FOOD DERIVED FROM GLUFOSINATE AMMONIUM TOLERANT COTTON LINE LL25

A SAFETY ASSESSMENT

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SUMMARY

Food derived from genetically modified (GM) cotton line LL25 has been assessed for its safety for human consumption. This cotton line has been genetically modified to be tolerant to the herbicide glufosinate ammonium and has been developed principally for cultivation in the United States and Canada. The line in this application is known commercially as LibertyLink® cotton.

A number of criteria have been addressed in the safety assessment including: a characterisation of the transferred gene, its origin, function and stability; changes at the DNA, protein and whole food levels; compositional analyses; evaluation of intended and unintended changes; and the potential for the newly expressed protein to be either allergenic or toxic to humans.

History of Use

Cotton is grown primarily for the value of its fibre with cottonseed and its processed products being a by-product of the crop. Humans have consumed cottonseed oil, the major product of cottonseed, for decades. Cottonseed oil is considered to be premium quality oil, valued for its high-unsaturated fatty acid content. The other food use of cottonseed is the linters, which are composed of greater than 99% cellulose. Cottonseed itself and the meal fraction are not presently used in Australia and New Zealand as a food for human consumption because they contain naturally occurring toxic substances. These toxins are essentially removed in the production of oil and linters, making them fit for human consumption. The types of food products likely to contain cottonseed oil are frying oils, mayonnaise, salad dressing, shortening, and margarine. After processing (NCPA, 1990), linters may be used as high fibre dietary products and thickeners in ice cream and salad dressings.

Nature of the Genetic Modification

Cotton line LL25 was generated through the transfer of the *bar* gene to the non-transgenic cotton line Coker 312. The *bar* gene encodes the phosphinothricin acetyltransferase (PAT), an enzyme that confers tolerance to glufosinate ammonium (phosphinothricin). The *bar* gene is derived from the soil bacterium *Streptomyces hygroscopius*.

No functional antibiotic resistance genes were transferred to cotton LL25. Detailed molecular and genetic analyses of cotton line LL25 indicate that the transferred *bar* gene is stably integrated into the plant genome at a single insertion site and is stably inherited from one generation to the next.

Characterisation of Novel Protein

Cotton line LL25 expresses a single novel protein – PAT. Protein expression analyses indicate that PAT is expressed at low levels or is undetectable in the cotton and their processed fractions and therefore exposure to the protein

through consumption of food derived from cotton line LL25 would be negligible, if at all. In cotton line LL25, PAT was present at levels ranging from 48 to 75 μg/g fresh weight (equivalent to 0.019% to 0.036% of the total crude protein) in fuzzy seed and from 0.13 to 1.4 μg/g fresh weight (equivalent to 0.001% to 0.006% of the total crude protein) in lint. Levels of PAT were much lower in the cotton hulls and meal and were undetectable in crude or deodorised oil, the main cottonseed products used in the human food supply.

The safety of PAT has been assessed on numerous previous occasions by FSANZ. In all instances it has been concluded that PAT is non-toxic to humans and has limited potential as a food allergen.

Comparative Analyses

Compositional analyses were done to establish the nutritional adequacy of cotton line LL25, and to compare it to non-transformed control lines and commercial varieties of cotton. The constituents measured were protein, fat, carbohydrate, ash, moisture, fibre, fatty acids, amino acids, minerals and the anti-nutrients, gossypol, cyclopropenoid acids and phytic acid, trypsin inhibitor, lectins, isoflavones, raffinose and stachyose.

No differences of biological significance were observed between the cotton line LL25 and its non-GM counterparts. Several minor differences in key nutrients and other constituents were noted however the levels observed represented very small percentage changes and do not indicate an overall pattern of change that would warrant further investigation. On the whole, it was concluded that food from cotton line LL25 is equivalent in composition to that from other commercial cotton varieties.

Nutritional Impact

The detailed compositional studies are considered adequate to establish the nutritional adequacy of the food and indicate that food derived from cotton line LL25 is equivalent in composition to food from non-GM cotton varieties. The introduction of food produced from cotton line LL25 into the food supply is therefore expected to have minimal nutritional impact. The nutritional adequacy of food produced from cotton line LL25 was also confirmed using a feeding study in rapidly growing broiler chicks. This demonstrated that the cottonseed meal from cotton line LL25 is equivalent to that from non-GM cotton in its ability to support typical growth and wellbeing.

Conclusion

No potential public health and safety concerns have been identified in the assessment of food from cotton line LL25. On the basis of the data provided in the present application, and other available information, food from this cotton line can be considered as safe and as wholesome as food produced from other cotton varieties.

FOOD DERIVED FROM HERBICIDE TOLERANT COTTON LINE LL25

A SAFETY ASSESSMENT

BACKGROUND

A safety assessment has been conducted on food derived from cotton that has been genetically modified to be tolerant to the herbicide glufosinate ammonium. The genetically modified (GM) cotton variety is known commercially as LibertyLink cotton.

Glufosinate ammonium (also referred to as phosphinothricin) is a non-selective, contact herbicide that provides effective post-emergence control of many broadleaf and grassy weeds. The mode of action of the herbicide is to inhibit the enzyme glutamine synthetase, an essential enzyme involved with ammonium accumulation and nitrogen metabolism in plants. The inhibition of glutamine synthetase results in an over accumulation of ammonia in the plant, which leads to cell death. Tolerance to glufosinate ammonium is conferred though the expression in the plant of the enzyme phosphinothricin acetyltransferase (PAT), encoded by the *bar* gene from the soil bacteria *Streptomyces hygroscopius.* The production of PAT by cotton line LL25 enables the post emergence use of glufosinate ammonium herbicides without risk of damaging the crop. The applicant has stated that development of GM glufosinate ammonium tolerant cotton will provide a selective use for glufosinate ammonium, creating a valuable new weed management tool for cotton producers. Glufosinate-ammonium is currently registered in Australia under the commercial name of Basta® for non-selective uses, or Finale® for turf and home garden uses, and as Buster® in New Zealand.

Cottonseed is processed into four major by-products: oil, meal, hulls and linters. Only the oil and the linters are used in food products in Australia and New Zealand. Cottonseed oil is used in a variety of foods including cooking, salad and frying oils: mayonnaise, salad dressing, shortening, margarine and packaging oils. Cottonseed oil is the third major vegetable oil produced in the U.S., behind soybean and corn oil (NCPA 1999). It is considered to be premium quality oil, due to its balance in unsaturated fatty acids and high tocopherol (Vitamin E) content and stability when used as frying oil. Cotton linters are used as a cellulose base in high fibre dietary products as well as viscosity enhancers in toothpaste, ice cream and salad dressing. Linter fibre is also used to improve the viscosity of dressings and is commonly used to bind solids in pharmaceutical preparations such as tablets. Linter pulp also has diverse uses in the paper industry, in fingernail polishes and printed electrical board circuits for use in the computer and electronics industry (NCPA, 1999). Cottonseed meal is primarily used for stock food and is not currently sold for human consumption in Australia or New Zealand.

Cotton line LL25 has been developed for cultivation in the United States, Canada and Australia. The Office of the Gene Technology Regulator (OGTR)

has granted the applicant a licence for field trials of this cotton in Australia (DIR038/2003). It is intended that cotton line LL25 will be used in conventional breeding programs and may therefore enter the Australian food supply as both domestic and imported food products, once it has been approved for commercial production. Cotton is not grown in New Zealand and therefore food from cotton line LL25 will enter the New Zealand food supply as imported, processed food products only.

HISTORY OF USE

Host Organism

Cotton (*Gossypium hirsutum* L.) is grown as a commercial crop worldwide and has a long history of safe use for both human food and stock feed. The cultivar Coker 312 was used as the parental variety for transformation. Coker 312 is a United States Protected Variety of SEEDCO Corporation (PVP 7200100). Coker 312 was developed from a cross of Coker 100 with (Delta and Pine Land) D&PL-15 and selected through successive generations of line selection.

Cotton is grown typically in arid regions of the tropics and sub-tropics. It is primarily grown as a fibre crop with the resulting cottonseed being processed as a by-product. Cottonseed is processed into four major by-products: oil, meal, hulls and linters (Figure 1), but only the oil and the linters are used in food products. Food products from cottonseed are limited to highly processed products due to the presence of the natural toxicants, gossypol and cyclopropenoid fatty acids in the seed. These substances are removed or reduced by the processing of the cottonseed into oil and linters.

Cottonseed oil is regarded as premium quality oil and has a long history of safe food use. It is used in a variety of foods including frying oil, salad and cooking oil, mayonnaise, salad dressing, shortening, margarine and packing oil. It is considered to be healthy oil as it contains predominantly unsaturated fatty acids. Cottonseed oil has been in common use since the middle of the nineteenth century (Jones and King 1990; Jones and King 1993) and achieved GRAS (Generally Recognised As Safe) status under the United States Federal Food Drug and Cosmetic Act because of its common use prior to 1958. In the US, it ranks third in volume behind soybean and corn oil, representing about 5-6% of the total domestic fat and oil supply.

Cotton linters are short fibres removed from the cottonseed during processing and are a major source of cellulose for both chemical and food uses. They are used as a cellulose base in products such as high fibre dietary products as well as a viscosity enhancer (thickener) in ice cream, salad dressings and toothpaste.

The other major processed products derived from cottonseed are meal and hulls, which are used as stock feed. Cottonseed meal is not used for human consumption in Australia or New Zealand. Although it has permission to be used for human food (after processing) in the US and other countries, it is

primarily sold for stock feed. Human consumption of cottonseed flour has been reported, particularly in Central American countries and India where it is used as a low cost, high quality protein ingredient in special products to help ease malnutrition. In these instances, cottonseed meal is inexpensive and readily available (Ensminger 1994, Franck 1989). Cottonseed flour is also permitted for human consumption in the US, provided it meets certain specifications for gossypol content, although no products are currently being produced.

In Australia, the area of cotton harvested in 2004 – 2005 was 315,000 hectares and the predicted harvested area for 2005 – 2006 is 341,000 hectares (ABARE, 2005.) Cotton is not grown in New Zealand.

