In the case of the *nptII* gene, which does not have a bacterial promoter, an additional step would need to occur before antibiotic resistance could be expressed:

- integration of the DNA fragment containing the *nptII* gene in the appropriate orientation with respect to a functional bacterial promoter.

The transfer of either the *nptII* or *aad* gene to microorganisms in the human digestive tract is therefore highly unlikely because of the number and complexity of the steps that would need to take place consecutively.

The second and most important issue that must be considered is the potential impact on human health in the unlikely event successful transfer of either the *nptII* or *aad* gene to microorganisms in the human digestive tract did occur.

In the case of transfer of either the *nptII* or the *aad* gene from glyphosate-tolerant cotton to microorganisms of the digestive tract, the human health impacts are considered to be negligible. Of the antibiotics that are inactivated by NPT II, both neomycin and kanamycin still have clinical use, although neomycin tends to be used topically due to its oral toxicity (Davis *et al* 1980). The *nptII* gene already occurs naturally in bacteria inhabiting the human gut (Kärenlampi 1996). Streptomycin resistance genes are common and can be found at high frequencies in natural populations of bacteria and clinical isolates (Shaw *et al* 1993). Streptomycin has mostly been replaced by newer aminoglycosides, however, it is still used for special indications, such as in the treatment of tuberculosis and brucellosis (Kärenlampi 1996).

Therefore, the additive effect of a *nptII* gene or an *aad* gene from the glyphosate-tolerant cotton being taken up and expressed by microorganisms of the human digestive tract would be insignificant compared to the populations of kanamycin- and streptomycin-resistant bacteria already naturally present.

In relation to transfer of novel genetic material from genetically modified food to human cells via the digestive tract, this is also equally unlikely to occur. 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 genetically modified foods comprise only a minute fraction of the total DNA in the food (generally less than 0.01%) and are therefore unlikely to pose any special additional risks compared with the large amount of DNA naturally present in all foods.

Absence of DNA in refined oil

Only refined oil and cellulose from processed linters of cottonseed are consumed by humans. Processed linters are essentially pure cellulose (>99%) and are subjected to heat and solvent treatment that would be expected to remove and destroy DNA. The refining process for cottonseed oil also includes heat, solvent and alkali treatments that would be expected to remove and destroy DNA, and intact fragments of the *nptII* or *aad* genes are unlikely to survive the processing steps making the chance of horizontal gene transfer even more unlikely. The processing steps can also lead to the release of cellular enzymes (nucleases) which are responsible for degrading DNA into smaller fragments.

Refined cottonseed oil from line 1445 was analysed to ascertain if any intact DNA could be detected using the Polymerase Chain Reaction (PCR). The primers used were designed to amplify a portion of the CP4 EPSPS gene, the E9 terminator and a portion of cotton genomic DNA. No DNA was detected in refined cottonseed oil. The assay was able to detect as little as one nanogram of purified genomic DNA from line 1445.

The lack of intact DNA in the intended food products, cottonseed oil and cellulose from linters also mitigates against any horizontal transfer of genetic material to cells in the human digestive tract as a result of the ingestion of these foods.

Conclusions regarding general safety issues

The CP4 EPSPS and *npt II* genes are expressed in glyphosate-tolerant cotton line 1445 and the protein products are expressed at relatively low levels in the seed. There is no significant protein present in the refined oil or processed linters which are the only cotton products used for human consumption. The CP4 EPSPS gene and protein have been well characterised and are considered similar to EPSPS genes that are present in plants and therefore regularly consumed in human foods. The transfer of these genes to cotton is not considered to be a risk public health and safety.

It is extremely unlikely that either the kanamycin or strepromycin resistance genes will transfer from foods derived from glyphosate-tolerant cotton to bacteria in the human digestive tract because of the number and complexity of steps that would need to take place consecutively. In the highly unlikely event that either resistance gene was transferred to bacteria in the human digestive tract the human health impacts would be

negligible because kanamycin- and streptomycin-resistant bacteria are already commonly found in the human gut and in the environment.

It is also equally unlikely that novel genetic material from the glyphosate-tolerant cotton will be transferred to human cells via the digestive tract. The novel genetic material comprises only a minute fraction of the total DNA in the glyphosate-tolerant cotton therefore it is unlikely to pose any special additional risks compared with the large amount of DNA naturally present in all foods.

The demonstrated absence of DNA in refined cottonseed oil from line 1445, and the probable degradation and removal of DNA through the processing steps for refined oil and processed linters further mitigate against any horizontal transfer of DNA from glyphosate-tolerant cotton to cells in the human digestive tract.

