FOOD PRODUCED FROM GLYPHOSATE-TOLERANT SUGAR BEET LINE 77

A Safety Assessment

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SUMMARY

Food derived from genetically modified (GM) sugar beet line 77 has been evaluated for 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 sugar beet line 77 has been developed to provide growers with a crop that is tolerant to applications of the broad-spectrum herbicide, glyphosate. This trait has been introduced into sugar beet plants by the addition of two new genes. One of these genes encodes the CP4-EPSPS protein, a key enzyme in the biosynthesis of aromatic amino acids in plants and microbes that is not sensitive to applications of glyphosate. The second gene, the *gox* gene, encodes the glyphosate oxidoreductase enzyme that can degrade the herbicide. However, this gene was truncated during transformation and 69% of the gene is fused to sugar beet DNA resulting in a chimeric gene. Although messenger RNA transcripts from this chimeric gox sequence are present in the sugar beet, no novel protein is translated and the sugar beet does not have GOX enzyme activity.

As well as the two genes conferring glyphosate tolerance, a third gene was transferred, the *uid*A gene, which encodes β–D-glucuronidase (GUS). GUS serves as a marker for plant transformation.

Single copies of the *cp4 epsps*, *uid*A and the chimeric *gox* gene were stably integrated at one insertion site in sugar beet. They were also inherited in a Mendelian manner, and always segregated together.

History of use

Sugar beet has a long history of safe use as a source of sugar and provides approximately one third of world sugar. The major food products are refined sugar and molasses. Sugar beet pulp may be used as food fibre. By-products from sugar beet (tops, leaves and post-processing trash) are used as cattle feed.

Characterisation of novel protein

Two new proteins are present in glyphosate-tolerant sugar beet line 77, namely the CP4-EPSPS and GUS proteins. These proteins were detected at very low levels in root tissue of sugar beet line77 (58ppm and 0.5ppm for CP4 EPSPS and GUS respectively). They were also present at higher levels in leaf and stem tissue (237ppm and 3ppm for CP4-EPSPS and GUS respectively). Neither protein was detected in the principal food fractions produced from sugar beet (refined sugar and molasses). The novel proteins were also detected at very low levels in sugar beet pulp. However the proteins are not expected to be present in the final product due to the extensive refining that pulp undergoes if it is processed into refined dietary fibre. Thus exposure to the novel proteins is likely to be extremely low.

The potential toxicity and allergenicity of the CP4 EPSPS and GUS proteins as well as the potential protein product from the chimeric *gox* gene were assessed. These proteins did not possess characteristics of known toxins and results from acute oral toxicity testing in mice did not indicate any toxic effects. The novel proteins were found to be rapidly digested in conditions that mimic human digestion. Additionally, they show no amino acid sequence similarity to known allergens and are not detectable in products refined from the glyphosate-tolerant sugar beet.

Comparative analyses

The compositional analyses were comprehensive and demonstrated that there are no substantial differences in the levels of major constituents or nutrients, between sugar beet line 77 and conventional sugar beet lines. The components measured were proximate (protein, fat, moisture, fibre, ash, and carbohydrates), invert sugar (glucose and fructose) content, sodium, amino nitrogen, polarisation (% sucrose) and potassium. No significant differences regarding nutritional and toxicological parameters were evident and thus no feeding studies were undertaken.

These analyses confirmed that glyphosate tolerant sugar beet line 77 is nutritionally and compositionally comparable to other sugar beet lines and that no health or safety risks are posed by consuming food derived from the GM sugar beet.

Conclusion

No public health and safety concerns have been identified in the assessment of glyphosate tolerant sugar beet. Based on the currently available data, food derived from the GM sugar beet line 77 is comparable to food derived from conventional sugar beet in terms of its safety and nutritional adequacy.

FOOD PRODUCED FROM GLYPHOSATE TOLERANT SUGAR BEET LINE 77:

A SAFETY ASSESSMENT

BACKGROUND

A safety assessment has been conducted on food derived from sugar beet that has been genetically modified to be tolerant to the herbicide, glyphosate. The modified sugar beet is referred to as glyphosate-tolerant sugar beet line 77.

Glyphosate is the active ingredient of the herbicide Roundup® 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. Biochemical studies on the EPSPS enzyme from a variety of different species have shown that a natural variation in glyphosate binding affinity exists, particularly across bacterial species (Schultz *et al*. 1985). Further studies on bacterial and plant EPSPS enzymes demonstrated that sequence changes at the active site of the enzyme, a highly conserved region across species, could alter substrate and inhibitor binding properties (Padgette *et al.*, 1991). Tolerance to glyphosate in plants can therefore be achieved by introducing a version of the EPSPS gene producing a protein with a reduced binding affinity for glyphosate, thus allowing the plant to function normally in the presence of the herbicide.

Glyphosate-tolerant sugar beet line 77 was developed through the introduction of the *cp4-epsps* gene derived from the soil bacterium *Agrobacterium sp.*CP4 (Padgette *et al.*, 1996). The *cp4-epsps* gene has been transferred into a number of other crop plants, including soybean, canola, corn, and cotton, to establish glyphosate tolerance. These plants have been assessed by Food Standards Australia New Zealand (FSANZ) under Standard 1.5.2 – Foods Produced Using Gene Technology of the Australian New Zealand Food Standards Code and have been found to be safe for human consumption (FSANZ, 2002).

Sugar beet is processed into two major food products, pure sucrose and molasses. Sugar beet pulp is a by-product of processing which has occasionally been purified and sold as food fibre. Waste products from both pre-processing (leaves and tops) and post-processing (trash) are used as cattle feed. Sugar beet currently accounts for approximately 1/3rd of world sugar production with some 35% being produced in the EU, 20% in Russia and 10% in the USA (Macrae *et al.* 1993). Sugar in Australia and New Zealand is entirely produced from sugar cane; while refined sugar from sugar beet is not specifically imported into Australia or New Zealand, it may occur as an element within ingredients used in locally produced processed foods or as an ingredient within imported processed foods.

HISTORY OF USE

Sugar beet has a long history of safe use as a food for both humans and other animals. Sugar beet root has been used as a source of sugar since ancient times, being initially cultivated in southern Europe and North Africa, although production was limited. The prominence of sugar beet in Europe rose when a practical method for extracting sugar was invented in Germany in the mid 18th century. Sugar beet was brought to the United States in the middle of the 19th century where it now accounts for approximately half of the sugar produced (one third of sugar consumed in the USA is imported).

Sugar beet is currently grown in many climates, from temperate (California, Spain and Italy) to cold climates (Dakota, Finland and Russia) and accounts for approximately 40% of world refined sugar production. Sugar beet is not grown in Australia or New Zealand - we obtain our sugar from sugar cane. However, sugar from sugar beet may enter the Australian and New Zealand food supply through imported processed foods.

Uses of purified beet sugar include soft drinks, chocolates and confectionery, yoghurts and other milk-based foods, pastries and biscuits, syrups, jams and preserved fruits, wines, breakfast foods, ice-creams and sorbets, liquors and spirits, concentrated and powdered milk, sweets and burnt sugar (used to dye and aromatise).

The main use of sugar beet pulp is in animal feedstuffs. Other products, representing a very small percentage of the total use, are processed from pulp. The food components which could be extracted from pulp include: *L-arabinose* (hemicellulose monomer) and *araban gel* used as a fat substitute, *pectins* (polymer of D-glutamic acid) used for specific food applications (emulsion stabilisation), and *fibre products* used as texturing agents and as a source of fibrin by the bakery and breakfast cereal industry.