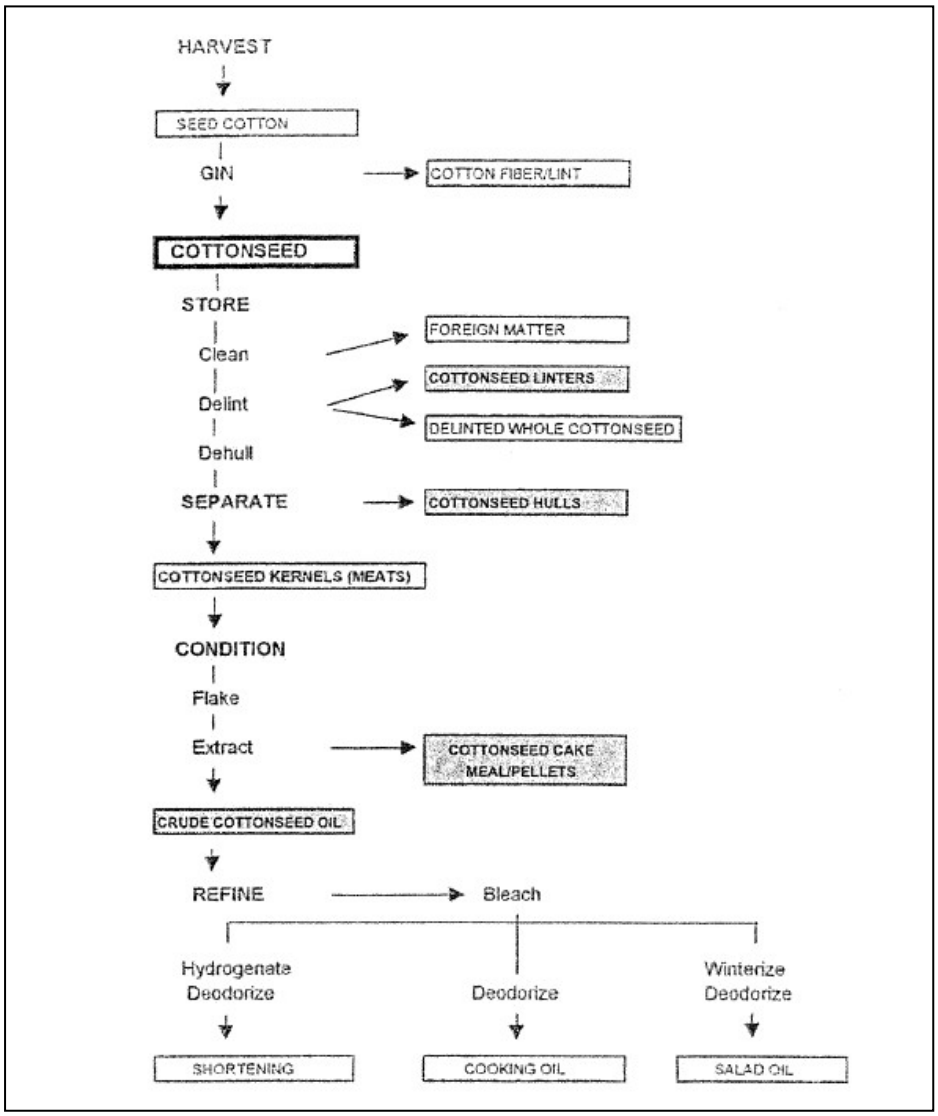

Figure 1: Processing steps of cottonseed, from harvest to products (NCPA, 2000)

Donor Organisms

Streptomyces hygroscopius

The source of the *bar* gene is the bacterium *Streptomyces hygroscopius*. *S. hygroscopius* belongs to the *Streptomyceta*, and is generally soil-borne, although it may be isolated from water. *Streptomyces* are not typically pathogenic to animals or humans, and few species have been shown to be phytopathogenic (Bradbury, 1986; Kutzner, 1981). A number of species within the genus produce highly active antibiotics and also effective mechanisms of defence against antibiotics. The source of the current *bar* gene was *S. hygroscopius*, strain ATCC21705 (Murkami et al., 1986).

Cauliflower mosaic virus (CaMV)

The *35S* promoter element is derived from the plant virus CaMV and controls the expression of the *bar* gene. CaMV is a double stranded DNA caulimovirus with a host range restricted primarily to cruciferous plants.

Although CaMV is a known plant pathogen, only a single DNA fragment of the CaMV genome corresponding to a promoter, has been transferred into cotton (Odell et al., 1985). No other DNA fragments, including the genes that code for the pathogenicity of the virus, have been transferred into cotton line LL25.

Agrobacterium tumefaciens

A. tumefaciens has been used as the source for the 3' *nos*, which terminates transcription and directs polyadenylation, of the *bar* gene in cotton line LL25.

The species *A. tumefaciens* is a Gram-negative, non-spore forming, rod-shaped bacterium commonly found in the soil. It is closely related to other soil bacteria involved in nitrogen fixation by certain plants.

Agrobacterium naturally contains a plasmid (the *Ti* plasmid) with the ability to enter plant cells and insert a portion of its genome into plant chromosomes. Normally therefore, *Agrobacterium* is a plant pathogen causing crown gall disease of a wide range of dicotyledonous (broad-leaved) plants, especially with sugar beets, pome fruit and viniculture crops. However, adaptation of this natural process has now resulted in the ability to transform a broad range of plant species without causing adverse effects in the host plant. *A. tumefaciens* has no known pathogenicity to humans.

DESCRIPTION OF THE GENETIC MODIFICATION

Method used in the genetic modification

The new gene was introduced into the cotton plant (*Gossypium hirsutum* L, Coker 312 var.), by *Agrobacterium* mediated transformation (Zambryski, 1992). This is achieved using a plasmid vector (pGSV71), which allows specific genes, integrated into the *Agrobacterium* T-DNA between regions known as the left and right borders, to be transferred to the plant. In this application, one plasmid carrying the required gene was used to generate line LL25.

Agrobacterium mediated transformation involves incubation of the bacteria carrying the particular plasmid with plant cells for a few hours to days, during which time T-DNA transfer takes place. The cells are then washed and cultured in the presence of the selection agent, glufosinate ammonium, and transformed shoots are regenerated and characterised.

Function and regulation of the novel gene

The section of plasmid (the expression cassette) transferred into cotton line LL25 is illustrated in Figure 2. This portion of the pGSV71 plasmid contains the DNA sequence that encodes the *bar* gene and the regulatory elements that control the expression of the *bar* gene in the transgenic cotton. All the genetic elements present in the expression cassette are described in Table 1.

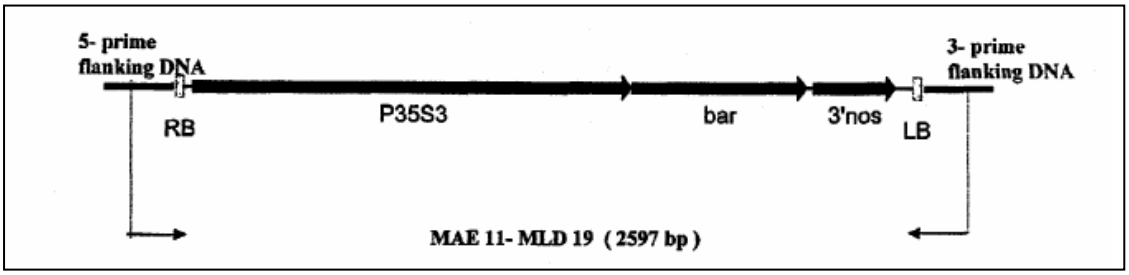

Figure 2: Linear map of insert in cotton line LL25 (MAE11-MLD19 fragment)

The bar gene

The *bar* (***b***ial***a***phos-***r***esistance) gene was isolated from *Streptomyces hygroscopius*, strain ATCC21705 (Murakami et al., 1986). It encodes the enzyme **p**hosphinothricin **a**cetyl**t**ransferase (PAT), which confers resistance to glufosinate ammonium.

The bacteria *Streptomyces hygroscopius,* also naturally produces the antibiotic bialaphos, which is an effective broad-spectrum herbicide. By acetylating the free amino group of phosphinothricin (PPT), the PAT enzyme prevents autotoxicity in the bacterial organism and generates complete resistance towards high doses of PPT, bialaphos or the synthetically produced glufosinate-ammonium.

Thus, the gene encoding the PAT enzyme serves as both an antibiotic-biosynthetic gene and an antibiotic-resistance gene (Murakami et al., 1986; Thompson et al., 1987).

Since the native *bar* gene has a GTG initiation codon, this was modified to the plant-preferred ATG initiation codon, to guarantee correct translation initiation and increased expression levels in plants (De Block et al., 1987). The amino acid sequence of the resultant PAT is not changed by this modification.

The *bar* gene is under the control of a plant viral promoter (P35S) which has been used for constitutive expression of the PAT protein in all tissues of the plant. Expression of the introduced transgene was analysed using Northern blot analysis, detailed in “Protein expression analysis” on p.32.

Table 1: Genetic elements present in the expression cassette in cotton line LL25

Symbol	Definition	Source	Size (bp)	Reference	Function
RB	Right border repeat	*Agrobacterium tumefaciens*	25	Gielen et al., 1984	Required for transfer of T-DNA in the plant cell. No function in the plant cell
	Polylinker sequence	synthetic	28		Plasmid cloning site
P35S3	Promoter	cauliflower mosaic virus	1385	Odell et al., 1985	High level constitutive expression of *bar* gene in the cotton plant.
bar	Glufosinate ammonium tolerance *bar* gene	*Streptomyces hygroscopius*	552	Thompson et al., 1987	Herbicide tolerance and selectable marker used to select for transformed plant cells.
	Polylinker sequence	synthetic	19		Plasmid cloning site
3'nos	Terminating signal of *bar* gene	*Agrobacterium tumefaciens*	261	Depicker et al., 1982	transcription termination signal
	Polylinker sequence	synthetic	51		Plasmid cloning site
LB	Left border repeat	*Agrobacterium tumefaciens*	25	Gielen et al., 1984	Required for transfer of T-DNA in the plant cell. No function in the plant cell

Characterisation of the transgene in the plant

Traditional molecular techniques were used to analyse the inserted DNA in cotton line LL25. Southern blot analysis was used to determine the insert copy number, intactness of the PAT coding region, intactness of the PAT expression cassette, and to assess whether vector backbone sequences were introduced during the transformation process.

Test material was taken from cotton line LL25 and from untransformed plants of the same cultivar, Coker 312, as a control. The transformed plants were characterised at the molecular and biochemical level using a range of laboratory techniques and procedures outlined below in Table 2.

Insert and copy number

Southern hybridisation was used to confirm the number and nature of the DNA insertions in cotton line LL25. Cotton line LL25 DNA, non-transgenic Coker 312 genomic DNA and pGSV71 plasmid DNA were digested with eleven different restriction enzymes, processed by gel electrophoresis, transferred by blotting to nylon membranes and probed with a probe covering the T-DNA sequence.

The copy number was determined by digesting cotton line LL25 genomic DNA with Eco R1, which cuts once only within the insert. Only one band was visible, indicating that a single insert was present.