TOXICOLOGICAL ISSUES

Levels of naturally occurring toxins

Studies evaluated:

Nida, D.L., Halsey, M., Jackson, T., Taylor, M., Ebert, C., Taylor, N. and Sims, S. 1994 Evaluation of cotton with Roundup Ready™ genes generated in 1993 US field test locations. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-13613

Nida, D.L., Rogan, G.J. and Taylor, M.L. 1995 Evaluation of cotton with Roundup Ready™ genes generated in 1994 US field test locations. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-14046

Gossypol

Gossypol is a biologically active terpenoid aldehyde that is present in discrete glands in various plant tissues, including seed. Gossypol has a number of toxic effects on mammals including: heart damage and heart failure (Poore and Rogers 1998, Randel *et al* 1992); mitochondrial uncoupling (Cuellar and Ramirez 1993); liver damage (Manabe *et al* 1991); disruption of oestrous cycles, pregnancy and embryo development (Randel *et al* 1992); and reduction of fertility because of sperm immotility and depressed sperm counts with specific mitochondrial damage evident (Randel *et al* 1992, Risco *et al* 1993). Whole cottonseed or cottonseed meal is not used in human foods and the presence of gossypol limits its use as in animal feed. As described above (3.1), ruminant animals (eg cattle and sheep) are able to tolerate gossypol more than other animals because of detoxification in the rumen.

The levels of gossypol were determined for control line C312 and line 1445 in cottonseed and refined oil (Nida *et al* 1996b). The effect of treatment with glyphosate on the gossypol content of cottonseed was also determined. The data are shown in Table 4.

The level of gossypol detected in cottonseed of line 1445 is statistically higher compared to line C312 (5% level, pairwise T test), but still within the ranges reported in the literature (Beradi & Goldblatt, 1980). The gossypol content observed in cottonseed for both C312 and line 1445 varied considerably between the six field test sites.

No free gossypol was detected in refined oil, the main food substance produced from cottonseed and consumed by humans.

Table 4. Gossypol levels in cottonseed from glyphosate-tolerant cotton line 1445 treated/untreated with glyphosate

Component	Control Coker 312	line 1445 - glyphosate	line 1445 + glyphosate	Literature Range %
%Total gossypol				
Seed[1]	1.19 (0-99-1.46)	1.32* (1.13-1.63)	ND	0.4 – 1.7
Seed[2]	0.902 (0.67-1.02)	1.023* (0.84-1.17)	1.047* (0.88-1.15)	0.4 – 1.7
Refined oil[1]	n.d.	n.d.	ND	<= 0.01
% Free gossypol				
Seed[1]	NA	NA	ND	0.4 – 1.7
Seed[2]	0.774 (0.55-0.86)	0.903* (0.75-1.01)	0.947* (0.80-1.06)	0.4 – 1.7
Refined oil[1]	n.d.	n.d.	ND	<= 0.01

n=6, 1: 1993 field trial data, 2: 1994 field trial data, *: statistically significant difference from control line C312 at the 5% level using a pairwise T test, ND=not determined, n.d.= not detected, NA=not applicable, ranges shown in parentheses

Cyclopropenoid fatty acids

The cyclopropenoid fatty acids, sterculic acid (C-17), dihydrosterculic acid (C-19) and malvalic acid (C-18), are unique fatty acids common in cotton and produce undesirable biological effects, including: the inhibition of biodesaturation of stearic to oleic acid affecting phospholipid biosynthesis (Rolph *et al* 1990; Cao *et al* 1993, Gunstone *et al* 1994); and has been reported to induce termination of embryo development in sheep through inhibition of progesterone production in the *corpus luteum* (Tumbelaka *et al* 1994).

The levels of sterculic acid, dihydrosterculic acid and malvalic acid were determined in cottonseed and refined oil. The data are shown in Table 5. No statistical difference was detected in the content of these fatty acids in cottonseed between line C312 and line 1445 with or without glyphosate treatment (5% level using a pairwise T test).

In refined oil (single samples processed from cottonseed pooled from the six field sites) the levels of dihydrosterculic and malvalic acids were virtually identical in line 1445 and the control line C312. The level of sterculic acid in refined oil from line 1445 was slightly higher than for the control line C312. However each of the cyclopropenoid fatty acids constitutes <1% of the total fatty acids and all the values observed were comparable to ranges observed for commercial cottonseed oil.