The nutrition and health aspects of sugar consumption have been extensively researched over the last 20 years and other than the contribution to dental caries, there is no conclusive evidence that demonstrates that sugar is a hazard to the general public when consumed at the levels and in the manner currently practiced. As a consequence sugar has GRAS (Generally Recognised as Safe) status.

DESCRIPTION OF THE GENETIC MODIFICATION

Studies evaluated:

Kolacz, K.H. and G.F. Barry 1996. Roundup® Ready Sugar Beet: Plant Transformation Vector. Monsanto Technical Report MSL-14678. Monsanto Company, St Louis, USA.

Mannerlof, M., Tuvesson, S., Steen, P. and P. Tenning. 1997. Transgenic sugar beet tolerance to glyphosate. Euphytica 94: 83-91.

Mannerlof, M. and J. Gielsen. 1996. Molecular analysis of Roundup Ready sugar beet line T9100152 (Note: this line is the same as GTSB77). Novartis Seeds, Technical Report.

Methods used in the genetic modification

Glyphosate-tolerant sugar beet line 77 was produced by *Agrobacterium*-mediated transformation of sugar beet line A1012. The *Agrobacterium*-mediated DNA transformation system is the basis of natural plasmid-induced crown-gall formation in many plants and 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 border sequences were isolated from the Ti plasmid of *Agrobacterium* and normally delimit the DNA sequence (T-DNA) transferred into the plant.

The plasmid used for transformation, plasmid PV-BVGT03, contains four gene cassettes each consisting of the gene of interest plus specific controlling sequences within the Left and Right Borders. Three of these gene cassettes were transferred to sugar beet line 77 – the *cp4-epsps* gene cassette, the *uidA* gene cassette, and the modified *gox* gene cassette. These are shown in table 1. The fourth gene cassette contained the *nptII* gene. This gene encodes neomycin phosphotransferase II, which confers resistance to the antibiotic kanamycin. This gene was not transferred to sugar beet.

Function and regulation of novel genes

Each of the genes of interest transferred from plasmid PV-BVGT03 to sugar beet requires regulatory sequences that promote and terminate gene transcription into messenger RNA and translation into a protein product targeted to the appropriate cellular compartment. A promoter sequence is the leading control element of a gene that dictates when, where and to what extent, the gene is transcribed into messenger RNA. A terminator is a DNA sequence that defines the terminal end of a gene by stopping the transcription of messenger RNA. These sequences can be unique in each organism and thus

regulatory elements derived from plants are often used in gene constructs to enable the functioning of novel genes derived from other organisms.

The regulatory and coding regions for each novel gene cassette that was transferred to sugar beet line 77 are summarised in Table 1 below.

Table 1: Description of gene cassettes for transfer from plasmid PV-BVGT03.

Cassette	Genetic Elements	Source	Function
EPSPS	Modified 35S promoter (35S)	figwort mosaic virus	Promoter of high level constitutive gene expression in plant tissues
	Chloroplast Transit Peptide (CTP2)	*Arabidopsis thaliana* *epsps* gene	Directs the EPSPS protein into the chloroplast where it is active
	CP4-EPSPS coding region (*cp4-epsps*)	*Agrobacterium* sp. Strain CP4	Coding sequence for 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS) which maintains aromatic amino acid synthesis through its insensitivity to glyphosate
	Pea E9 3' terminator (E9-3')	*Pisum sativum* *rbcS* gene	Contains signal sequences for termination of transcription and directs polyadenylation
GUS	Modified cauliflower mosaic virus 35S promoter (CaMV)	cauliflower mosaic virus	Promoter for high level constitutive gene expression in plant tissues
	UidA coding region (*uidA*)	Protein coding sequence of the enzyme β-glucuronidase (*uidA* gene) from *Escherichia coli*	Colourimetric marker enzyme used for selection of transformed plant lines
	Pea E9 3' terminator (E9-3')	*Pisum sativum* *rbcS* gene	Contains signal sequences for termination of transcription and directs polyadenylation
GOX	Modified 35S promoter (35S)	figwort mosaic virus	Promoter for high level constitutive gene expression in plant tissues
	Chloroplast Transit Peptide (CTP1)	Chloroplast transit peptide sequence from small subunit 1A of Ribulose bisphosphate carboxylase from *Arabidopsis thaliana*	Directs the GOX protein into the chloroplast which is the site of action
	Gox coding region (*gox*)	Synthetic glyphosate oxidoreductase gene based on sequence from the bacterium *Ochromobactrum anthropii* strain LBAA	Metabolises glyphosate to amino-methyl phosphonic acid (AMPA) and glyoxylate which are not active on EPSPS
	NOS 3' terminator	From nopaline synthase gene from *Agrobacterium* sp.	Contains signal sequences for termination of transcription and directs polyadenylation

The cp4 epsps gene cassette

EPSPS is an essential enzyme involved in the biosynthesis of aromatic amino acids via the shikimate metabolic pathway. This metabolic pathway is present in all plants, bacteria and fungi (Haslam, 1993). Plant variants of the EPSPS enzyme are inhibited by the herbicide glyphosate, however, bacterial variants of the EPSPS enzyme are, in general, not inhibited due to reduced binding affinity to the herbicide (Schültz *et al*, 1985). One such low binding-affinity variant is the *cp4-epsps* gene derived from the common soil bacterium *Agrobacterium*. The *cp4-epsps* gene was transferred to sugar

beet to confer tolerance to glyphosate.

In the EPSPS cassette the *cp4-epsps* coding sequence from *Agrobacterium* was fused between a modified version of the 35S promoter from a figwort mosaic virus (P-CMoVb), which promotes constitutive expression of the gene in plant tissues, and the 3' end of the pea rbcS E9 gene (E9 3'), which terminates transcription and contains sequences that will direct the polyadenylation of the messenger RNA. The bacterial *cp4 epsps* gene was modified to create a synthetic gene, which allows for higher expression in plants. These changes to the DNA sequence do not affect the functional activity of the expressed proteins.

The bacterial EPSPS enzyme was targeted to the chloroplast, the active site of the enzyme in higher plants (della Ciopa *et al*, 1986), by the chloroplast transit peptide sequence (CTP2) derived from the *Arabidopsis thaliana epsps* gene. This sequence was fused between the 35S promoter and the *cp4-epsps* coding region.

The GUS gene cassette

The *uidA* gene from the bacterium *Escherichia coli* (*E. coli*) codes for the enzyme β-glucuronidase (GUS), an acid hydrolase that cleaves β-glucuronides (Jefferson *et al.,* 1987). The *uidA* gene was introduced into sugar beet line 77 to act as a visible marker in plant transformation. When present, GUS is capable of hydrolysing the chemical p-nitrophenyl-β-D-glucuronide into a colour-forming compound that enables visual scoring of transgenic events. GUS activity also occurs naturally in vertebrates and has been detected in a number of plant species including sugar beet where it can be differentiated from the *uidA* derived GUS due to a different pH activity optimum (Hu *et al.* 1990; Wozniak and Owens 1994).

In the GUS gene cassette the *uidA* coding sequence was fused between an enhanced 35S promoter derived from cauliflower mosaic virus (which promotes high-level constitutive gene expression in plant tissues), and the 3' non-translated region of the *rbcS* E9 gene from pea, which directs polyadenylation of messenger RNA.

The GOX gene cassette

The *gox* gene from the common soil bacterium *Ochromobactrum anthropii* strain LBAA [formerly *Achromobacter sp*] encodes the enzyme glyphosate oxidoreductase (GOX), which 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 and is readily degraded by several micro-organisms. Glyoxylate is commonly found in plant cells and is broken down by the glyoxylic acid pathway for lipid metabolism.