PCR and sequence analysis

A discriminating polymerase chain reaction (PCR) protocol was used to amplify the complete insert of cotton line LL25. The fragment obtained was then purified and sequenced for comparison with the pGSV71 plasmid sequence. A few small differences (a 1 bp insertion, 2 bp deletions and 1 bp substitution) were noted between the consensus sequence obtained from the PCR fragment and the pGSV71 plasmid sequence.

The sequence of the inserted fragment was verified by re-amplification and subsequent sequence determination of the corresponding region of the LL25 insert and the transforming plasmid. The alignment of the transgenic LL25 sequence and the pGSV71 plasmid sequence resulted in an exact alignment of the functional elements contained within the T-DNA.

The determination of inserted sequences in cotton line LL25 confirmed the presence of one copy of the T-DNA.

Table 2: Outline of molecular and biochemical methods used for identification of glufosinate-ammonium tolerant cotton line LL25

Analysis method	Purpose
Southern Hybridisation	- Detection of the gene cassette in the cotton plant genome - Quantification of the insertions in the plant genome - Verification of the physical linkage of the introduced genes - Verification that inserted DNA corresponds with plasmid DNA - Investigation of T-DNA borders - Identification of the transgenic line by its hybridisation pattern.
Polymerase Chain Reaction (PCR)	- Verification of the presence of the introduced gene - Characterisation of plant DNA sequence flanking the inserted DNA
Northern Blotting	- Analysis of the expression of the transgene in different plant tissues (seeds, leaves, stem and root) - Analysis of cryptic expression in flanking plant DNA/insertion junction regions.
Bioinformatics	- evidence of novel transcripts arising from transformation event (at either junction of the insert)
PAT assay	- Quantification of enzymatically active PAT enzyme

Flanking regions and Open Reading Frame analysis

PCR analysis was used to determine the presence/absence of the right and left-hand borders of the inserted sequence. This analysis showed that the complete right border direct repeat sequence had not been inserted in cotton line LL25 and that the endpoint of the T-DNA was situated within the left border terminal repeats.

Southern blot analysis, with four overlapping probes, confirmed the absence of vector backbone sequences in the transformed cotton line LL25. The nature of the right and left border flanking sequences of cotton line LL25 was confirmed, both by PCR and Southern blot analysis, as being of *Gossypium hirsutum* plant origin.

Northern Blot analysis was used to confirm the absence of cryptic expression at the right and left-hand border junctions. DNA fragments containing plant DNA/insert junction sequences were isolated and subcloned in a pGEM-T vector. Plasmid DNA of relevant transformant clones was used as a template to synthesise sense and antisense transcripts homologous to plant DNA/insertion junction sequences. The transcripts obtained were used as probes in Northern blot analysis. Probes were prepared to cover 677 bp upstream of the right border flanking plant DNA and 412 bp downstream of the left border flanking plant DNA. The analysis showed that the experimental set-up gave detection limits of 0.2 and 0.5 pg of right and left border RNA transcripts (incoming and outgoing signals) respectively, and that no positive signal could be detected with the cryptic expression probes in seeds, leaves, roots or stems of cotton line LL25 and it's non-transgenic counterpart.

In addition to Northern blot analysis, bioinformatics analysis was conducted to confirm the absence of cryptic expression in the flanking plant DNA/insertion junction regions of cotton line LL25. BLASTn and BLASTx sequence similarity searches revealed that there were no meaningful sequence similarities with published sequences. The Open Reading Frame (ORF) tool identified twenty-six putative cryptic ORFs in the right and left border integration sequences of cotton line LL25 and BLASTp sequence similarity searches were carried out on each sequence. Further bioinformatics analysis showed that none of the ATG codons encoding the first amino acid of each putative cryptic ORF were considered as potential initiation codons. Despite three of the ORF sequences sharing sequence similarity with known proteins, no regulatory motifs, which comprise core promoter structures, were found upstream from these ORFs. As this DNA region cannot be considered as potentially involved in transcription initiation, these ORFs are not considered to be biologically meaningful. Additionally, none of the core promoter motifs and 3'untranslated region regulatory signals identified in the right and left-hand border integration sequences of cotton line LL25, were considered to be functional.

The bioinformatics analysis indicated overall that the identified putative ORFs lack the appropriate upstream transcriptional regulatory sequences and are unlikely to be expressed.

Conclusion

Detailed molecular analyses have been carried out on cotton line LL25 to characterise the inserted DNA. Results indicate that one copy of the T-DNA was introduced at a single locus in the cotton genomic DNA.

The *bar* gene was intact and no significant changes occurred to the DNA sequences of the insert during transformation. The small differences noted between the PCR fragment consensus sequence and the plasmid sequence are unlikely to alter PAT protein expression or encode for any novel protein.

Stability of the genetic changes

Breeding process

Following transformation, the transformed cotton line LL25 was backcrossed with its isogenic non-transgenic parental line, Coker 312. The progeny of this backcross were tested for expression of the *bar* gene using a standard Liberty® spray; 51.4% of the plants were found to be susceptible to glufosinate ammonium, indicating a segregation pattern of approximately 1:1 as expected for simple gene inheritance.

Segregation analysis

Segregation data comparing the frequency of the observed-to-expected numbers of progeny expressing the PAT protein were analysed statistically using the Chi-squared analysis. The ratio of resistant: susceptible plants for all generations segregated as expected for a single insertion site (Table 3).

Table 3: Segregation analysis of cotton line LL25

Parents and zygosity for the *bar* locus	Generation	Ratio R:S	Observed		Expected		χ square values	
			R	S	R	S	Calc. [1]	p =0.05 1df
Self-pollinated hemizygous T1 plants [(bar/-)x(bar/-)]	Individual T2 plants [2]	3:1	2959	957	2937	979	0.66	3.84
Self-pollinated hemizygous T1 plants [(bar/-)x(bar/-)]	T2 boll rows [3]	1:2	89	145	78	156	2.33	3.84
Hemizygous T0, or T1 crossed with elite recurrent parent [(bar/-)x(-/-)]	F1 [4]	1:1	659	597	628	628	3.06	3.84
1st back-cross of F1 plants with recurrent parent [(bar/-)x(-/-)]	BC1 [4]	1:1	166	172	169	169	0.11	3.84
1st self-pollinartion of hemizygous BC1 [(bar/-)x(bar/-)]	BC1F2 [4]	3:1	824	270	820	274	0.08	3.84

1. Assumes a one locus model. There was no significant difference (p=0.05) for the χ square goodness-of-fit test for the hypothesis of one locus. To reject the null hypothesis, the χ square value must be greater than 3.84, with one degree of freedom;
2. Every plant counted in every row, data pooled for this analysis;
3. Segregation of entire versus partially resistant T2 boll rows derived from resistant T1 plants. Homozygous boll rows (no segregation for resistance) were the source of the lines that were used in early event agronomic and stability studies; and
4. Data pooled across genetic backgrounds (no background effect evident).

R=resistant; S=susceptible; T0= primary Coker312 transformant; T1= hemizygous (bar/-), progenies of self-pollinated T0 plant surviving the Liberty treatment (zygosity confirmed by subsequent T2 progeny tests).

Genetic Stability

To demonstrate the stability of the insertion event (in cotton line LL25) in different backgrounds and environments, genomic DNA was isolated from roughly twenty individual plants of different genetic backgrounds and across multiple generations.

Table 4 lists three generations of cotton line LL25 crossed with Coker 312 as the recurrent parent (i.e. T4, T5 and T6); other backgrounds tested were FiberMax 966, FiberMax 832 (two seed lots), FiberMax989, HS26 and AVS9023.

Table 4: Overview of the tested generations and backgrounds of cotton line LL25.

Generation	Background
T4	Coker 312
T5	Coker 312
BC3/F3	FM966
BC3/F3 (A)	FM832
BC3/F3 (B)	FM832
BC3/F3	FM989
BC3/F3	HS26
BC3/F3	AVS9023
T6	Coker 312

Southern blot analysis demonstrated that the internal T-DNA fragment and right border integration fragment of cotton line LL25, resulting from restriction enzyme cleavage in the integrated T-DNA and in the adjacent plant DNA, were identical in all tested samples. The T6 generation of cotton line LL25 was grown at eleven different locations in the USA; Southern blot analysis consistently showed the T-DNA to be stable in all cases.

Conclusion

The transformation event in cotton line LL25 was shown to be stable over several generations and in different genetic backgrounds. The integrated T-DNA in cotton line LL25 was also shown to be stable when plants were grown in different environments.

Antibiotic resistance genes

Antibiotic resistance genes can be present in some transgenic plants as a result of their use as marker genes in the laboratory or in the field. It is generally accepted that there are no safety concerns with regard to the presence in the food of the 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 micro-organisms present in the human digestive tract and that this could compromise the therapeutic use of some antibiotics.

Cotton line LL25 does not contain an antibiotic resistance marker gene. The *bar* gene confers tolerance to glufosinate-ammonium herbicides both in culture (during the initial selection stages of transgenic plants in the laboratory) and when applied to whole plants in the field and therefore no other selectable marker gene was required.

Presence of DNA in food fractions

PCR analysis was used to determine if novel DNA, comprising the DNA insertion event of cotton line LL25, could be detected in raw agricultural commodities (seed and lint) as well as the processed fractions (seed cotton, de-linted seed, linters, cottonseed hulls, toasted meal, crude oil and deodorised oil).

Novel DNA was detectable in all the raw commodities (as expected) and most processed fractions of cotton line LL25. Deodorised oil, the most highly processed product (NCPA, 2000), did not contain any detectable DNA, including novel DNA.

CHARACTERISATION OF THE NOVEL PROTEIN

Biochemical function and phenotypic effects

The mode of action of glufosinate-ammonium (or phosphinothricin) is to inhibit the plant enzyme glutamine synthetase (GS), an essential enzyme in nitrogen metabolism and amino acid biosynthesis in plants. The result of GS inhibition is the over accumulation of inorganic ammonia leading to the death of plant cells.

The only novel protein in cotton line LL25 is PAT.

PAT

Phosphinothricin acetyl transferase (PAT) is encoded by the *bar* gene and is the enzyme responsible for detoxification of the herbicide phosphinothricin (L-PPT) in cotton line LL25. PPT is a potent inhibitor of the enzyme glutamine synthetase (GS) in both bacteria and plants, where it apparently binds competitively to the enzyme by displacing L-glutamate from the active site. GS converts glutamate and ammonia into glutamine and the binding of L-glufosinate-ammonium (L-GA) to GS results in the build-up of ammonia that inhibits photophosphorylation in photosynthesis (Wild and Wendler, 1990). Ammonia, although a plant nutrient and metabolite, is toxic in excess and leads to death of plant cells.