Table 5. Cyclopropenoid levels in glyphosate-tolerant cotton line 1445 treated/untreated with glyphosate

Component	Coker 312 untreated	line 1445 untreated	line 1445 + glyphosate	Literature Range (%)
Malvalic acid (C-17)				
Seed [1,3]	0.43 (0.25-0.58)	0.41 (0.21-0.58)	NA	
Seed [2,3]	0.334 (0.31-0.36)	0.336 (0.29-0.4)	0.357 (0.31-0.38)	
Refined oil [1,4]	0.35	0.56	NA	0.08-0.56
Sterculic acid (C-18)				
Seed [1,3]	0.71 (0.52-0.92)	0.70 (0.56-0.98)	NA	
Seed [2,3]	0.237 (0.12-0.77)	0.183 (0.11-0.37)	0.172 (0.12-0.26)	
Refined oil [1,4]	0.44	0.50	NA	0.22-1.44
Dihydrosterculic (C-19)				
Seed [1,3]	1.12 (0.34-3.39)	0.58 (0.27-1.07)	NA	
Seed [2,3]	0.128 (0.11-0.15)	0.128 (0.10-0.15)	0.122 (0.10-0.14)	
Refined oil [2,4]	0.23	0.23	NA	

n=6 for seed values, single values for refined oil, 1: 1993 field trial data, 2: 1994 field trial data, 3: seed values are % of total lipid, 4: oil values are % of total fatty acids, ND=not determined, NA=not applicable, ranges in parentheses

Aflatoxin

Aflatoxins are a group of mycotoxins produced by the *Aspergillus flavus* and *A. parasiticus* and are potent animal toxins and carcinogens and have been epidemiologically implicated as environmental carcinogens in humans. Cottonseed is one of the commodities most commonly contaminated by aflatoxins. Cotton that is damaged by moth larvae is more susceptible to infection by *Aspergillus* fungi. This infection is often initiated through larval damage that occurs in the field rather than in storage.

None of the four primary aflatoxins of cottonseed was detected at a sensitivity of 1 ppb in cottonseed from either line C312 or line 1445 from any of the experimental sites (Nida *et al* 1996b).

Potential toxicity of novel proteins

Studies evaluated:

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, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-12949

CP4 EPSPS

The toxicity of the CP4 EPSPS protein has previously been tested by acute gavage exposure in mice and no deleterious effects were observed (Harrison *et al* 1996). The CP4 EPSPS protein was administered at levels 1000 fold of those in anticipated consumption of food products (high dose: 572 mg/kg body weight).

The CP4 EPSPS protein solution was administered at three dosages up to 572 mg/kg to 10 male and 10 female mice in a single one ml dose at day zero and observations made for possible toxic effects twice daily for seven days. No mortality or moribundity resulted and there were no significant differences in terminal body weights between the treated and control groups. Upon necropsy, body cavities were opened and organs examined *in situ* and removed. No pathological findings attributable to the treatment were observed.

NPT II

The toxicity of the NPT II protein has previously been tested by acute gavage exposure in mice and no deleterious effects were observed (Fuchs *et al* 1993). The NPT II protein was administered in three cumulative target dosages of up to 5000 mg/kg body weight. Dissolved NPT II protein was administered to 10 male and 10 female mice in a split dose, in two equal doses over a four hour period. Mice were observed over a seven day period. No mortality or moribundity was observed and there were no significant differences in terminal body weights between the treated and control groups. No abnormalities were observed in internal organs upon post mortem examination.

Potential allergenicity of existing proteins

Allergic reactions to foods arise from an immune reaction to a particular protein which may be present in the food in very small amounts. Some common foods are known to elicit an allergic response in susceptible individuals. Foods such as cow's milk, soybeans and tree nuts are some of the better known sources of food allergies.

Refined cottonseed oil and cellulose from linters are devoid of protein and, given that most allergens are proteins, their consumption is unlikely to result in an allergic reaction. In addition, many refined oils have been shown not to be allergenic even if the source can be allergenic (eg peanuts, Taylor *et al* 1981). There have been reported incidences of allergic reaction in humans in response to consumption of foods containing cottonseed protein (Atkins *et al* 1988, Malanin and Kalimo 1988). However, whole cottonseed, cottonseed meal and cottonseed flour are not used for human consumption.

Therefore the potential for cottonseed oil or linters from glyphosate-tolerant cotton line 1445 to constitute a source of allergens is extremely low.

Potential allergenicity of novel proteins

Studies evaluated:

Burnette, B.L., Taylor, M.L., Lakemeyer, L.L., Smith, C.E., Bailey, M. and Nida, D.L. 1993 Characterisation of 5-enol-pyruvyl-shikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) produced by glyphosate-tolerant cotton and assessment of equivalence relative to CP4 EPSPS produced by *E. coli*. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-13166

Heeren, R.A., Padgette, S.R. and Gustafson, M.E. 1993 The purification of recombinant *Escherichia coli* CP4 5-enolpyruval-shikimate-3-phosphate synthase for equivalence studies. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-12574

Mitsky, T.A. 1993 Comparative alignment of CP4 EPSPS to known allergenic and toxic proteins using the FASTa algorithm. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-12820

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, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-12949

Sims, S.R., Nida, D.L., Segalini, L.L., Leach, J.N., Ebert, C.C., Fuchs, R.L. and Berberich, S.A. 1995 Analysis of expressed proteins in fiber fractions from insect-protected and glyphosate-tolerant cotton varieties. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-14289

Although there are no predictive assays available to definitively assess the allergic potential of proteins, a number of characteristics are common among many of the allergens that have been characterised. Known allergens tend to be glycosylated proteins with a molecular weight of 10–70 kD. Protein 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 NPT II proteins were evaluated for potential allergenicity against these criteria: size; glycosylation; resistance to heat and digestive degradation; and sequence similarity to known allergens.