The *gox* gene was intended for transfer to sugar beet to augment its resistance to glyphosate. In the GOX gene cassette the *gox* coding region was fused, between a modified 35S promoter sequence from a figwort mosaic virus (which promotes constitutive expression in plants) and a terminator sequence derived from the 3' non-translated region of the nopaline synthase gene from *Agrobacterium*. The GOX protein was targeted to the chloroplast, the site of action of glyphosate, by a chloroplast transit peptide (CTP1) sequence fused between the 35S promoter and the *gox* coding region. The CTP1 sequence was derived from the *Arabidopsis thaliana rubisco* gene (Timko *et al*, 1988).

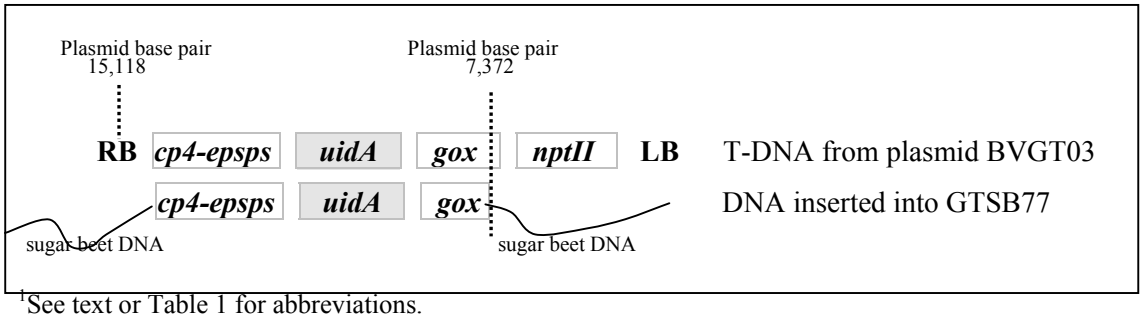
Characterisation of the genes in the plant

Molecular analysis of glyphosate-tolerant sugar beet line 77 was used to detect the presence of transferred DNA sequences and to determine the copy number and stability of the inserted DNA.

Using DNA hybridisation probes specific to each gene cassette, Southern blot analysis revealed that a single insertion event occurred with complete copies of the *cp4-epsps* and *uidA* genes and a partial copy of the *gox* gene being transferred from the T-DNA of plasmid PV-BVGT03.

A genomic library of sugar beet line 77 was made to characterise the border sequences of the insertion event with the sugar beet genome. DNA sequencing revealed that the integrated DNA commenced 25 base pairs (bp) downstream of the right border prior to the figwort mosaic virus promoter sequence of the *cp4-epsps* gene cassette and terminated 897 bp downstream of the *gox* gene start codon (at base pair 7372 of the T-DNA) within the coding region of the *gox* gene (Figure 1). The upstream regulatory elements of the *gox* gene, the constitutive promoter 35S and the *A. thaliana* SSU1A gene chloroplast transit peptide (CTP1), were found to be intact. Sequencing downstream of the truncated *gox* gene into the sugar beet genomic DNA revealed that 897 bp of the *gox* gene had been transferred (representing 69% of the complete *gox* coding sequence). Two translational stop codons were identified in the sugar beet genome 130 bp and 234 bp downstream of the fusion junction in frame with the *gox* gene reading frame. A transcription termination signal was also identified within the sugar beet genomic DNA 650 bp downstream of the junction point.

Figure 1: Schematic diagram of the T-DNA from PV-BVGT03 and the DNA inserted into sugar beet GTSB77[1]



[1]See text or Table 1 for abbreviations.

Stability of genetic changes

The stability of the inserted DNA was demonstrated using Southern blot analysis of tissues from the R_2, R_3 and R_4 generations of sugar beet line 77. The right junction region was probed using an internal fragment of the *cp4-epsps* gene and the left border region was probed using an internal fragment of the *gox* gene. Segregation analysis based on this molecular analysis showed that a single dominant insertion event had occurred that segregated as a single locus according to Mendelian principles. The analysis further showed that the chimeric *gox* gene was stably maintained through generations.

Phenotypic segregation analysis was also undertaken of the glyphosate-tolerant trait and GUS activity in these generations and confirmed an inheritance pattern consistent with the stable transfer of a single dominant locus for these genes. These phenotypes were also shown to be stable over multiple generations in successive cropping years.

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

The potential human health impact of transfer of novel genetic material to cells of the human digestive tract depends 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 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 micro-organisms is in respect to antibiotic resistance genes. No antibiotic resistance gene is present in sugar beet line 77, so in this case transfer of antibiotic resistance to micro-organisms in the digestive system is not an issue.

In relation to the transfer of other novel genetic material from GM food to bacteria in the human digestive system, this is extremely unlikely to occur 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 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 sugar beet line 77 is not considered to pose any risk to public health and safety.

CHARACTERISATION OF NOVEL PROTEIN

Biochemical function and phenotypic effects

Two novel proteins were expected to be expressed in glyphosate-tolerant sugar beet line 77: CP4-EPSPS and GUS. As a truncated version of the *gox* gene had also been transferred, a possibility also existed that chimeric GOX-like proteins may also be expressed.

CP4-EPSPS Protein

CP4-EPSPS is an essential enzyme in the biosynthesis of the aromatic amino acids via 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 is 47.6 kD in size and consists of a single polypeptide of 455 amino acids.

Plant EPSPSs are localised in the chloroplast. In sugar beet line 77, the CP4-EPSPS gene has been fused to the *Arabidopsis thaliana* EPSPS chloroplast transit peptide (CTP) that targets the protein to the chloroplast. *In vitro* chloroplast uptake assays have shown that the *A. thaliana* EPSPS CTP delivers CP4-EPSPS to the chloroplast where it is subsequently cleaved from the pre–protein, yielding mature CP4-EPSPS with no CTP amino acids retained (della Ciopa *et al*, 1986). 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 is predicted to be expressed in sugar beet line 77.

[1] Food and Agriculture Organization.

CP4-EPSPS has previously been introduced into soybeans, potato, canola, corn and cotton. Products of these transgenic commodities are variously permitted for sale in Australia, New Zealand, the EU, USA, Canada and Japan.

GUS protein

The β-glucuronidase protein, GUS, has an experimentally determined molecular weight of 68.2kD. GUS catalyses the hydrolysis of a wide range of glycosides including synthetic p-nitrophenyl-β-D-glucuronide. Hydrolysis of this chromogenic compound produces a blue colour that has proved a versatile visual marker in a range of plant transformation systems (Jefferson *et al.* 1987). GUS is naturally present in a wide range of microbes, animals and plants including sugar beet (Wozniak and Owens, 1994). The *E. coli* variant of GUS expressed in sugar beet line 77 was distinguishable from endogenous sugar beet GUS due to differential pH specificity for the chromogenic substrate.

Chimeric GOX-like proteins

A truncated version of the *gox* gene was inserted into sugar beet line 77. The truncation incorporated 897 bp of the *gox* gene cassette (representing 69% of the complete *gox* coding sequence). Sequencing into the sugar beet genome identified two translational stop codons in the sugar beet genome 130 bp and 234 bp downstream of the fusion junction in frame with the *gox* translational frame. A transcription termination signal was also identified within the sugar beet genome 650 bp downstream of the junction point.

Because the sequence data revealed the possibility for a functional gene a thorough investigation of *gox*-like expression products was undertaken.