The PAT protein catalyses the conversion of L-GA to N-acetyl-L-GA, which does not inactivate GS. Therefore, plants expressing the PAT enzyme are tolerant to glufosinate ammonium herbicides. The *bar* gene in *S. hygroscopius* encoding for PAT, functions both as an integral part of the

biosynthetic pathway of bialaphos and as an enzyme which confers resistance (Kumada, 1988).

The P35S promoter is used to express the *pat* gene constitutively throughout the plant. Transgenic plants expressing the PAT protein are tolerant to high doses of commercial formulations of glufosinate-ammonium (eg. Basta®, Buster®, Harvest ® and Liberty ®).

The PAT enzyme is an acetyl transferase consisting of 183 amino acids; it has a molecular weight of 22 kDa, and exhibits enzyme specificity for both L-glufosinate (phosphinothricin, L-PPT) and demethylphosphinothricin (DMPT) in the acetylation reaction (Thompson, 1987). In the presence of acetyl-CoA, the PAT protein catalyses the acetylation of the free amino group of L-PPT, to N-acetyl-L-PPT, a compound that does not inactivate glutamine synthetase. The PAT enzyme has also been shown to have a very low affinity to related compounds and amino acids; even excess glutamate is unable to block the PPT-acetyltransferase reaction (Thompson et al., 1987). Acetyl-transferase activity is heat- and pH-dependent (Wehrmann et al., 1996); PAT shows its maximum activity at 40-45^{o}C, and complete thermoinactivation occurs at 60^{o}C (10 min) and above. The optimum pH for PAT is 8.5, but it is active over a pH range of 6 to 11.

Protein expression analysis

In cotton line LL25 the only novel protein expected to be expressed is the PAT protein. Expression of this protein was determined using Enzyme Linked Immunoabsorbent Assay (ELISA), which quantifies the amount of the PAT enzyme and Northern blot analysis, in which the PAT mRNA transcript was quantified.

Studies evaluated:

Kowite, W.J. and Currier, T.C. (2001) PAT protein content in raw agricultural commodities of transgenic cotton event LL25, USA 2000. Aventis CropScience, Biotechnology Support Department 2T. W. Alexander Drive, Research Triangle Park, NC 27709, USA, Unpublished Aventis report # BK00B001.

Kowite, W.J. and Currier, T.C. (2002) PAT protein content in processed agricultural commodities of transgenic cotton event LL25, USA, 2000. Aventis CropScience, Biotechnology Support Department 2T. W. Alexander Drive, Research Triangle Park, NC 27709, USA, Unpublished Aventis report # BK00B003.

From a food safety viewpoint, it is important to determine the tissues and level of expression of the novel protein in cotton line LL25 in order to determine potential dietary exposure to this protein.

Six field trials were established in the southern U.S. states of Mississippi (two trials), Arkansas, Missouri, Texas and North Carolina. There were six transgenic plots (cotton line LL25) and three non-transgenic plots (Coker 312) at each test site and cotton was grown using typical production practices. The content of the PAT protein in cotton line LL25 was determined in cottonseed

harvested from cotton which had either been treated with Liberty® or a conventional herbicide control program. Cottonseed from four field trials was collated and subsequently separated into four raw agricultural commodities (fuzzy seed - ginned cottonseed, cleaned seed, lint coat and lint) for total extractable protein and PAT analysis (Aventis report # BK00B001). Cottonseed from the other two field trials were utilised for a separate processing study (Aventis report # BK00B003) and for compositional analysis (Aventis report # BK00B002).

An Aventis in-house sandwich immunoassay (ELISA), with PAT specific polyclonal antibodies, was used to assess the amounts of PAT. The sensitivity of the ELISA assay ranged from 1.23 to 18.75 ng/g, in raw and fractionated cotton commodities. The PAT protein content ranged from 48 to 75 μg/g fresh weight (equivalent to 0.019% to 0.036% of the total crude protein) in fuzzy seed and from 0.13 to 1.4 μg/g fresh weight (equivalent to 0.001% to 0.006% of the total crude protein) in lint. For each of the cotton fractions, the PAT concentration showed statistically significant differences according to the cotton production site (i.e. environmental effects). PAT concentrations in the fuzzy seed, cleaned seed and in the lint also varied significantly according to the herbicide regime; the Liberty®-sprayed plants having slightly higher PAT concentrations than the non-sprayed plants. This could be due to an increase in metabolism of PAT induced by the presence of Liberty®. PAT was not found in the non-transgenic control line Coker 312.

In a parallel study, the amount of PAT protein was traced in different fractions of both unprocessed and processed cottonseed. ELISA indicated that the level of PAT in the final product was reduced as processing stringency increased (refer to Figure 1). Low levels of PAT were found in the cotton linters (6.17 ± 0.79 μg/g), however this was determined in the unprocessed matrix.

When linters are used in food products, they undergo processing (for example, alkaline washing at high temperatures (NCPA, 1990), which would effectively denature and/or remove any protein present. No PAT was detected in either crude or deodorised oil, the main cottonseed products used in the human food supply.

Table 5: PAT content in unprocessed and processed cotton products as detected by ELISA

Matrix	PAT protein content (μg/g) ± SD	Crude protein content (mg/g) ± SD	PAT protein content as % of crude protein
Whole, linted cottonseed	66.5 ± 8.6	23.45	0.029
Cotton lint	0.64 ± 0.54	2.13	0.003
Delinted cottonseed	114 ± 10	243 ± 1	0.047
Linters	6.17 ± 0.79	43.2 ± 12.4	0.014
Cottonseed hulls	11.0 ± 2.1	59.8 ± 0.1	0.018

Solvent extracted meal	0.03 ± 0.01	452 ± 35	7×10^{-6}
Toasted meal	0.02 ± 0.003	450 ± 16	5×10^{-6}
Crude oil	not detected	not analysed	-
Deodorised oil	not detected	not analysed	-

To demonstrate the expression of the introduced transgene, Northern blot analysis was performed on leaf, stem, root and seed tissues, using sense and antisense *bar* probes. The analysis showed that the *bar* sequences present in cotton line LL25 were expressed in all tissues tested. Expression levels ranged from between 4 and 8 pg/μg total RNA in leaf and stem samples, and between 2 and 4 pg/μg total RNA in seeds. No cryptic transgene expression was found using the antisense *bar* probe.

In summary, the levels of PAT detected in seeds of cotton line LL25 were very low. Given the absence of any detectable protein in the refined oil and very low amounts in linters, human exposure to the PAT protein through the consumption of oil and linters derived from cotton line LL25 would be unlikely and if it did occur, the levels of protein would be negligible.

Potential toxicity of the novel protein

Studies evaluated:

Kennel, P. (2002) Acute toxicity by intravenous injection in the mouse. Aventis CropScience. Study # SA01352. Aventis CropScience, 355, rue Dostoievski, BP 153, F-06903 Sophia Antipolis Cedex. Unpublished Aventis report.

Herouet, C. (2002) Overall amino acid sequence homology with known toxins and allergens. Aventis CropScience. Study # SA02198. Aventis CropScience, 355, rue Dostoievski, BP 153, F-06903 Sophia Antipolis Cedex. Unpublished Aventis report.

Acute oral toxicity

The potential toxicity of the PAT protein has been investigated by FSANZ on numerous previous occasions where acute oral toxicity studies in mice have been evaluated:

For example, A380 – glufosinate ammonium tolerant corn; A372 - glufosinate ammonium tolerant canola; A375 - glufosinate ammonium tolerant corn; A481 - glufosinate ammonium tolerant soybean. These studies do not indicate any evidence for toxicity and there is now general consensus that the PAT protein is not toxic to either humans or other animals (OECD, 1999).

An intravenous study (study # SA01352) examining the intravenous toxicity of the PAT protein was submitted in support of the absence of toxicity of the PAT protein, however FSANZ considers administration of novel proteins via the oral route to be more informative in relation to acute toxicity.

Similarity to known protein toxins

In addition to consideration of acute oral toxicity, the amino acid sequence of the PAT protein has also been compared to that of known protein toxins. The complete amino acid sequence of the PAT protein was compared with all protein sequences present in seven large reference databases: SwissProt, trEMBLE, GeneSeq-Prot, PIR, PDB, DAD and GenPept databases (Herouet, 2000). The algorithm used was BLASTP 2.2.2 (release Jan 08 2002) as a standard method for rapid and sensitive pairwise comparison of a query sequence to entire protein databases (Altschul et al., 1997).

The scoring matrix used was BLOSUM62, a series capable of directly examining multiple alignments of distantly related protein regions (Henikoff and Henikoff, 1992), which has been found to be optimal for detecting low level protein similarities with protein sequence lengths of more than 85 amino acids.

The BLOSUM62 matrix also enables a sequence comparison with no less than 62% divergence, thus avoiding over-emphasis of closely (evolutionary) related family members. The criterion used to indicate potential toxicity or allergenicity was a 35% identity with a toxin or allergenic protein on a window of 80 amino acids.

The overall homology search indicated significant homology only with other PAT proteins, especially the *pat* gene product (85% homology) from *Streptomyces* sp. Based on the overall homology search, the PAT protein encoded by the *bar* gene from *Streptomyces hygroscopius* does not have any significant homology with any known protein toxins.

Potential toxicity of glufosinate ammonium metabolites

Two metabolic pathways operate in glufosinate-ammonium tolerant plants to inactivate glufosinate-ammonium: N-acetylation of L-glufosinate producing N-acetyl-L-glufosinate (NAG) and the deamination of glufosinate and its subsequent conversion to 3-[hydroxyl (methyl) phosphinoyl] propionic acid (MPP). NAG is generally the main metabolite that is formed. As these metabolites are a by-product resulting from the activity of an introduced enzyme it is important that a consideration of its safety be included in any evaluation of glufosinate-ammonium tolerant cotton.

NAG is considered non-toxic to plants, invertebrates, rodents and mammals, including humans (OECD official use document, 1999; Hoerlein, 1994).

The International Programme on Chemical Safety (IPCS, 1999) has also reported that the toxicity of metabolites resulting from the interaction of glufosinate-ammonium with PAT can be considered less toxic or comparable to that of the parent compound. An ADI (acceptable daily intake) level of 0 – 0.2 mg/kg body weight was established for glufosinate-ammonium, and its metabolites NAG and MPP (IPCS, 1999).

In accordance with these results and other available evidence, an exemption from the requirement to establish a maximum permissible level for residues of

PAT, and the genetic material necessary for its production, was granted by the United States Environmental Protection Agency in April 1997 (USEPA, 1997).

Potential allergenicity of the novel protein

Studies evaluated:

Herouet, C. (2002) Epitope homology and glycosylation searches. Aventis CropScience. Study # SA02199. Aventis CropScience, 355, rue Dostoievski, BP 153, F-06903 Sophia Antipolis Cedex. Unpublished Aventis report.