CP4 EPSPS

The CP4 EPSPS gene was derived from *Agrobacterium* strain CP4. *Agrobacterium* is not a food substance but is a common soil bacterium found on and around plant produce. The CP4 EPSPS protein also shares 50% homology with plant EPSPS proteins which are normally consumed in plant foods without any reported allergenic effects. The EPSPS proteins are naturally present in foods derived from plants and microbes and have no history of being allergenic.

The molecular weight of the CP4 EPSPS protein is 48 kD, and thus within the size range of typical allergens. However, previous studies have demonstrated that the CP4

EPSPS protein is rapidly degraded in a simulated digestive system (Harrison *et al*, 1996) and is inactivated by heat treatment (Padgette *et al* 1996b).

The CP4 EPSPS protein sequence does not contain typical glycosylation sequences (eg histidine-aspartate-glutamate-leucine, Gomord *et al* 1997). Western blots indicated that the relative mobility of CP4 EPSPS protein in crude extracts from cottonseed of line 1445 did not differ from purified CP4 EPSPS. This supports the conclusion that the CP4 EPSPS is not glycosylated *in planta*.

Direct testing of CP4 EPSPS protein isolated from genetically modified cotton line 1698 was negative for glycosylation. line 1698 is a glyphosate-tolerant line generated by transformation with PV-GHGT06 (see 2.3 above) and the CP4 EPSPS expression cassette is identical to that contained in PV-GHGT07 used to generate line 1445. The glycosylation data obtained for CP4 EPSPS from line 1698 may therefore be considered highly predictive for line 1445.

Amino acid sequence similarity with known allergens is a useful indicator of allergenic potential. The amino acid sequence of the CP4 EPSPS protein was compared to the amino acid sequences of known allergens present in public domain databases (GenBank, EMBL, Swissprot, PIR). No biologically significant homology was found with any of these known allergens.

Comparison of the biochemical profile of the CP4 EPSPS enzyme to known protein allergens also provides a basis for allergenic assessment. It has been shown previously that purified CP4 EPSPS protein is rapidly degraded in simulated mammalian digestive systems (Harrison *et al* 1996). No CP4 EPSPS protein was detectable by Western blot after 15 seconds incubation in simulated gastric fluid and greater than 50% of CP4 EPSPS protein was removed by 15 minutes incubation in simulated intestinal fluid (Harrison *et al* 1996).

It can be concluded that the CP4 EPSPS gene introduced into cotton does not encode a known allergen and that the CP4 EPSPS protein does not share immunologically significant amino acid sequences with known allergens.

NPT II

The *npt II* gene is derived from Transposon Tn5 of *Escherichia coli*, a bacterium commonly found in the human digestive tract. NPT II is a 29 kD protein and thus within the size range of typical allergens. However NPT II has previously been shown to be rapidly degraded under simulated mammalian digestive conditions, being completely removed after 10 seconds in simulated gastric fluid and with 50% digested after five minutes in simulated intestinal fluid (Fuchs *et al* 1993).

The use of the NPT II enzyme as a processing aid has previously been evaluated by the Food and Drug Administration of the USA. The FDA concluded that this enzyme does not have any of the recognised characteristics of food allergens or any attributes that would distinguish it toxicologically from other phosphorylating enzymes in the food supply (US FDA 1994).

Residues of glyphosate or metabolites

Glyphosate is a herbicide commonly used on crops in the USA and Australia and is also used to desiccate plant tissues prior to harvest of grain. Maximum residue limits (MRLs) for glyphosate in grain crops have been set in the Food Standards Code (used by Australia, Standard A14 – Maximum Residue Limits, ANZFA 1999b) and Codex (used by New Zealand). MRLs are set at levels well below those which would represent a safety concern. Glyphosate has very low acute toxicity to mammals with an oral LD50 of >10,000 mg/kg in mice and >5,000 mg/kg in rats (Smith and Oehme 1992).

The MRL set for glyphosate in crude cottonseed oil is 0.1 mg/kg in the Food Standards Code and 0.05 mg/kg for edible cottonseed oil in Codex (FAO/WHO Codex 2000).