Northern blot analysis of RNA recovered from sugar beet line 77 tissue showed that two transcripts of differing abundance hybridised to random primed DNA probe. The high abundance transcript was approximately 1.5-1.7 kilobases (kb) in size. The lower abundance transcript was approximately 2.0 kb in size. The different transcripts were concluded to represent transcription of the chimeric *gox* fusion gene through to the two alternative transcription termination sites. Both transcripts however were concluded to be of sufficient length to code for GOX-like proteins and further studies to identify the expression of these proteins were undertaken.

Protein expression analyses

As the expression of all three genes; *cp4-epsps*, *uidA*, and chimeric *gox*, are under the control of constitutive promoters it is expected that respectively expressed proteins would be found in all tissues of sugar beet line 77. Expression levels of CP4-EPSP, GUS and putative GOX-like proteins were measured using either ELISA or Western blotting. ELISA is a highly sensitive technique that can detect the presence of a protein generally to a sensitivity of 10-100 pg.

ELISAs for CP4-EPSPS and GUS protein were conducted using antibodies raised to each protein expressed in *E. coli* cultures into which the *cp4-epsps* and *uidA* genes were cloned. The *E. coli*-derived proteins were determined to be equivalent to the plant-expressed forms through a number of analyses (see Potential toxicity of newly expressed proteins below). Analyses were performed on early and mature leaf tissue (referred to as top) and processed root (referred to as brei), from segregating populations of sugar beet line 77 in separate field trials. Segregating plants not expressing the glyphosate-tolerant phenotype were used as controls. Trials were conducted in six different geographic sites in Europe in 1995 and 1996, and in five different geographic sites in the USA in 1996. Results are presented in Table 2.

Table 2. Summary of expression levels of CP4-EPSPS and GUS in sugar beet 77*

Tissue Type	CP4-EPSPS protein[1] (ng/mg tissue fresh wt)			GUS protein[1] (ng/mg tissue fresh wt)		
	EU 1995[2]	EU 1996[3]	US 1996[4]	EU 1995[2]	EU 1996[3]	US 1996[4]
Early Leaf						
Mean	145	n.a.	n.a.	2	n.a.	n.a.
Range	130 - 179			0.8 - 3.6		
Top Untreated						
Mean	285	190	172	3.0	3.4	2.78
Range	249 - 370	134 - 273	126 - 193	2.4 - 3.6	2.4 - 3.6	2.35 - 3.35
Treated						
Mean	n.a.	n.a.	151	n.a.	n.a.	2.69
Range			130 - 167			2.29 - 3.18
Brei						
Untreated						
Mean	54	63	47	0.6	0.5	0.39
Range	46 - 64	50 - 76	32 - 60	0.4 - 0.8	0.08 - 0.6	0.28 - 0.55
Treated						
Mean	n.a.	n.a.	50	n.a.	n.a.	0.48
Range			32 - 60			0.41- 0.64

n.a. not available

* Treated values represent plants sprayed with glyphosate at the recommended agronomic rate: 3 applications at 0.75 lb (active equivalent) per acre.

[1] No expression products were detected in untransformed negative control plants for either protein.

2 Mean and range were calculated using n=6 with one sample being provided from 6 different geographic sites.

[3] Mean and range were calculated using n=12 with two samples being provided from 6 different geographic sites.

[4] Mean and range were calculated using n=10 with two samples being provided from 5 different geographic sites.

The results outlined in Table 2 show that expression of CP4-EPSPS and GUS proteins are generally at low levels in sugar beet with highest expression occurring in young leaves and mature tops and the lowest in brei. The level of CP4-EPSPS protein is near two orders of magnitude higher than the level of GUS in nearly all tissues. Expression levels for both proteins in mature tops and brei from glyphosate-tolerant sugar beet line 77 plants treated with glyphosate levels representative of commercial conditions were comparable to levels in untreated GTSB77 plants. Similar results were obtained in studies run over two successive years in a range of geographical locations. These data indicate that expression of *cp4-epsps* and *uidA* genes is consistent over generations and reflects that both genes are stably inherited. They also indicate that spraying with glyphosate does not influence the expression of these two genes.

The level of GOX-like proteins potentially expressed in sugar beet line 77 tissue was analysed by Western blot analysis using antibodies raised to protein expressed in *E. coli* cultures into which the chimeric *gox*-fusion gene had been cloned. Cloning was undertaken using a PCR-based strategy to amplify the *gox*-fusion sequence from sugar beet line 77. The *gox*-fusion cloned into *E. coli* included the CTP1 sequence, the truncated *gox* sequence and 130 bp of fused sugar beet genomic DNA ending at an identified translational stop codon. The plasmid transformation vector containing the *gox*-fusion sequence was named pMON34550.

Protein extracted from refractile bodies in cultures of *E. coli* transformed with pMON34550 were

purified and run on SDS-PAGE against GOX protein standards (from a transgenic sugar beet line expressing GOX). A protein – designated Protein 34550 – ran concurrent with the GOX standard at approximately 46kD. Amino acid sequencing of Protein 34550 indicated it was composed of the 89 amino acids of the CTP1 sequence, 299 amino acids from the N-terminus of the GOX protein, and 43 amino acids encoded by sugar beet genomic DNA.

Western blots using antibodies raised and affinity purified to Protein 34550 were undertaken on plant tissue extracts from three different sugar beet line 77 varietal lines grown at three different locations in the USA. Tissues analysed included seeds, whole plants (at the two leaf stage and at three months of age), and extracts from leaves and roots from plants at the four, six and eight leaf stage. For all samples, no Protein 34550 was shown to be present at a limit of detection in plant tissue of 180 parts per billion (ppb).

Further, analysis of messenger RNA transcripts from the truncated *gox* gene in sugar beet line 77 tissues showed that the messenger RNA transcripts contain a C-terminal coding region and a 3' untranslated region that is rich in AU nucleotide sequence. Such AU motifs are associated with messenger RNA degradation (Di Noia *et al.,* 2000). Translation from such transcripts may be inhibited or the translation products are highly unstable polypeptides, which results in their rapid degradation and thus a lack of detectable Protein 34550 in sugar beet line 77 tissues.

To confirm that Protein 34550 does not exhibit GOX-like activity an ancillary study was undertaken by monitoring the production of glyoxylate, the breakdown product of glyphosate associated with GOX activity. No detectable GOX-like activity could be demonstrated for Protein 34550 purified from *E. coli*. A similar study confirmed that sugar beet line 77 does not display GOX activity.

Protein levels in sugar products produced from sugar beet

The high temperatures and precipitation methods used in the production of sugar from sugar beets are known to reduce protein levels significantly with the highest level of protein detected in refined sugar being 1.2 µg per g (Potter, *et al.,* 1990). Since CP4-EPSPS and GUS proteins are expressed at low levels in the sugar beet root (CP4-EPSPS ranging between 32-76 µg per g fresh weight and GUS ranging between 0.08-0.8 µg per g fresh weight), the level of these proteins in refined sugar is extremely low or absent (i.e. less than 2 ppb and 4 ppb respectively, as discussed in Section 3.4).

It is concluded that little if any CP4-EPSPS or GUS protein would be consumed as a consequence of eating food containing sugar derived from sugar beet line 77.

Potential toxicity of novel protein

Studies evaluated:

Astwood, J.A. 1996. β-D-glucuronidase (GUS) shares no significant sequence similarity with protein toxins found in the public domain databases. Monsanto Technical Report MSL-14633. Monsanto Company, St. Louis, USA, 63198.