Esdaile, D.J. (2002) *In Vitro* digestibility study in simulated gastric fluid. Aventis CropScience. Study # SA02173. Aventis CropScience, 355, rue Dostoievski, BP 153, F-06903 Sophia Antipolis Cedex. Unpublished Aventis report.

Esdaile, D.J. (2002) *In Vitro* digestibility study in simulated intestinal fluid. Aventis CropScience. Study # SA02174. Aventis CropScience, 355, rue Dostoievski, BP 153, F-06903 Sophia Antipolis Cedex. Unpublished Aventis report.

Van der Klis, R-J. (2003) Equivalence between the Phosphinothricin acetyl transferase (PAT) enzymes produced in cotton (*G. hirsutum*) and bacteria (*E. coli*). Bayer BioScience N.V., Molecular and Biochemical Analytical services, Protein characterisation, Nazerethsesteenweg 77, B-9800 Astene-Deinze, Belgium. Unpublished Bayer report.

Similarity to known allergens

In addition to the broad amino acid sequence homology study (Herouet, C., 2002) described above, it was necessary to analyse the established database in finer detail for the existence of shared allergenic epitopes (or immunoreactive sequences) which may have been missed during the broad homology analysis. This approach evaluated the potential amino acid sequence similarity of the PAT protein with epitopes (eight linearly contiguous identical amino acids, which is the minimum peptide length for a T-cell binding epitope) belonging to known allergens.

No similarities between the PAT protein and epitope of known allergens based on a “100% identity over a linear contiguous 8 amino acid segment” matching criteria were found. An *in silico* search using specific consensus sequences of potential glycosylation sites, often found in allergenic proteins, revealed no N- and O-glycosylation motifs. It is thought unlikely that the PAT protein will be glycosylated in plants.

Based on the overall homology search and the epitope homology search, the PAT protein encoded by the bar gene from *Streptomyces hygroscopius* does not share any significant homology with known allergens.

In vitro digestibility

Stability to digestion in simulated gastric and intestinal fluids has been considered an essential endpoint in assessing potential allergenicity, since

several allergens are known to be stable for up to 24 hours in simulated gastric fluid.

The simulated human gastric fluid method described in the U.S. Pharmacopeia has been used to systematically compare the relative stability of a number of common food allergens with common safe food proteins and with proteins engineered into plants (Fuch and Aswood, 1996). Allergens remain stable for at least 2 minutes with the major allergens being stable for at least 60 minutes in simulated gastric fluids, as demonstrated by gel electrophoresis.

Digestion experiments were performed according to the hypothesis that food allergens must exhibit sufficient gastric stability (at least 15 minutes) in order to potentially reach the intestinal mucosa where absorption and sensitisation can occur. To ensure that these results were meaningful in cotton line LL25, an equivalency study comparing the PAT protein produced by bacteria (*Escherichia coli*) and the PAT protein produced in the leaf of cotton line LL25, was confirmed using SDS-PAGE and Western blot analysis. SDS-PAGE showed that the molecular weight of the PAT protein from *E. coli* was about 20 – 22kDa. SDS-PAGE was not sensitive enough to detect the PAT protein extracted from leaf samples of cotton line LL25, even though several other protein bands were observed in cotton leaf samples from cotton line 15 and Coker 312 plants (non-transgenic control plants), indicating the correct extraction of the proteins. The sensitive Western blot analysis indicated no significant difference in molecular weight between the *bar* gene-encoded PAT proteins produced by *E. coli* or leaf material of cotton line LL25. Both proteins showed a molecular weight of 20 – 22kDa. The proteins from the non-transgenic Coker 312 variety did not show any immunoreactivity. Based on the Western blot analysis it can be concluded that the PAT protein produced in *E. coli* is equivalent to the PAT protein from leaf material of cotton line LL25 under the experimental conditions used.

The PAT protein solutions were incubated with simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) for different periods of time and then analysed by SDS-PAGE and Western blot analysis. The PAT protein was digested very rapidly with no residual protein visible after 30 seconds of incubation with SGF, in the presence of pepsin, at pH 2. Similarly, the PAT protein was totally digested within seconds when incubated with SIF and pancreatin, at pH 7.5. The proteases pepsin and pancreatin, enable protein degradation, and in their absence, the PAT protein remained practically intact.

Another study demonstrated that the PAT protein was no longer detectable by a silver-stained SDS-PAGE analysis after a brief incubation in simulated human gastric fluid (Wehrmann et al., 1996). This study also confirmed that when pepsin was omitted, no degradation of the PAT protein occurred.

These *in vitro* digestion experiments demonstrate that the PAT protein encoded by the *bar* gene has an extremely short structural and functional stability under simulated gastric and intestinal conditions.

Stability to heat and processing

When the PAT protein was subjected to temperatures of 60, 75 and 90 °C for up to 60 minutes, it remained detectable by Western blot analysis, indicating that the protein's tertiary structure was intact.

Summary and conclusion

Cotton line LL25 expresses one novel protein, PAT. The expression levels of the PAT protein in cotton line LL25 ranged from undetectable (in the oil) to 121 μg PAT protein/g dry weight (delinted cottonseed).

A number of studies have been done with the PAT protein to determine its potential toxicity and allergenicity. The PAT protein does not exhibit sequence homology with known protein toxins or allergens, and does not exhibit any of the physicochemical characteristics of known allergens. There is no evidence of acute toxicity from animal studies and the protein demonstrates digestive lability in conditions that mimic human digestion. The protein demonstrates heat stability and this result is inconsistent with previous studies on stability to heat and processing, however, given the digestive lability, it does not raise any safety concerns regarding potential allergenicity. Taken together, the evidence indicates that the PAT protein is unlikely to be either toxic or allergenic to humans.

COMPARATIVE ANALYSES

A comparative approach focussing on the determination of similarities and differences between the GM food and its conventional counterpart aids in the identification of potential safety and nutritional issues and is considered the most appropriate strategy for the safety and nutritional assessment of GM foods (WHO 2000). The critical components to be measured are determined by identifying key nutrients, key toxicants and anti-nutrients for the food source in question (FAO 1996). The key nutrients and toxicants/anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. These may be major constituents (e.g., fats, proteins, carbohydrates) or minor components (e.g., minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in the plant, such as those compounds whose toxic potency and level may be significant to health (e.g., solanine in potatoes if the level is increased). The key components of cottonseed that have been considered in this comparison include proximates, amino acids, fatty acids, minerals, and the toxicants gossypol and cyclopropenoid fatty acids.

Nutrient analysis

Compositional analyses were undertaken of whole linted cottonseeds, cotton lint as well as different processed cottonseed products. The constituents analysed were selected on the basis that they comprise the important basic nutrients of cotton. These are proximates, micro-nutrients such as minerals and vitamin E, amino acids and fatty acids.

Transgenic cotton line LL25 and its non-transgenic control, were grown over fifteen different field trials carried out in 2000 and 2001 in the main cotton growing regions of the USA (North Carolina, Mississippi, Arkansas, Missouri and Texas). In every trial, three plots of non-transgenic control (Coker 312) and six plots of the transgenic cotton line LL25 were planted.

All the plots in each field trial were planted and cultivated under the same conditions except for those transgenic lines sprayed with glufosinate-ammonium (Liberty®). Three, out of the six transgenic plots, were sprayed at a normal application rate of 0.58 kg active ingredient/ha. To compensate for the environmental effects within a single location, replicate plots of single treatments were established.

In total, 135 cottonseed samples from 15 sites taken over two years were analysed for 52 components; the statistical analysis of the data was carried out using a commercially available statistical package (SAS version 6.12). Comparisons of the levels and variations of the components were made between the transgenic line (both sprayed and not-sprayed), its non-transgenic counterpart, and the natural range of variation for the respective characteristics in the standard. A discrepancy range of 20% was taken as acceptable, meeting most of the natural variation ranges for the measured components (TemaNord, 1998). The relative treatment difference between product averages was taken based on the “Guidance for Industry Concerning

Statistical Procedures for Bio-equivalence Studies Using a Standard Two Treatment Crossover Design" by the FDA (FDA, Div of Bio-equivalence, Office of Generic Drugs, 1997).

For each component and each site, mean values (mean), standard deviation (SD) and the coefficient of variance ((SDx100)/mean) were calculated. If the coefficient of variance was larger than 20%, the standard equivalence criterion was thought to be too strict due to high natural variation of the non-transgenic material. The variance components "between sites" and "within sites" were also estimated for each component to determine the reason for observed variance. If different results were found for a component between replicates of a single site, then the variance was found "within sites"; if the results for the single sites differed, the variance was found "between sites".

An analysis of equivalence was then performed for each component according to EC regulation guidelines for novel foods and novel food ingredients 258/97; the analysis was made first for each site and then over all sites. An analysis of variance (ANOVA) was calculated to assess the effect of treatment and site factors, both separately and interactively; a significant interaction was indicated at probability ($p < 0.05$). Based on the ANOVA, 2-sided confidence intervals (95%) were calculated for the treatment differences. Two treatments were considered as equivalent, if the 95% confidence interval of the difference was within $\pm$ 20% of the mean value of the non-transgenic reference treatment.

Cottonseeds and Cotton lint

A summary of the outcomes of pooled comparisons of compositional data for non-transgenic samples and transgenic non-Liberty®- sprayed samples and between non-transgenic samples and transgenic Liberty®-sprayed samples, for all sites, is given in Tables 6 – 10.

Taking the two years' data sets as a whole, there were many instances of significant treatment x environmental (site) interactions (i.e. $p < 0.05$) and the coefficient of variance was also found to vary from 5 – 95% for different parameters both within and between sites (Table 6). This table indicates the tendency of the control, non-transgenic crop towards natural variation both between sites and within sites.

Furthermore, there were significant interactions between spraying regime and trial location, such variations and interactions can confound the data, but also aid in the interpretation of subsequent discrepancies in the results.

Summary tables of the compositional analyses (Tables 7 – 12) show a comparison of results for pooled data from all sites. The standard values reported were collated from different sources (OECD, 2002; FAO/WHO Food Standards. Codex Alimentarius, 2001) and are inherently restricted to natural variations in the cotton variety, the environment, the analytical method used, the number of samples tested and the statistical evaluation of the results achieved.

Proximate analysis

A summary of the proximate analyses for whole, linted cottonseed is shown in Table 7. No statistically significant differences were found between cotton line LL25 and the control line Coker 312 for the 15 sites analysed over two years. The transgenic line was found to be equivalent to the control line, both under a non-spraying and spraying regime, for all proximates. Also, all the values fell within the literature range (standard) values. There were significant site*treatment interactions for all the proximate parameters except for moisture (Table 6).