The levels of glyphosate residues in refined cottonseed oil and processed linters would be expected to be very low because of removal in processing. Glyphosate is a very hydrophilic molecule (Malik *et al* 1989) and this would be expected to contribute to its removal in processing. The residue levels in foods derived from glyphosate-tolerant cotton line 1445 would have to comply with either the Australian or CODEX MRL, depending on the jurisdiction.

Reports in the literature concentrate on the fate of glyphosate in weed plants killed by herbicide application, with data indicating that the primary removal of glyphosate is by bacterial activity (Smith and Oehme, 1992). Bacterial degradation results in the production of aminomethylphosphonate (AMPA) and glyoxylate, both non-toxic compounds (Smith and Oehme, 1992). An alternative pathway of degradation exists in many bacteria with glyphosate being converted to sarcosine and then to glycine and inorganic phosphate, all of which are non-toxic (Pipke *et al* 1987, Jacob *et al* 1988, Dick and Quinn 1995).

Data from the literature also suggest that glyphosate is not metabolised in plant tissues (Malik *et al* 1989, Wigfield *et al* 1994). CP4 EPSPS does not metabolise glyphosate. Therefore the novel CP4 EPSPS protein will not result in the production of any novel metabolites of glyphosate that would not otherwise be produced in a conventional plant sprayed with glyphosate.

Conclusions regarding toxicological issues

The data and analyses on the potential for toxicity or allergenicity of the CP4 EPSPS or NPT II proteins support the conclusions that neither protein is derived from an allergenic or toxic food source nor exhibits the characteristics of known protein allergens. CP4 EPSPS does not exhibit sequence similarity with known toxins or allergens. Furthermore, the CP4 EPSPS and NPT II proteins are present in relatively low abundance in cottonseed and both have been demonstrated to be degraded in conditions that mimic human digestion.

Refined cottonseed oil and cellulose from processed linters are the two components of cotton used in human foods. Refined oil is generally accepted as being devoid of protein. CP4 EPSPS protein was detected in raw linters but could not be detected in linters after the first processing step (brown linter stock, Sims *et al* 1996). From these

data it can be concluded that the food products derived from glyphosate-tolerant cotton should pose no greater threat as a source of allergic reaction than food products from conventional cotton.

NUTRITIONAL ISSUES

Studies evaluated:

Jennings, J.C., Doherty, S.C., Hamilton, K.A. and Lirette, R.P. 2000 Assessment of processed oil from Roundup Ready^{R} and Bollgard^{R} cottonseed for the presence of transgenic DNA. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-16554

Nida, D.L., Halsey, M., Jackson, T., Taylor, M., Ebert, C., Taylor, N. and Sims, S. 1994 Evaluation of cotton with Roundup Ready^{TM} genes generated in 1993 US field test locations. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-13613

Nida, D.L., Rogan, G.J. and Taylor, M.L. 1995 Evaluation of cotton with Roundup Ready^{TM} genes generated in 1994 US field test locations. Monsanto Company, 700 Chesterfield Parkway North, St Louis, MO, USA 63198. MSL-14046

Compositional analysis

Cottonseed used for compositional analyses was derived from field trial experiments conducted in 1993 and 1994. Cotton was planted at six sites for each trial. The six sites used in 1994 were different to those used in 1993. In order to assess the impact, if any, of application of glyphosate on the composition of line 1445, compositional analyses were made from plants subjected to glyphosate treatment or free from glyphosate treatment. These analyses were made at six field sites during trials in 1994. Treated plants were sprayed four times during the growing season: preemergent, postemergent (3-4 leaf stage), post-directed and preharvest (6-8 days prior) with applications of 3.36, 1.26, 1.26 and 1.68 kg/ha respectively.

The compositional analyses of cottonseed included proximate, amino acid, and aflatoxin, and of oil included tocopherols. Toxicant analyses evaluated gossypol and cyclopropenoid fatty acids in cottonseed and refined oil. The components measured included proximates (protein, fat, ash, carbohydrates, moisture, crude fibre), amino acid composition, fatty acids profile, and levels of tocopherols and of the toxicants sterculic and malvalic acid and gossypol. References were provided for all methods used in the analyses and the majority of methods were derived from standard methodology in *Official Methods of Analysis*, Association of Official Analytical Chemists (AOAC) and are validated AOAC International Methods, or American Oil Chemists Society (AOCS) Methods.

Proximate analysis

The proximate analyses of cottonseed from line 1445 did not vary markedly from the control line C312 (Tables 6 and 7). However some statistically significant differences were observed (5% level using a pairwise T test).

The protein content of cottonseed of line 1445 obtained from two separate growing seasons (untreated with glyphosate) was be ~6.5% higher than that of C312 (see Tables 6 and 7, 6.1% higher from 1993 and 6.5% higher from 1994). The protein content of

cottonseed of line 1445 treated with glyphosate was also higher than that of the C312 control but was not statistically significant. All of the protein content values were within the ranges reported in the literature. The level of carbohydrates was also less in all samples of line 1445 analysed. It should be noted that the carbohydrate content is calculated by subtraction rather than directly, and the reduction reflects the increased protein level.