Astwood, J.D. 1997. Protein 34550 has no significant sequence similarity to known allergens and toxins. Monsanto Technical Report MSL-14988. Monsanto Company, St. Louis ,USA, 63198.

Harrison, L.A., Bailey, M.R., Nida, D.L., Taylor, M.L., Holden, L.R. and S.R. Padgette. 1993. Preparation and confirmation of doses for an acute mouse feeding study with CP4-EPSPS. Monsanto Technical Report MSL-12900. Monsanto Company, St. Louis, USA, 63198.

Harrison, L.A., Biest, N.A., Leimburger, R. and S.R. Padgette. 1996. Equivalence of plant- and microbially-expressed proteins: β-D-glucuronidase from glyphosate-tolerant soybean and *E. coli*. Monsanto Technical Report MSL-12881. Monsanto Company, St. Louis, USA, 63198.

Harrison, L.A., Biest, N.A., Leimburger, R. and S.R. Padgette. 1996. Preparation, characterisation and confirmation of doses for an acute mouse feeding study with β-D-glucuronidase. Monsanto Technical Report MSL-12979. Monsanto Company, St. Louis, USA, 63198.

Lee, T.C. and G. Go. 1997. Preparation and confirmation of doses for an acute mouse toxicity study (EHL-96210) with Protein 34550. Monsanto Technical Report MSL-14982. Monsanto Company, St. Louis, USA, 63198.

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

Naylor, M.W. 1992. Acute oral toxicity study of β-D-glucuronidase (GUS) protein in albino mice. Monsanto Technical Report MSL-12485. Monsanto Company, St. Louis, USA, 63198.

Naylor, M.W. and F. Ruecker. 1997. Acute oral toxicity study of Protein 34550 in albino mice. Monsanto Technical Report MSL-15042. Monsanto Company, St. Louis ,USA, 63198.

Taylor, M.L., Go,G., Mahadeo, D.A. Rochester, D.E. and T.E. Nickson. 1997. Assessment of equivalence of Protein 34550 expressed in Roundup Ready® sugar beet (Line #77) and *E. coli*. Monsanto Technical Report MSL-14870. Monsanto Company, St. Louis, USA, 63198.

The detailed protein expression analyses have demonstrated that three new proteins are expressed in sugar beet line 77; the CP4 EPSPS protein, the truncated GOX protein (Protein 34550) and the GUS protein. The potential toxicity and dietary exposure of these three novel proteins were assessed through four different approaches;

1. potential human exposure;
2. homology to known protein toxins;
3. digestive fate in simulated gastric and intestinal fluids; and
4. acute mouse toxicity studies.

The toxicity of the CP4-EPSPS protein expressed in sugar beet line 77 has also been addressed by FSANZ in other safety assessments of foods assessed under Standard 1.5.2 of the Australia New Zealand Food Standards Code (see Roundup Ready soybeans, Roundup Ready cotton, Roundup Ready corn and Roundup Ready canola). The safety of the GUS protein and Protein 34550 have not been addressed in other applications. Certain aspects of the toxicity data have been published in the scientific literature as cited in the text.

The *cp4-epsps, uidA* and *chimeric-gox* genes were cloned into *E. coli* in order to obtain sufficient amounts of each respective protein to assess its safety. CP4-EPSPS and GUS proteins derived from *E. coli* were shown to be equivalent for safety assessment purposes to the respective plant expressed proteins on the basis that:

- comparative Western blot analysis demonstrated similar immunoreactivity and equivalent molecular weights (the latter also shown by SDS-PAGE analysis);
- positive correlation occurred between quantity and immunological dose-response in ELISA assays;
- comparative functional enzyme assays demonstrated correlative activities (in the case of CP4-EPSP and GUS); and
- homology of the N-terminal sequence of amino acids through 15 positions; and
- there are no differences in glycosilation patterns.

As no Protein 34550 was identified in sugar beet line 77 tissue, no equivalence of the *E. coli* cloned Protein 34550 could be established. PCR analysis using primers specific to the *truncated-gox* gene did, however, show that the sizes of the genes in both sugar beet line 77 and *E. coli* are equivalent at approximately 1273 bp.

Patterns of amino acid sequence or shared regions between proteins may provide insight into the biological significance of a protein including its toxicity and allergenicity. When compared to the amino acid sequences of protein toxins in the PIR, EMBL, SwissProt and GenBank databases, the amino acid sequence of the CP4-EPSPS and GUS proteins, and Protein 34550, showed no significant similarities to any of the 1,935 protein toxins, or toxin-associated proteins, listed in these databases.

History of human exposure to CP4-EPSPS

The CP4-EPSPS protein has a specific catalytic function in the aromatic acid shikimate pathway of plants, bacteria and fungi. This pathway is not present in mammals. The CP4-EPSPS protein shows high amino acid sequence homology to the other EPSPS enzymes found in common food crops, (for example, soybean and tomato) which have a long history of safe human consumption, or that are present in fungal and microbial food sources such as Baker's yeast (*Saccharomyces cerevisiae*) or *Bacillus subtilis*. Thus, CP4-EPSPS is a member of a family of closely related proteins from plants and microbes that are commonly found in human foods.

History of human exposure to GUS

The *uidA* gene was originally isolated from *E. coli* which is a commensal bacterium found in the gut microflora of many animals including humans (Jefferson *et al.,* 1986). The GUS protein is an acid hydrolase that catalyses the cleavage of certain β-glucuronides and has been used extensively as a visible marker in evaluating putative genetic transformation events (Jefferson *et al.*, 1987). Apart from its expression in animals, GUS activity has also been detected in a number of plant tissues including sugar beet (Wozniak and Owens, 1994). The GUS protein is thus a common component of bacteria native to humans and animals regularly associated with a range of foods and feeds.

History of human exposure to the chimeric gox gene product; Protein 34550

The native *gox* gene is derived from *Ochromobactrum anthropii* strain LBAA [formerly *Achromobacter sp*], a commonly found bacterium in soil and is likely to occur on food plants. Southern and PCR analyses identified that a truncated chimeric version of the *gox* gene was present in sugar beet line 77. When cloned into *E. coli* the truncated chimeric *gox* gene expressed a new protein, which was labelled Protein 34550. Protein 34550 is composed of the 89 amino acids of the CTP1 genetic element, 299 amino acids of the N-terminus of the GOX protein and 43 amino acids encoded

by sugar beet DNA. Protein 34550 has no GOX enzymatic activity. If Protein 34550 were expressed in sugar beet line 77 it would represent a novel protein not previously found in the human diet.

Western and ELISA analysis for the presence of Protein 34550 in sugar beet line 77 revealed that it is not expressed in either leaves, roots or in derivative food components (refined sugar, molasses and dietary fibre). While no evidence of the presence of Protein 34550 was found in sugar beet line 77, toxicity studies of the protein were still carried out.

Sequence similarity to known protein toxins

Patterns of amino acid sequence or shared regions between proteins may provide insight into the biological significance of a protein including its toxicity and allergenicity. When compared to the amino acid sequences of protein toxins in the PIR, EMBL, SwissProt and GenBank databases, the amino acid sequence of the CP4-EPSPS and GUS proteins, and Protein 34550, showed no significant similarities to any of the 1,935 protein toxins, or toxin-associated proteins, listed in these databases.

Digestive fate of novel proteins in simulated gastric and intestinal fluids

Most proteins are readily degraded upon exposure to gastric and intestinal fluids in the digestive tract with 50% of solid food emptying from the human stomach in 2 hr (Sleisenger and Fordtran, 1989). The rate of degradation of novel proteins in simulated gastric (SGF) and intestinal fluids (SIF) thus enables a prediction of their fate in digestion.