Table 6: Analysis of control group (non-transgenic, non-sprayed) plants. (n=45 for seed samples; n=18 for lint samples)

Parameter	p value for Treatment*Site interaction [a] (n=15)	% coefficient of variance (cv) between sites[b]	%cv within sites
Proximates (seed samples)			
Moisture	0.08	57.75	42.25
Fat	0.00	71.84	28.16
Protein	0.00	85.42	14.58
Ash	0.00	93.37	6.6
Total Carbohydrates	0.00	45.23	54.77
Crude Fibre	0.01	8.99	91.01
Acid detergent fibre	0.01	40.85	59.15
Neutral detergent fibre	0.01	14.45	85.55
MINERALS, VITAMINS			
Calcium	0.03	91.95	8.05
Phosphorus	0.01	76.27	23.08
Magnesium	0.51	36.79	63.21
Potassium	0.00	87.66	12.34
Iron	0.6	83.86	16.14
Zinc	0.1	43.21	56.79
Vitamin E	0.53	27.42	72.58
Anti-nutrients			
Gossypol - free	0.19	73.55	26.45
Gossypol - total	0.74	53.51	46.49
Phytic acid	0.00	87.35	12.65
Malvalic	0.6	62.39	37.61
Sterculic acid	0.45	38.56	61.44
Dihydrosterculic acid	0.14	46.83	53.17

[a] interactions exist at $p < 0.05$

[b] values taken from analysis of control group (non-transgenic, non-Liberty® sprayed) plants.

Table 6: (continued): Analysis of control group (non-transgenic, non-sprayed) plants. (n=45 for seed samples; n=18 for lint samples)

Parameter	p value for Treatment*Site interaction [a] (n=15)	%cv between sites[b]	%cv within sites
Total Amino acids			
Alanine	0.00	74.33	25.67
Arginine	0.00	84.25	15.75
Aspartic acid	0.00	80.1	19.9
Cysteine	0.64	68.08	31.92
Glutamic acid	0.00	83.69	16.31
Glycine	0.00	78.88	21.12
Histidine	0.00	80.25	19.75
Isoleucine	0.00	78.03	21.97
Leucine	0.00	80.0	20
Lysine	0.01	72.14	27.86
Methionine	0.65	41.11	58.89
Phenylalanine	0.00	83.44	16.56
Proline	0.00	81.21	18.79
Serine	0.00	2.15	17.85
Threonine	0.00	80.69	19.31
Tryptophan	0.03	87.96	12.04
Tyrosine	0.00	76.42	23.58
Valine	0.00	81.29	18.71
Total fatty acids (wt method)			
C14:0 Myristic	0.00	99.05	0.95
C16:0 Palmitic	0.00	99.61	0.39
C16:1 Palmitoleic	0.00	95.59	4.41
C18:0 Stearic	0.00	97.33	2.6
C18:1 Oleic	0.00	98.62	1.38
C18:2 Linoleic	0.00	99.49	0.51
C18:3 Linolenic	0.00	70.79	29.21
C20:0 Arachidic	0.00	98.58	1.42
C22:0 Behenic	0.00	88.82	11.18
C24:0 Lignoceric	0.00	97.64	2.36
Proximates (lint samples, n=18)			
Moisture	0.04	77.87	22.13
Fat	0.07	82.61	17.39
Protein	0.03	47.43	52.57
Ash	0.05	89.76	10.24
Total Carbohydrates	0.05	89.3	10.7
Crude Fibre	0.00	77.17	22.83
Acid detergent fibre	0.05	73.26	26.74
Neutral detergent fibre	0.46	5.41	97.59

[a] interactions exist at $p < 0.05$

[b] values taken from analysis of control group (non-transgenic, non-Liberty® sprayed) plants.

Table 7: Proximate analysis in whole, linted cottonseed of cotton line LL25 and non-transgenic counterpart (n=45) compared to commercial cotton varieties (standard values)

Parameter	Coker 312	Cotton line LL25	Cotton line LL25 sprayed	Standard values	Coker 312/ Cotton line LL25 #	Coker 312/ Cotton line LL25 sprayed ##
Moisture %fw	8.31 ± 1.25	8.89 ± 1.34	8.75 ± 1.34	7.0 – 11.0	Yes[b]	Yes
Fat %dm	19.93 ± 2.47	19.2 ± 2.89	19.05 ± 2.18	12 - 32	Yes	Yes
Protein %dm	23.96 ± 2.64	24.95 ± 3.87	24.86 ±3.3	11.8 – 26.8	Yes	Yes
Ash %dm	4.14 ± 0.4	4.3 ± 0.51	4.27 ± 0.41	3.34 – 4.9	Yes	Yes
Total Carbohydrates %dm [a]	54.98 ± 2.85	51.55 ± 5.46	51.82 ± 4.14	36.3 – 67.8	Yes	Yes
Crude Fibre	28.46 ± 2.48	28.27 ± 4.25	28.45 ± 3.75	20.8 – 33.0	Yes	Yes
Acid detergent fibre	37.00 ± 3.03	36.41 ± 5.40	37.16 ± 4.67	33.9 – 49.6	Yes	Yes
Neutral detergent fibre	43.5 ± 2.64	42.98 ± 5.63	43.48 ± 3.95	39.32 – 63.4	Yes	Yes

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) not Liberty®- sprayed, from all sites.

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) Liberty® sprayed over all sites

[a] Total carbohydrates calculated as 100% - (protein %dm + %dm +ash %dm)

[b] "yes" refers to two equivalent treatments

Table 8 shows a summary of proximate analyses in lint of cotton line LL25 compared to the non-transgenic counterpart, Coker 312. The proximate levels in lint contain greatly reduced fat, protein and, to a lesser extent, ash levels compared to those in whole, linted cotton. No significant differences were found between transgenic and non-transgenic lines for total carbohydrates, crude fibre, acid and neutral detergent fibre. However, the site-by-site analysis of the moisture, fat, protein and ash components of lint indicated statistically significant differences between transgenic and non-transgenic samples. However, there was no clear tendency for variations (from the mean) in the samples. The Applicant attributed this to the difficulty in analysing the lint's high fibre matrix, as indicated by the high coefficient of variance calculated in the control samples for protein, fat and ash (Table 6).

Table 8: Proximate analysis in lint of cotton line LL25 and non-transgenic counterpart (n=45)

Parameter	Coker 312	Cotton line LL25	Cotton line LL25 sprayed	Coker 312/ Cotton line LL25 #	Coker 312/ Cotton line LL25 sprayed ##
Moisture %fw	7.5 ± 0.78	8.34 ± 2.2	8.24 ± 7.72	No (-)[b]	Yes
Fat %dm	1.34 ± 0.83	1.33 ± 0.89	1.38 ± 0.97	No (+)	No (-)
Protein %dm	2.02 ± 0.58	2.56 ± 1.36	2.63 ± 1.6	No (-)	No (-)
Ash %dm	2.82 ± 1.51	3.1 ± 1.91	2.95 ± 1.6	No (-)	No (-)
Total Carbohydrates %dm [a]	93.82 ± 2.72	93.02 ± 3.81	93.09 ± 3.72	Yes	Yes
Crude Fibre	86.5 ± 6.09	80.94 ± 11.36	81.75 ± 8.43	Yes	Yes
Acid detergent fibre	94.71 ± 3.78	80.97 ± 8.37	91.58 ± 6.31	Yes	Yes
Neutral detergent fibre	99.03 ± 4.97	97.05 ± 7.53	97.64 ± 5.4	Yes	Yes

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) not Liberty® - sprayed over all sites

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) Liberty® sprayed over all sites

[a] Total carbohydrates calculated as 100% - (protein %dm + %dm +ash %dm)

[b] "yes" refers to two equivalent treatments. If the 95%-confidence interval of the difference exceeded 20% of the mean border of the respective reference treatment (non-transgenic, not Liberty® sprayed), a "no (+)" was marked. If the 95% confidence interval of the difference was below as well as beyond the bio-equivalence range, a "no (+-)" was set.

Mineral and Vitamin E analysis

A summary of the major minerals found in cottonseed from transgenic cotton line LL25 and the non-transgenic line Coker 312, is given in Table 9. No statistically significant differences were found between transgenic and non-transgenic lines for phosphorus, potassium, magnesium, iron and zinc content, regardless of spraying regime. However, a statistically significant difference was found for calcium content between the transgenic and non-transgenic lines. This may be due to the high variance found between sites (cv%=91.95; table 6); it is unlikely to be due to the significant treatment*site interaction found ($p<0.03$) as no significant differences were found between sprayed transgenic cotton and the non-transgenic line (Table 9). Overall, this cannot be considered as having a significant impact on the nutritional value of the food as the values still fall within the standard values for calcium content in cottonseed.

Table 9: Minerals and Vitamin E in whole, linted cottonseed of cotton line LL25 and non-transgenic counterpart (n=45) compared to commercial cotton varieties (standard values)

Parameter	On dry matter basis				Coker 312/ Cotton line LL25 #	Coker 312/ Cotton line LL25 sprayed ##
	Coker 312	Cotton line LL25	Cotton line LL25 sprayed	Standard values		
Calcium %	0.12 ± 0.03	0.14 ± 0.06	0.13 ± 0.04	0.11 – 0.21	No (-)	Yes
Phosphorus %	0.65 ± 0.12	0.65 ± 0.12	0.67 ± 0.1	0.45 – 0.75	Yes	Yes
Potassium %	1.12 ± 0.08	1.14 ± 0.08	1.15 ± 0.09	0.99 – 1.28	Yes	Yes
Magnesium %	0.4 ± 0.06	0.4 ± 0.04	0.4 ± 0.04	0.31 – 0.46	Yes	Yes
Iron mg/kg	66 ± 34	67 ± 34	63 ± 26	37.9 - 151	Yes	Yes
Zinc mg/kg	31.0 ± 4.5	32.1 ± 5.8	32.4 ± 6	24.9 - 42	Yes	Yes
Vitamin E IU/kg	161 ± 48	165 ± 42	160 ± 38	23.9 – 269.2 [a]	Yes	Yes

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) not Liberty®- sprayed over all sites

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) Liberty® sprayed over all sites

[a] range calculated from the alpha-tocopherol content in refined cottonseed oil (202.7 – 1004.5 IU/kg (136 – 674 mg/kg; f=0.671) FAO/WHO Food Standards, Codex Alimentarius, 2001) and an oil content in whole cottonseed (11.8 - 26.8%dm) y multiplication with f1=0.118 and f2=0.268.

Fatty Acid Analysis

The following fatty acids were analysed and compared in cotton line LL25 (sprayed and not sprayed) and the control line Coker 312: myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (C16:1), stearic acid (18:0), oleic acid (C18:1), linoleic acid (18:2), linolenic acid (18:3), arachidic acid (20:0), behenic acid (C22:0) and lignoceric acid (C24:0). A summary of the fatty acid analyses is shown in Table 10.