Other statistically significant differences observed for line 1445 (untreated with glyphosate) were reduced ash content and increased fat content, but these differences were not evident in line 1445 treated with glyphosate (Table 7), or in cottonseed from the previous season (Table 6). However it should also be noted that there was significant overlap in the ranges of individual values for protein, fat, ash and moisture, treated or untreated with glyphosate, and all were well within reported ranges.

The results of the proximate analyses indicate that there are no substantial differences in these components between cottonseed from the control line C312 and line 1445 either treated or untreated with glyphosate.

Table 6. Compositional analysis of Cottonseed from line 1445 not treated with glyphosate[#]

Component	**Coker 312**	**line 1445**	**Literature Range (%)**
Protein %	27.76	*29.59**	12-32
Total fat %	23.35	**23.79**	23.2-25.7
Ash %	4.35	**4.70**	4.1-4.9
Carbohydrates %	44.35	*41.91**	
Calories/100g	498.59	500.17	
Moisture %	11.55	11.05	5.4-10.1

n=6, [#]from plants grown in field trials in 1993, *: statistically significant difference from control line C312 at the 5% level using a pairwise T test

Table 7. Compositional analysis of cottonseed from line 1445 treated with glyphosate[#]

Component	Coker 312 untreated	line 1445 untreated	line 1445 + glyphosate	Literature Range (%)
Protein %	28.80	*30.55**	30.03	12-32
Total fat %	24.43	*25.57**	25.09	23.2-25.7
Ash %	4.35	*4.53**	4.46	4.1-4.9
Crude Fibre %	13.83	13.59	13.66	
Carbohydrates %	42.40	*39.62**	*40.38**	
Calories/100g	504.7	508.2	507.8	
Moisture %	6.74	7.46	6.06	5.4-10.1

n=6, [#]from plants grown in field trials in 1994, *: statistically significant from control line C312 at the 5% level using a pairwise T test

Amino acid composition of cotton line 1445

A modified version of an AOAC International method was used to determine the amino acid composition of cottonseed from line 1445 (Nida *et al* 1996b). Eighteen individual amino acids were quantitated using an automated amino acid analyser. The analysis did not distinguish the related amino acids glutamate and glutamine or aspartate and asparagine. Seed from plants treated and untreated with glyphosate application was compared with the control line 312. The data are shown in Table 8.

No statistically significant differences between cottonseed of control line C312 and untreated or treated line 1445 were noted in the values for any of the amino acids tested. In particular, it was evident that expression of CP4 EPSPS, an enzyme in the aromatic amino acid biosynthetic pathway, in line 1445 had no effect on the levels of the aromatic amino acids tryptophan, phenylalanine and tyrosine.

Lipid and fatty acid composition of cotton line 1445

Cottonseed

Cottonseed from line 1445, cultivated with or without glyphosate treatment, was compared with control C312 samples for fatty acid composition (Nida *et al* 1996b). Total lipid content and composition of different fatty acid types were determined and the data are shown in Table 9 (1993 field trial data, no glyphosate treatment) and Table 10 (1994 field trial data, including treatment with glyphosate).

No statistically significant differences (5% level in a pairwise T test) were observed between line 1445 and the control line C312 for fatty acid composition of cottonseed grown in the 1993 field trial (Table 9).

Table 8. Amino acid content of cottonseed from line 1445 treated with glyphosate[#]

Component	Coker 312 untreated	line 1445 untreated	line 1445 + glyphosate	Literature Range[1]
Aspartate (& asparagine)	9.36	**9.41**	9.478	8.8-9.5
Threonine	3.441	**3.442**	3.487	2.8-3.2
Serine	4.734	**4.705**	4.721	3.9-4.4
Glutamate (&glutamine)	19.78	19.28	19.73	20.5-22.4
Proline	3.688	**3.640**	**3.635**	3.1-4.0
Glycine	4.362	4.295	4.455	3.8-4.5
Alanine	3.771	3.718	3.733	3.6-4.2
Cysteine	1.548	1.548	1.603	2.3-3.4
Valine	4.018	3.960	4.121	4.3-4.7
Methionine	1.511	1.450	1.484	**1.3-1.8**
Isoleucine	2.866	2.817	2.920	3-3.4
Leucine	5.899	5.835	5.932	5.5-6.1
Tyrosine	2.722	2.690	2.743	2.8-3.3
Phenylalanine	5.151	5.127	5.190	5-5.6
Lysine	4.573	4.512	4.614	3.9-4.1
Histidine	3.014	2.963	3.017	2.6-2.8
Arginine	10.98	11.10	11.34	10.9-12.3
Tryptophan	1.089	1.087	1.080	1-1.4

n=6, values are g/100g protein, [#]from plants grown in field trials in 1994, 1: Lawhon *et al* 1977, *: statistically significant from control line C312 at the 5% level using a pairwise T test

No statistically significant differences (5% level in a pairwise T test) between cottonseed from line 1445 (either treated or untreated with glyphosate) and the control line C312 for fatty acid composition of cottonseed grown in the 1994 field trial, with three exceptions (Table 10).