The degradation of C4 EPSPS, GUS and Protein 34550 were followed, either through Western blot or enzyme activity assays, in both SGF and SIF at 37^{o}C. CP4-EPSPS protein was shown to have a half-life of 15sec in SGF and 10min in SIF. No detectable GUS protein was present after 15sec exposure to SGF and over 50% had been degraded in SIF after 60 to 120min. Over 90% of GUS activity had dissipated after 4h in SIF. Protein 34550 was degraded within 15 seconds exposure to SGF and within 60 seconds exposure to SIF, moreover no intermediate stable protein fragments larger that 2kD were generated by digestion of Protein 34550 in either SGF or SIF.

On the basis of these data all three novel proteins were demonstrated to be readily digestible.

Acute oral toxicity of novel proteins mice

To directly assess the potential toxicity of the CP4-EPSPS, GUS and 34550 proteins acute gavage tests were undertaken in mice using purified forms of each protein. The dosage of administration, treatment-related findings, and the safety factors relative to the highest potential human consumption are shown in Table 4. Note the safety factors were calculated on the basis of the level which humans would be exposed if each protein were expressed in soybean, corn, tomato and potato assuming no loss of protein due to processing.

Despite the level of all three proteins being well in excess of the likely human dietary exposure factor, no mortality or morbidity 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 with any protein were observed.

Table 4. Acute gavage studies of CP4-EPSPS, GUS and Protein 34550 toxicity in mice and exposure safety factor.

Protein	Max. dosage (mg/kg body wt)	Significant treatment-related effects[4]	Human exposure safety factor[5]
CP4-EPSPS[1]	572	None	1,300
GUS[2]	100	None	>1,000
34550[3]	20	None	10^{10}- 10^{11}

[1] Administered as a single dose by gavage to groups of 10 mice per sex at dosages of 49, 154 and 572 mg/kg (Harrison *et al.,* 1996).
[2] Administered as a single dose by gavage to groups of 10 mice per sex at dosages of 1, 10 and 100 mg/kg.
[3] Administered as a single dose by gavage to groups of 10 mice per sex at dosages of 0.2, 2 and 20 mg/kg.
[4] In-life observations included body weights, food consumption and signs of toxicity. Post-mortem observations included internal and external examinations, kidney weights and kidney histopathology. No statistical differences were observed outside those expected by chance alone at $p \leq 0.05$.
[5] Based on the potential exposure to humans if the protein were expressed in soybean, corn, tomato and potato assuming no loss due to processing.

Potential allergenicity of novel proteins

Studies evaluated

Astwood, J.A. 1995. CP4-EPSPS synthase shares no significant sequence similarity with proteins associated with allergy and coeliac disease. Monsanto Technical Report MSL-14174 Monsanto Company, St. Louis, USA, 63198.

Astwood, J.A. 1996. β-D-glucuronidase (GUS) shares no significant sequence similarity with protein associated with allergy or coeliac disease. Monsanto Technical Report MSL-14632 Monsanto Company, St. Louis, USA, 63198.

Leach, J.N. and J.D. Astwood. 1997. Assessment of the digestibility and fate of purified Protein 34550 *in vitro* using mammalian digestive fate models. Monsanto Technical Report MSL-14973. Monsanto Company, St. Louis, USA, 63198.

Ream, J.E., Bailey, M.R., Leach, J.N. and N. Biset. 1993. Assessment of the *in vitro* digestive fate of CP4-EPSPS synthase. Monsanto Technical Report MSL-12949. Monsanto Company, St. Louis, USA, 63198.

Ream, J.E. 1996. Assessment of the in vitro digestive fate of β-glucuronidase. Monsanto Technical Report MSL-14607. Monsanto Company, St. Louis, 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. Known allergens tend to be:

- glycosylated proteins with a molecular weight of 10–70 kDa,
- heat stable,
- resistant to proteolytic digestion and the acidic conditions of the stomach, and
- expressed as major proteins in commonly consumed foods.

The CP4-EPSPS, GUS and 34550 proteins were evaluated for potential allergenicity against well-accepted criteria for allergens (Taylor, 1992a; Taylor *et al.*, 1987; 1992b). These were whether:

- the source organism(s) has a history of allergenicity;
- the proteins are stable to digestion;
- the proteins are stable to food processing;
- the proteins are similar in amino acid sequence to known protein allergens; and
- the proteins are a principal component of the food.

Allergenicity of source organisms

The CP4-EPSPS protein was obtained from the naturally occurring soil-borne plant-symbiotic bacterium *Agrobacterium sp.* strain CP4. The *gox* coding region was obtained from the common soil bacterium *Ochrobactrum anthropi.* This coding region is responsible for the production of protein 34550. The *uidA* gene was obtained from *E. coli,* a bacteria prevalent in the gastrointestinal tract of animals including humans. None of these source organisms are known to be allergenic to humans.

Stability of protein to digestion

As reported above, the CP4-EPSPS, GUS and 34550 proteins are degraded rapidly after exposure to simulated gastric fluid. Such rapid digestion would severely limit the amount of protein absorbed via the intestine and thus restrict any potential immunological response.

Stability of the protein to food processing

The levels of CP4-EPSPS and GUS proteins in sugar beet line 77 are extremely low in the root (0.005% and 0.00006% respectively) and Protein 34550 was undetectable at a limit of detection of 100ppb. None of these proteins were detectable in molasses and refined sugar components derived from sugar beet line 77 at limits of detection ranging from 2 – 100ppb. CP4-EPSPS and GUS were detected at levels of 50ppm and 1ppm respectively in sugar beet pulp. Beet pulp is used to a limited degree in the manufacture of refined soluble food fibre that could potentially be used as an additive at less than 1% in some specific foods (breakfast cereals etc.). While no direct analysis of these proteins was undertaken in soluble fibre derivatives, it is expected that they would be at negligible concentrations due to the processing steps involved.

Sequence similarity to known protein allergens

Amino acid sequence similarity with known allergens is a useful indicator of the allergenic potential of novel proteins. The amino acid sequence of the CP4-EPSPS, GUS and 34550 proteins were compared to the amino acid sequences of 219 known allergens present in public domain databases (eg GenBank, EMBL, Swissprot, PIR). Sequence similarity was defined as a sequence identity of greater than seven contiguous amino acids. No biologically significant similarity was found between any of the novel proteins with any of allergens listed in these databases.

Level of novel protein in the final food

Allergenic proteins in known allergenic foods (such as milk, soybeans and peanuts) exist as major proteins (Metcalfe *et al.*, 1996). There is very limited potential for the novel proteins from sugar beet line 77 to act as allergens in final food products because:

- none of the novel proteins could be detected in either sugar or molasses derived from sugar beet line 77, and
- dietary fibre refined from pulp derived from sugar beet line 77 would contain extremely low levels of CP4-EPSPS and GUS as these are expressed in the crude pulp at very low levels (50ppm and 1ppm respectively). Protein 34550 was not detected in pulp samples.

COMPARATIVE ANALYSES

Studies evaluated

Andersen, A., Dideriksen, T.B., Knudsen, D. and E. Smed. 1996. Compositional analysis of beet with Roundup Ready gene from 1995 field trials. Danisco Technical Report RR SB 01. Holeby, Denmark.

Andersen, A., Dideriksen, T.B., Knudsen, D. and E. Smed. 1997. Compositional analysis of beet with Roundup Ready gene from 1996 field trials. Danisco Technical Report RR SB 02. Holeby, Denmark.