No significant differences between the control, non-transgenic lines and the transgenic lines (regardless of spraying treatment) were found for any of the fatty acids measured. All values for palmitic acid, including the values for the non-transgenic control, were consistently slightly lower than the standard lower range value and conversely, arachidic acid values were consistently slightly higher than the standard higher range values. The analytical nutrient reports from two independent laboratories concurred that linoleic acid values for both non-transgenic and transgenic (sprayed and non-sprayed) were all significantly higher than the standard high range values. These differences though, do not have any bearing on the results because no differences were found between the transgenic lines and their non-transgenic counterpart.

Amino acid analysis

Eighteen amino acids were analysed in cotton line LL25 and compared with the non-transgenic control, Coker 312. No significant differences were observed between the transgenic and non-transgenic lines, for any of the amino acids analysed (Table 11). There were significant site* treatment interactions for all the amino acids except for methionine and cysteine (Table 6), however this was regardless of whether the cotton lines were transgenic or not.

Cottonseed Products

The composition of cottonseed products was determined from two trials, performed in 2000 and 2001 in typical cotton growing regions in south-eastern U.S. Cotton line LL25, and its non-transgenic counterpart Coker 312, was grown in triplicate at ten sites in total. The herbicide treatments were the same as those described above.

A review of the compositional data provided for cotton hulls, cottonseed meal and crude cottonseed oil, indicated there were no statistically significant differences between the transgenic and non-transgenic control products for any of the constituents measured. If discrepancies were observed, they were all within the standard range of values and therefore are not considered to affect the nutritional value of the food . A summary of the data obtained for refined, deodorised oil and tocopherols is presented in Table 12.

Table 10: Total fatty acids in whole, linted cottonseed of cotton line LL25 and non-transgenic counterpart (n=45) compared to commercial cotton varieties (standard values)

	% Relative					Coker 312/ Cotton line LL25 #	Coker 312/ Cotton line LL25 sprayed ##
Fatty Acid	**Coker 312**	**Cotton line LL25**	**Cotton line LL25 sprayed**	**Standard values[a]**	**Standard values[b]**		
Saturated							
C14:0 Myristic	0.70 ± 0.1	0.67 ± 0.14	0.67 ± 0.12	0.89 – 1.2	0.4 – 2.5	Yes	Yes
C16:0 Palmitic	23.64 ± 1.6	23.34 ± 1.69	23.33 ± 1.63	25.2 – 28.6	16.2 - 29	Yes	Yes
C18:0 Stearic	2.4 ± 0.2	2.42 ± 0.18	2.43 ± 0.2	2.43 – 3.4	1.0 – 5.0	Yes	Yes
C20:0 Arachidic	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.04	0.21 – 0.29	0 – 1.0	Yes	Yes
C22:0 Behenic	0.17 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.1 – 0.27	0 – 0.6	Yes	Yes
C24:0 Lignoceric	<0.1 – 0.15[c]	<0.1 – 0.14[c]	<0.1 – 0.14[c]	No data	0 – 0.1	Yes	Yes
Total Saturated[d]	27.21	26.9	26.9		25.9		
Mono-unsaturated							
C16:1 Palmitoleic	0.56 ± 0.05	0.55 ± 0.05	0.55 ± 0.05	0.56 – 0.8	0 – 1.5	Yes	Yes
C18:1 Oleic	14.78 ± 1.4	14.65 ± 1.7	14.63 ± 1.2	13.94 – 15.8	12.4 - 44	Yes	Yes
Total Mono-unsaturated	15.34	15.15	15.18		17.8	Yes	Yes
Polyunsaturated							
C18:2 Linoleic	55.92 ± 3.05	56.44 ± 3.07	56.46 ± 2.97	36.32 – 47.3	33 – 60.5	Yes	Yes
C18:3 Linolenic	0.51 ± 0.06	0.5 ± 0.07	0.5 ± 0.06	0.08 – 0.3	0 – 2.1	Yes	Yes
Total Polyunsaturated	56.43	56.94	56.96				
Grand Total	98.98	98.99	99.04		51.9		

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) not Liberty®- sprayed over all sites

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) Liberty® sprayed over all sites

[a] Standard values for de-linted and linted cottonseed

[b] Standard values for cottonseed oil

[c] a calculation of the mean value for C24:0 is not possible as some values are not quantifiable; the range of means at a single site are presented

[d] Total saturated fatty acid values calculate with C24:0 <0.10%

Table 11: Total amino acids in whole, linted cottonseed of cotton line LL25 and non-transgenic counterpart (n=45) compared to commercial cotton varieties (standard values)

g/kg Dry matter					Coker 312/ Cotton line LL25 #	Coker 312/ Cotton line LL25 sprayed ##
AMINO ACID	Coker 312	Cotton line LL25	Cotton line LL25 sprayed	Standard values[a]		
Ala	0.85 ± 0.09	0.9 ± 0.14	0.87 ± 0.11	0.83 – 1.51	Yes	Yes
Arg	2.36 ± 0.35	2.51 ± 0.5	2.43 ± 0.41	2.51 – 4.4	Yes	Yes
Asp	2.12 ± 0.23	2.27 ± 0.34	2.21 ± 0.3	2.02 – 3.55	Yes	Yes
Cys	0.37 ± 0.04	0.39 ± 0.06	0.39 ± 0.04	0.41 - 0.86	Yes	Yes
Glu	4.22 ± 0.53	4.46 ± 0.79	4.34 ± 0.68	4.72 – 8.16	Yes	Yes
Gly	0.9 ± 0.1	0.95 ± 0.15	0.92 ± 0.13	0.87 – 0.13	Yes	Yes
His	0.61 ± 0.07	0.64 ± 0.1	0.62 ± 0.1	0.60 – 1.03	Yes	Yes
Ile	0.69 ± 0.07	0.72 ± 0.12	0.7 ± 0.1	0.69 - 1.17	Yes	Yes
Leu	1.25 ± 0.14	1.31 ± 0.21	1.28 ± 0.18	1.27 – 0.18	Yes	Yes
Lys	0.97 ± 0.1	1.02 ± 0.15	0.99 ± 0.13	0.99 – 1.65	Yes	Yes
Met	0.35 ± 0.03	0.36 ± 0.05	0.36 ± 0.03	0.3 – 0.53	Yes	Yes
Phe	1.11 ± 0.15	1.18 ± 0.22	1.15 ± 0.18	1.15 – 2.03	Yes	Yes
Pro	0.8 ± 0.09	0.84 ± 0.14	0.82 ± 0.12	0.71 – 1.39	Yes	Yes
Ser	0.95 ± 0.11	1.00 ± 0.15	0.97 ± 0.13	0.9 – 1.63	Yes	Yes
Thr	0.73 ± 0.07	0.77 ± 0.11	0.75 ± 0.1	0.64 – 1.21	Yes	Yes
Trp	0.32 ± 0.04	0.34 ± 0.06	0.34 ± 0.05	0.23 – 0.49	Yes	Yes
Tyr	0.49 ± 0.06	0.52 ± 0.09	0.5 ± 0.08	0.64 – 1.17	Yes	Yes
Val	0.96 ± 0.11	1.02 ± 0.18	0.99 ± 0.15	0.99 – 1.67	Yes	Yes

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) not Liberty® - sprayed over all sites
Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) Liberty® sprayed over all sites

Processing of whole cottonseed did not significantly alter fatty acid levels in transgenic or non-transgenic plants. However, linolenic acid levels were halved, as a result of processing, in both the transgenic plants and non-transgenic plants (Table 10 and Table 12).

There were no statistically significant differences in fatty acid levels in the refined oil extracted from transgenic (herbicide-treated and untreated) and non-transgenic control plants. Furthermore, all the values obtained fell within the standard range of values (Table 12).

The cottonseed oil refining process had a significant overall effect on alpha tocopherol levels, causing an 4 –5 factor increase (using a conversion factor, f=0.671, Table 9 and Table 14). This was true for oil derived from both transgenic and non-transgenic plants. No statistically significant difference was found in tocopherol levels between transgenic cotton line L25 (sprayed and not-sprayed) and the non-transgenic control, Coker 312.

Table 12: Total fatty acids and tocopherols in refined, deodorised seed oil of cotton line 25 and its non-transgenic counterpart compared to commercial cotton varieties (standard values) (n=2)

Fatty Acid		Coker 312	Cotton line LL25	Cotton line LL25 sprayed	Standard values
Saturated	C14:0 Myristic	0.66 ± 0.05	0.66 ± 0.02	0.68 ± 0.01	0.5 – 2.5
	C15:0 Pentadecanoic	<0.1 – 0.12[a]	<0.1[b]	<0.1[b]	NF
	C16:0 Palmitic	20.68 ± 0.57	20.39 ± 0.57	20.47 ± 1.21	16.2 – 29
	C18:0 Stearic	2.35 ± 0.74	2.29 ± 0.72	2.22 ± 0.7	1.0 – 5.0
	C20:0 Arachidic	0.22 ± 0.07	0.21 ± 0.06	0.21 ± 0.08	0 – 0.5
	C22:0 Behenic	0.11 ± 0.01	<0.1[a]	<0.1[a]	0 – 0.6
	C24:0 Lignoceric	<0.1[b]	<0.1[b]	<0.1[b]	NF
Total Saturated[c]		24.02	23.55	23.58	
Mono-unsaturated	C16:1 Palmitoleic	0.62 ± 0.03	0.63 ± 0.04	0.64 ± 0.05	0 – 1.5
	C18:1 Oleic	14.92 ± 0.07	15.48 ± 0.64	15.35 ± 0.45	12.4 - 44
	C20:1 Gadoleic	<0.1 – 0.12[b]	<0.1 – 0.10[b]	<0.1[b]	NF
Total Mono-unsaturated		14.52	14.72	14.88	
Polyunsaturated	C18:2 Linoleic	59.19 ± 1.42	59.03 ± 2.04	59.17 ± 2.59	33 – 60.5
	C18:3 Linolenic	0.23 ± 0.04	0.24 ± 0.05	0.22 ± 0	0 – 2.1
	C22:5 Docosapentaenoic	<0.1 – 0.16[b]	<0.1 – 0.15[b]	<0.1 – 0.15[b]	NF
Total Polyunsaturated		59.88	60.05	59.77	-
Grand Total		99.16	99.18	99.1	-
Tocopherols (ppm)					
Alpha tocopherol		528 ± 100	521 ± 108	512 ± 87	136 – 674
Gamma tocopherol		427 ± 63	425 ± 15	410 ± 10	138 ± 746
Delta tocopherol		<1.0[d]	<1.0[a]	<1.0[a]	0 – 2.1
Total tocopherols		955 ± 163	944 ± 122	922 ± 97	380 – 1200

[a] the calculation for the mean is not possible for C15:0, C20:0, C20:1 and C22:5 as the values are only detectable in some samples

[b] values were not obtained by calculation of the mean, since all results are below the limit of quantification

[c] total saturated fatty acid values calculated with C15:0 < 0.10% and for the transgenic samples with C22:0 <0.10%

[d] values were not obtained by calculation of the mean, since all results are below the limit of quantification

Key toxicants

Cotton contains two naturally occurring toxic compounds – gossypol and cyclopropenoid fatty acids. These compounds have been analysed in cottonseed from cotton line LL25 and compared with the non-transgenic parental line Coker 312 (Table 12).