The total lipid content in glyphosate treated line 1445 was slightly higher (5.6%) than the control. However the lipid contents of cottonseed from the 1994 trial for the control line C312 and line 1445 (+/- glyphosate) were less than those determined for either line in the 1993 trial (Tables 9 and 10). Therefore this small difference is not considered significant because the small variation in lipid content observed for line 1445 treated with glyphosate was less than the interseasonal variation for all lines.

The level of myristic acid (14:0) was lower in cottonseed of line 1445 treated with glyphosate (21.5%) than the control line C312 (1994 trial, Table 10). The level of arachidic acid (20:0) in untreated line 1445 is higher (16%) than the C312 control (1994 trial, Table 10). However these differences are not considered significant because

levels of myristic and arachidic acid each constitute less than 1% of the total lipid, and the values were within the limits adopted by FAO/WHO Codex Alimentarius (1993) and values reported in the literature (Lawhon *et al* 1977, Cherry 1983, Gunstone *et al* 1994).

Table 9. Fatty acid content of cottonseed from line 1445[#]

Component	**Coker 312 untreated**	line 1445 untreated	**Literature Range[3]**
Lipid[1]	32.65	**32.24**	
Myristic (14:0)[2]	0.97	**0.95**	**0.64-1.3**
Pentadecanoic (15:0)[2]	1.00	**0.56**	
Palmitic (16:0)[2]	27.70	26.76	22.18-27.76
Palmitoleic (16:1)[2]	0.64	**0.65**	**0.66-1.3**
Margaric (17:0)[2]	0.16	**0.18**	
Stearic (18:0)[2]	2.68	2.67	2.14-3.23
Oleic (18:1)[2]	15.28	15.49	13.95-21.16
Linoleic (18:2)[2]	43.18	45.90	45.84-57.83
Linolenic (18:3)[2]	0.16	0.21	
Arachidic (20:0)[2]	0.24	0.29	
Behenic (22:0)[2]	0.15	0.17	

n=6, 1: % of sample weight, 2: % total lipid, 3: Cherry 1983, [#]from plants grown in field trials in 1993, no statistically significant differences from control line C312 at the 5% level using a pairwise T test

Refined oil

Single samples of refined oil from cottonseed of lines C312 and 1445 were assessed for fatty acid composition. The data are shown in Table 11. The oil samples were refined from cottonseed pooled from all six sites from the 1993 field trial. No data was presented for the composition of oil from cottonseed treated with glyphosate. There were no significant differences in the major fatty acid components of refined oil observed between line 1445 and the control line C312 for. The levels of some low percentage fatty acids differed between line 1445 and the control line C312, but all values were within ranges reported in the literature (Lawhon *et al* 1977, Cherry 1983, Rogers 1990, Gunstone *et al* 1994). It can therefore be concluded that the composition of refined oil from glyphosate-tolerant cotton line 1445 is equivalent to oil derived from conventional cotton varieties.

Table 10. Fatty acid content of cottonseed from line 1445 treated with glyphosate[#]

Component	Coker 312 untreated	line 1445 untreated	line 1445 + glyphosate	Literature Range[3]
Lipid[1]	26.26	**26.74**	*27.68**	
Myristic (14:0)[2]	0.779	**0.693**	*0.611**	**0.64-1.3**
Pentadecanoic (15:0)[2]	0.163	**0.147**	0.173	
Palmitic (16:0)[2]	24.60	24.82	25.30	22.18-27.76
Palmitoleic (16:1)[2]	0.388	**0.354**	**0.383**	**0.66-1.3**
Stearic (18:0)[2]	2.003	2.256	2.072	
Oleic (18:1)[2]	15.33	14.99	14.78	2.14-3.23
Linoleic (18:2)[2]	55.33	55.38	55.33	13.95-21.16
Linolenic (18:3)[2]	0.138	0.130	0.137	
Arachidic (20:0)[2]	0.178	*0.212**	0.194	
Behenic (22:0)[2]	0.104	0.106	0.105	

n=6, 1: % of sample weight, 2: % total lipid, 3: Cherry 1983, [#]:from plants grown in field trials in 1994, *: statistically significant from control line C312 at the 5% level using a pairwise T test

Alpha-tocopherols

Tocopherols are naturally present in cottonseed oil and serve as antioxidants and confer good storage properties. Alpha-tocopherols in particular have vitamin E potency. They are affected by processing and lost during refining and deodorising. The levels of α-tocopherol in refined oil from lines C312 and 1445 were 588 mg/kg and 670 mg/kg respectively (Table 11) within the ranges reported in the literature (Dicks 1965, Rogers 1990, Rossell 1991, Gunstone *et al* 1994).