Mueth, M. 1996. Compositional analysis of Roundup Ready™ sugar beet (line #77) from 1996 field trials. Monsanto Company/CEREGEN;Environmental Sciences. Study No. 96-01-49-01. Monsanto Company, St. Louis ,USA, 63198.

Nickson, T.E. and M.T. Gies. 1996. Analysis of roots, leaves and tops from glyphosate-tolerant sugar beet from the 1995 field trial. Monsanto Technical Report MSL-14561. Monsanto Company, St. Louis, USA, 63198.

Taylor, M.L., Mueth, M.G. and T.E. Nickson. 1997. Analytical and compositional analyses of Roundup Ready™ sugar beet (line #77) from 1996 US field trials. Monsanto Technical Report MSL-15048. Monsanto Company, St. Louis ,USA, 63198.

Nutrient analysis

The safety assessment of foods produced using gene technology entails, in this case, evaluating compositional data from the transgenic sugar beet plant in comparison with equivalent data from the parental (or untransformed) plant line or literature values for the particular crop species. This process involves identifying the key components, including nutrients and any toxicants, characteristic of sugar beets and also takes into account the variation in composition due to genetic variability, environmental factors, and post-harvest handling and processing.

In order to determine the equivalence of sugar beet line 77 to conventional sugar beet, a broad range of compositional analyses were undertaken on samples of sugar beet line 77 root and top tissue obtained from five trials in the USA in 1996 and 20 trials across Europe in 1995 (6 trials), 1996 (6 trials) and 1997 (8 trials). As root tissue is the only component of sugar beet used in food production, only compositional and quality data for this tissue is presented. Roots were processed into brei – shredded roots used in the first step of sugar processing.

The analyses included proximate values for:

- crude ash;
- crude fibre;
- crude protein;
- carbohydrate;
- dry matter
- crude fats were also determined in tops.

Additional quality components were measured included:

- invert sugar (glucose + fructose) content;
- sodium;
- amino nitrogen.
- polarisation (% sucrose);
- potassium;

Data on saponins, the principal toxicant in sugar beet root, were considered previously under Toxicological Issues.

The effect of applications of glyphosate on the level of these components was also assessed in all trials. Glyphosate was applied at the suggested agronomic concentration of 0.75 lb (active equivalents) per acre.

Proximate and quality component analysis

Mean values and ranges of proximate constituents and quality components for root/brei tissue from all field trials, both untreated and treated with glyphosate, are summarised in table 5.

Table 5: Mean values and ranges for the proximate and quality component analyses of sugar beet GTSB77 roots/brei from various field trials*.

	Control		GTSB77 – untreated		GTSB77 – treated[2]		Literature Range[3]
ROOTS/BREI	Mean	Range	Mean	Range	Mean	Range	
Crude Ash							
1995 Europe	3.4	2.7-4.9	3.4	2.7-5.1	3.0	2.3-4.0	1.1-17.7
1996 Europe	2.5	2.0-3.2	2.5	2.1-3.4	2.7	2.3-3.2	
1996 USA	5.5	4.6-6.3	6.6	4.8-9.0	8.8	4.9-15.6	
1997 Europe	2.7	2.0-3.8	2.7	2.0-4.0	2.8	2.0-4.4	
Crude Fibre							
1995 Europe	4.1	3.5-5.2	4.0	3.1-5.3	3.6	3.0-4.8	2.9-7.4
1996 Europe	4.2	3.9-4.6	4.2	3.9-4.6	4.2	3.6-4.8	
1996 USA	4.1	2.8-5.0	4.0	3.3-4.7	4.1	3.3-4.8	
1997 Europe	4.2	3.7-4.7	4.2	3.5-5.1	4.1	3.7-4.9	
Invert Sugar							
1995 Europe	1.7	0.3-3.7	1.8	0.4-4.24	1.0	0.3-1.9	0.3-2.7
1996 Europe	0.4	0.3-0.5	0.4	0.3-0.5	0.4	0.3-0.6	
1996 USA	n/d	n/d	n/d	n/d	n/d	n/d	
1997 Europe	0.6	0.3-1.7	0.7	0.3-2.6	0.5	0.3-1.0	
Amino Nitrogen							
1995 Europe	2.8	2.0-4.0	2.9	2.0-3.9	2.5	0.6-4.2	0.9-5.1
1996 Europe	1.6	0.7-2.8	1.6	0.8-2.5	2.0	0.7-2.8	
1996 USA	5.6	2.7-7.6	5.7	3.4-7.2	5.9	4.3-7.7	
1997 Europe	2.6	1.0-4.3	2.5	0.8-3.8	2.5	0.9-4.0	
Crude Protein							
1995 Europe	6.2	4.8-8.2	6.3	4.9-7.9	5.3	3.4-7.0	1.2-12.4
1996 Europe	4.3	3.0-5.4	4.3	3.0-5.2	4.8	3.1-5.9	
1996 USA	6.3	3.4-9.5	5.6	2.4-8.0	5.8	3.9-8.0	
1997 Europe	5.0	3.1-6.9	4.9	3.0-6.6	4.8	3.2-6.6	
Dry Matter							
1995 Europe	20.5	14.1-23.5	20.5	13.6-23.1	21.3	14.5-23.8	19.8-23.0
1996 Europe	23.9	19.2-26.4	23.9	19.5-26.2	23.5	18.9-26.0	
1996 USA	19.4	17.8-22.6	21.1	19.4-22.6	21.0	19.1-22.8	
1997 Europe	22.7	20.9-24.9	22.4	20.2-24.4	22.5	21.3-24.6	
Carbohydrate							
1995 Europe	86.3	81.7-88.9	86.3	81.7-88.7	88.2	86.6-90.0	67.3-91.0
1996 Europe	89.0	87.1-91.1	89.0	87.6-90.9	88.3	86.5-91.1	
1996 USA	84.1	80.3-87.2	84.1	79.0-88.1	82.0	74.0-86.0	
1997 Europe	88.1	84.9-91.0	88.2	85.1-91.1	88.3	85.2-91.1	
Polarisation							
1995 Europe	14.4	8.4-17.4	14.5	7.9-17.2	15.6	9.9-18.2	10.8-20.7
1996 Europe	17.3	13.8-19.4	17.3	14.1-19.4	16.8	13.1-18.9	
1996 USA	14.8	12.9-17.1	14.6	12.7-16.2	14.7	13.4-15.9	
1997 Europe	16.6	14.7-18.9	16.2	14.3-18.5	16.4	14.7-18.7	

Table 5:continued

Sodium							
1995 Europe	1.7	0.5-3.1	1.8	0.4-3.5	1.1	0.4-2.2	0.4-5.5
1996 Europe	0.5	0.3-0.8	0.5	0.2-0.8	0.5	0.3-1.2	
1996 USA	1.5	1.0-2.3	1.5	1.3-1.9	1.6	0.8-2.2	
1997 Europe	0.7	0.3-1.6	0.9	0.4-2.2	0.8	0.3-0.6	
Potassium							
1995 Europe	5.3	4.6-5.9	5.3	4.2-6.0	5.2	3.4-6.6	4.2-10.2
1996 Europe	4.9	4.1-6.0	5.0	4.0-6.4	5.2	3.8-5.9	
1996 USA	8.2	6.8-11.7	8.0	6.7-11.5	8.4	6.2-12.5	
1997 Europe	4.6	3.8-6.2	4.7	3.9-6.3	4.7	3.3-6.3	

*All units in g/100g dry weight except dry matter and polarisation (g/100g fresh weight). Sodium, Potassium, invert sugar (glucose+fructose) and Amino Nitrogen expressed as mmol/100g fresh weight. Analyses performed according to published methods.
[1] Samples taken from single plots at six (Europe '95&'96), five (USA '96) or eight (Europe '97) geographically different field trials. Non-expressing isogenic lines grown adjacent to trial plots acted as controls. Analyses undertaken according to standard published methods.
[2] Treated with three applications of 0.75lb active equivalents per acre glyphosate as recommended for agronomic purposes.
[3] See references Marlander *et al.,* 1996; Smed *et al.,* 1996; Augustinussen and Smed, 1979, and DLG, 1991.

Two-sided pooled-variance *t*-tests of proximate and quality values were undertaken for the European trials comparing values of non-transgenic control sugar beet to sugar beet line 77 both untreated or treated with glyphosate at recommended agronomic levels (three applications of 0.75lb active equivalents per acre glyphosate). No statistical analysis was performed on the US trial.

In the European trials no statistically significant differences were found for any value at the 5% level of significance between untreated sugar beet line 77 and its non-transgenic control within any trial year. Only two significant differences were found in glyphosate-treated sugar beet line 77 compared to non-transgenic control plants; crude fibre in 1995 trials (-13.2%), and protein in 1996 trials (+12.0%). As no broadly consistent differences occur in compositional or quality parameters for glyphosate-treated sugar beet line 77 compared to the non-transgenic control these values are most probably outliers. Given that these data show no consistent effect, and that the component types affected are not significant to food products derived from sugar beet, it is concluded that applications of glyphosate to sugar beet line 77 pose no human health and safety issue with respect to proximate or quality components.

None of the mean values for either non-transgenic control sugar beet or sugar beet line 77 either untreated or treated with glyphosate falls outside the literature range except for higher amino nitrogen levels in the 1996 US trial. These outliers occurred for both the non-transgenic control and sugar beet line 77 (both untreated and treated with glyphosate). As both the isogenic control line and the transgenic line were affected, this anomaly may reflect a differential agronomic practice being applied in the US trial. Given that this outlier occurs for only one characteristics (amino nitrogen) which is not significant to the food products derived from sugar beet, this finding is not considered significant to the human health and safety of sugar or ancillary food products derived from sugar beet line 77 under different conditions.

On the basis of the data provided it is concluded that sugar beet line 77, both untreated or treated with glyphosate at recommended agronomic application rates, is equivalent to conventional sugar beet with respect to composition and quality values relevant to the human health and safety of final food derivatives.

Naturally occurring toxicants

Sugar beet varieties naturally contain low levels of toxic saponins, which are a group of compounds with properties resembling soap and detergents. Saponins are a complex and chemically diverse group incorporating both triterpenes and steroids linked to one or more sugar groups. Saponins are found naturally, and in significant amounts, in commonly used food and forage plants such as clover,

alfalfa, soybeans, chickpeas, eggplant silver beet and spinach (Oakenfull and Sidhu, 1989) and are characterised by having a bitter and astringent taste. Saponins have been generally well characterised with the predominant sapogenic form in sugar beet being oleanolic acid. Due to their surface-active properties saponins have been implicated in foaming and turbidity problems during sugar production from sugar beet and efforts are made to limit saponin levels through processing.

The wide range of chemical and physical properties of saponins is matched by the extent and range of their physiological and pharmacological properties. In general they have been shown to interact with biological membranes, due to their detergent qualities, and to both inhibit and stimulate enzymes and metabolic activity (Oakenfull and Sidhu, 1989). Whilst there has been a tendency to treat saponins exclusively as antinutritional or toxic constituents, more recent work has shown several beneficial dietary effects of saponins, including an enhancement of nutrient absorption in digestion and an ability to lower blood cholesterol levels (Oakenfull and Sidhu, 1989).

Levels of saponin were analysed in the roots and tops of non-transgenic control and GTSB77 sugar beet plants, both untreated and treated with glyphosate at agronomically recommended rates, in European field trials conducted in 1995, 1996 and 1997 and in European and US field trials conducted in 1996. The results of the saponin analysis are presented for root tissue (Table 3), as this is the only tissue used in food production.

Table 3. Mean values and ranges of saponin levels in roots for control and sugar beet line 77 (GTSB77) (both untreated and treated with Roundup) from all field trials*.

	Control		**GTSB77 - untreated**		**GTSB77 – treated[1]**		**Literature Range[2]**
Trial/Year	Mean	Range	Mean	Range	Mean	Range	
EU 1995	151	72-233	137	60-261	134	91-197	75-965
EU 1996	529	304-999	484	293-846	365[3]	215-609	
USA 1996	215	111-304	208	128-260	180	116-255	
EU 1997	446	290-720	422	305-689	338[3]	217-496	

* Values are given on a mg/kg fresh weight basis. Values are means of samples analysed from 6 (Europe 1995, Europe and USA; 1996) or 8 (Europe 1997) sites. Analysis determined according to published methods.
[1] Treated with three applications 0.75lb active equivalents per acre Roundup.
[2] Reference Lüdecke *et al.,* 1958.
[3] Significantly less than control at the 5% significance level.

Two-sided pooled-variance statistical *t*-tests were undertaken in the European trials to determine whether significant differences occur in saponin level between conditions and treatment. No statistical differences, at the 5% level of significance, were observed for saponin levels between untreated sugar beet line 77 (GTSB77) and the non-transgenic control in trials covering multiple growth seasons and geographic regions. No significant difference was found for saponin levels between glyphosate-treated GTSB77 and control sugar beet except for the 1996 and 1997 European field trials were saponin level was reduced in treated GTSB77. These differences are, however, not biologically or nutritionally significant as they were inconsistent across seasons and sites (suggesting that it is not an influence of the genetic modification *per se*). Furthermore, all mean saponin values are within the range established for conventional sugar beet in the literature. On this basis, saponin levels in the root of sugar beet GTSB77, both untreated and treated with glyphosate at recommended agronomic application rates, are similar to commercially available sugar beet and are not considered to pose a risk to human health since:

- saponins are, in general, at very low levels in sugar beet tissues;
- sugar beet processing aims to eliminate saponins and other extraneous material from refined sugar products; and
- saponin levels in both glyphosate-treated and untreated line 77 do not differ significantly across seasons and sites, and fall within the range described for traditional sugar beet varieties in the literature.

Naturally occurring allergens

Allergic reactions to foods arise from an immune reaction to a particular protein that 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.

Very rare instances in the literature describe allergic response to beet sugar taken orally and to beet sugar solutions administered by injection (Randolf and Rollins, 1950; Richter *et al.*, 1976). One report (Richter *et al.*, 1976) pointed to certain polysaccharide components as the potential allergen. However, the identity of the immunogenic substances in sugar beet sugar has not been positively established. The instances of sugar beet sugar induced allergic responses are very rare and as refined sugar is generally recognised as safe, further investigation of putative naturally occurring allergens is not considered necessary.

NUTRITIONAL IMPACT

Animal feeding studies

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and 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 sugar beet line 77, no significant differences regarding nutritional and toxicological parameters were evident and no feeding studies were thus undertaken.

Another important factor in the assessment of sugar beet line 77 to support growth and well-being is that the principal human food derivative is highly refined sugar composed of 96-99% sucrose and 0.6-1.2% other sugars such as glucose and fructose. Refined sugar from any source has a history of safe use and is generally recognised as safe for human consumption.

Conclusions

On the basis of the compositional data evaluated in regard to sugar beet line 77, it was concluded that this herbicide tolerant sugar beet is equivalent to other commercially available sugar beet in terms of its composition and nutritional adequacy.

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