Table 11. Fatty acid content of refined cottonseed oil from line 1445[#]

Component	Coker 312 untreated	line 1445 untreated	Literature Range[3]
Myristic (14:0)[1]	0.95	**0.84**	0.5-2.5
Pentadecanoic (15:0)[1]	0.40	**0.43**	
Palmitic (16:0)[1]	25.54	25.14	17-29
Palmitoleic (16:1)[1]	0.63	**0.61**	0.5-1.5
Margaric (17:0)[1]	0.16	**0.20**	
Stearic (18:0)[1]	2.46	2.41	1.0-4.0
Oleic (18:1)[1]	15.03	14.53	13-44
Linoleic (18:2)[1]	50.10	51.27	33-58
Linolenic (18:3)[1]	0.14	0.16	0.1-2.1
Arachidic (20:0)[1]	0.26	0.27	**<0.5**
Behenic (22:0)[1]	0.12	0.08	<0.5
α-tocopherol[2]	580	670	74-660[4]

[#]single values for oil of cottonseed refined from pooled samples of plants grown in 6 field sites in 1993, 1: % total lipid, 2: mg/kg, 3: Lawhon *et al* 1977, Cherry 1983, Rogers 1990, Gunstone *et al* 1994, 4: Dicks 1965, Rossell 1991,

Conclusions from compositional analyses

Comprehensive data from a range of compositional analyses conducted on cottonseed from both untreated and treated cotton line 1445 and the control line C312 were presented for assessment. The compositional components measured included proximates (protein, fat, ash, carbohydrates, moisture, and crude fibre), amino acid composition, fatty acids profile, gossypol and α-tocopherol levels.

The results of the compositional data do not indicate that there are any substantial differences between glyphosate-tolerant cotton line 1445, either untreated or following treatment, and the non-transgenic control line Coker C312 for any of the parameters measured. Some small statistically significant differences were observed in protein, fat, ash and carbohydrate content for line 1445, but the various values were within ranges previously reported for cotton and were not considered to be of either biological relevance for commercially grown cotton varieties or of significance in terms of food safety.

Levels of anti-nutrients

In addition to its toxic effects the terpenoid gossypol, naturally occurring in cottonseed, has antinutritive characteristics through reducing the availability of lysine (Yannai and Bensai, 1983). The level of gossypol in line 1445 is described above (4.1 and Table 4). No gossypol was detected in refined oil.

Ability to support typical growth and well-being

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 line 1445, no data was presented on feeding studies to animals. The compositional and other data provided in the application is considered adequate to establish the nutritional adequacy and safety of refined cottonseed oil and processed linters from glyphosate-tolerant cotton. Cellulose derived from linters would not be expected to support typical growth and well-being from either the modified or unmodified cotton line.

The nutritional profile of cotton line 1445 was determined by compositional analysis of the major components of cottonseed and refined oil and these were found to be comparable to the conventional control line Coker C312. Refined oil and cellulose from processed linters are the only human food products derived from cottonseed. The composition of refined cottonseed oil from line 1445 was equivalent to, and would be expected to be equally nutritious as, the control parent line C312.

Conclusions regarding nutritional issues

The nutritional qualities of glyphosate-tolerant cotton line 1445 were determined by compositional analyses of the major components of the seed and processed fractions and these were found to be comparable in all respects to the conventional control line Coker 312. Genetically modified cotton plants that were treated with the herbicide glyphosate (Roundup®) during cultivation were also analysed and found to be comparable to the parent line.

The applicant has demonstrated that the levels of the antinutrients sterculic acid, malvalic acid and dihydrosterculic acid in cottonseed and refined oil from glyphosate-tolerant cotton line 1445 are not substantially different from the parent line and are within normal ranges. Gossypol was not detected in refined oil.

There is a long history of safe use of cottonseed oil and cellulose derived from processed linters. Based on the data submitted in the present application, refined oil and processed linters derived from glyphosate-tolerant cotton line 1445 are nutritionally and compositionally comparable to that from conventional cotton and are not considered to pose a risk to human health and safety.

ACKNOWLEDGEMENT

ANZFA gratefully acknowledges Associate Professor Richard Roush, Director, Centre for Weed Management Systems, Waite Institute, University of Adelaide for expert comments on this safety assessment report.

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