Gossypol

Gossypol is a biologically active terpenoid aldehyde that is present in discrete glands in all plant tissues, including seed (Abou-Donia, 1976; Jones, 1991). Gossypol can cause a number of toxic effects on mammals including reduced appetite, body weight loss, and dyspnoea (difficult and laboured breathing) (Berardi and Goldblatt 1980), adverse effects on the protein nutritive value of food by rendering lysine metabolically unavailable (Yannai and Bensai, 1983) and damage to normal mitochondrial functioning (Cuellar and Ramirez, 1993; Randel *et al.,* 1992, Risco *et al.,* 1993).

The levels of gossypol and related terpenoids in cottonseed varies with variety and environmental conditions, which can include factors as diverse as soil and air temperature, disease infections, moisture stress and the presence of chemicals (Bell, 1991).

Any 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 are able to detoxify gossypol (Randel *et al.,* 1992; Poore and Rogers, 1998; Nikokyris *et al.,* 1991). Processing of cottonseed is therefore essential for it to have feed or food value.

Gossypol exists in two forms, free and bound. The free form is toxic, while the bound form is considered non-toxic since it is not released in the animal rumen. In whole unprocessed cottonseed almost all of the gossypol is in the free form. During processing, gossypol partitions into the meal and oil components. Although some of the gossypol in meal remains as the free form, much of it becomes bound to proteins and therefore detoxified. Gossypol in oil is eliminated during the refining process.

The amount of free and total gossypol in the transgenic and non-transgenic cotton lines (sprayed and not-sprayed) was found to be comparable across all the sites. The levels of free gossypol recorded were at the upper limits of the standard values and in some cases, exceeded these values. The level of free gossypol in the non-transgenic line for example, was significantly higher than that found in both the sprayed and not-sprayed transgenic cotton lines.

Phytic acid and Cyclopropenoid fatty acids

Cyclopropenoid fatty acids are unique fatty acids that are naturally present in cotton, crude cottonseed oil and in the meal (because of the residual oil in the meal fractions). Refinement of cottonseed oil includes deodorisation and bleaching, which greatly reduces the cyclopropenoid fatty acid content of the oil due to extreme pH and temperature conditions.

The major types are sterculic acid (C-17), malvalic acid (C-18) and dihydrosterculic acid (C-19). Cyclopropenoid fatty acids are considered to be undesirable, anti-nutritional compounds of concern for food safety.

They have unfavourable 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 have been reported to induce termination of embryo development in sheep through inhibition of progesterone production in the *corpus luteum* (Tumbelaka *et al.,* 1994).

The cyclopropenoid fatty acids are destroyed either by hydrogenation or by heating the oil in the presence of free fatty acids for deodorisation purposes (Gunstone *et al.,* 1994).

Phytic acid (inositol hexaphosphoric acid, chelates with calcium, zinc, iron and magnesium in the digestive tract. The phytate-mineral complexes formed are generally insoluble at physiological pH, making the minerals biologically unavailable to monogastric animals and humans. Phytic acid is therefore regarded as an anti-nutrient in cottonseed and derived products (Amann, 1999).

No significant differences were found in the levels of the cyclopropenoid fatty acids, malvalic acid and sterculic acid, between cotton line LL25 and the parent line Coker 312 across all the sites. No significant differences were also found in phytic acid levels despite a highly significant site* treatment interaction ($p<0.00$). Statistically significant differences in dihydrosterculic acid levels were found between the non-transgenic and unsprayed transgenic lines (Table 12), however the value for the transgenic line was lower than that for the control line and both values fell well within the standard values, therefore this difference does not raise any safety concerns.

The amount of total gossypol in refined cottonseed was reduced by a factor of at least 600, compared to the levels found in whole cottonseed (Table 13 and Table 14). The levels of the other anti-nutrients, malvalic, sterculic, dihydrosterculic acid, were also slightly lower in refined oil than in whole cottonseed.

However, there were no significant differences in the anti-nutrient levels between transgenic and non-transgenic plants, other than a reduction in the amount of dihydrosterculic acid in the transgenic plants (sprayed and not sprayed, Table 14).

Table 13: Anti-nutrients in whole, linted cottonseed of cotton line LL25 and non-transgenic counterpart (n=45) compared to commercial cotton varieties (standard values)

Parameter	On dry matter basis				Coker 312/ Cotton line LL25 #	Coker 312/ Cotton line LL25 sprayed ##
	Coker 312	Cotton line LL25	Cotton line LL25 sprayed	Standard values		
Free gossypol %	0.92 ± 0.2	0.84 ± 0.21	0.82 ± 0.19	0.47 – 0.68	Yes	Yes
Total gossypol %	1.3 ± 0.22	1.22 ± 0.27	1.19 ± 0.22	0.71 – 1.46	Yes	Yes
Phytic acid %	1.9 ± 0.38	1.98 ± 0.41	1.98 ± 0.31	2.57	Yes	Yes
Malvalic acid % rel.	0.45 ± 0.11	0.43 ± 0.11	0.41 ± 0.1	0.17 – 1.5	Yes	Yes
Sterculic acid % rel.	0.28 ± 0.05	0.27 ± 0.1	0.25 ± 0.08	0.13 – 0.92	Yes	Yes
Dihydrosterculic acid % rel.	0.16 ± 0.03	0.14 ± 0.02	0.15 ± 0.06	0.11 – 0.34	No (+)	Yes

Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) not Liberty® - sprayed over all sites
Summary of the evaluation for non-transgenic (Coker 312) vs. transgenic (cotton line LL25) Liberty® sprayed over all sites

Table 14: Anti-nutrients in refined, deodorised cottonseed oil of cotton line LL25 and its non-transgenic counterpart (n=2)

Parameter	On dry matter basis			
	Coker 312	Cotton line LL25	Cotton line LL25 sprayed	Standard values
Total gossypol %	<0.002[a]	<0.002[a]	<0.002[a]	0.01 – 0.09
Malvalic acid % rel.	0.40 ± 0.06	0.41 ± 0.02	0.4 ± 0	0.015 – 1.44
Sterculic acid % rel.	0.24 ± 0.03	0.23 ± 0.02	0.23 ± .02	0.005 – 0.58
Dihydrosterculic acid %rel.	0.21 ± 0.12	0.17 ± 0.05	0.17 ± 0.07	0.22 – 0.23

[a] values were not obtained by calculation of the mean, since all results are below the limit of quantification

Conclusion

Detailed compositional analyses of key nutrients, anti-nutrients and toxicants were done on cottonseed and processed products, including refined oil, from cotton line LL25 (both sprayed and unsprayed) and compared to the parental control, Coker 312, as well as commercial cotton varieties. No meaningful differences were observed in the levels of key constituents, indicating that food from cotton line LL25 is compositionally equivalent to food from conventional cotton varieties.

NUTRITIONAL IMPACT

In assessing the safety and suitability of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and wellbeing. 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.

To date, all approved GM plants with modified agronomic production traits (e.g. herbicide tolerance) have been shown to be compositionally equivalent to their conventional counterparts. Feeding studies with feeds derived from the approved GM plants have shown equivalent animal performance to that observed with the non-GM feed. Thus the evidence to date is that for GM varieties shown to be compositionally equivalent to conventional varieties, feeding studies with target livestock species will add little to a safety assessment and generally are not warranted.

For plants genetically modified with the intention of significantly changing their composition/nutrient bioavailability and thus their nutritional characteristics, however, suitable comparators may not be available for a nutritional assessment based solely on compositional analysis. In such cases feeding trials with one or more target species may be useful to demonstrate wholesomeness for the animal.

In the case of cotton line LL25, the extent of the compositional and other available data is considered adequate to establish the nutritional adequacy of the food. However, a feeding study in broiler chickens has been conducted on cottonseed meal from cotton line LL25 and is evaluated below as additional supporting information.

Broiler chicken feeding study

The growing broiler chicken is an appropriate test system to detect potential differences in nutrient quality of transgenic cotton. During the first 21 days of life, the growing broiler chicken is sensitive to nutritional intake and undergoes an approximate 15-fold increase in body weight, and 33-fold increase during the total course of the study (33 days).

The experiment was designed so that a total of 140 Ross#508 chickens (14 replicates, half male and half female) were given the same feed supplemented with 10% cottonseed meal from four different cotton plant sources: FiberMax™ (a current commercial non-transgenic variety), Coker 312 (non-transgenic isogenic control), as well as sprayed and unsprayed cotton line LL 25 (the transgenic line).

There were no statistically significant differences in total feed consumption, total weight gain, feed conversion to body weight rate (Table 15) and mean chilled carcass weight among the cottonseed meal types tested.

Table 15: Feed consumption and feed conversion for broiler chickens

	Total mean feed consumption (g)							
Cotton plant source	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Total	Mean weight gain[1](g)	Feed conversion[2]
FiberMax™	101.5 ± 5	282.8 ± 15.8	552.6 ± 75.9	666.5 ± 49.6	356.5 ± 27.6	1963. 6± 144	1114 ± 45.3	1.8
Coker 312	90.6 ± 6.8	280 ± 10.1	550.4 ± 77.6	688 ± 50.1	397.1 ± 50	2006. 1± 119	1087.2 ± 93.3	1.9
LL25 – not sprayed	104.6 ± 105	295.2 ± 295	587 ± 73.6	649.1 ± 48.2	364.9 ± 17.4	2000. 7± 94	1071.5± 64.3	1.9
LL25 - sprayed	99.8 ± 4.7	281.7 ± 12.1	533.8 ± 47.9	644 ± 33.4	360.4 ± 24.3	1919. 8 ± 79	1097.7 ± 50.4	1.8

[1] mean weight gain calculated as (live weight day 33-live weight day 0)
[2] Feed conversion calculated as (total feed consumption)/(total weight gain), based on average values per bird.

Statistical analyses indicated that the mean breast weight of chickens fed unsprayed cotton line LL25, was on average 8.9% lower than that of those fed the commercial variety FiberMax™. The analyses also showed that the thigh-weight of birds fed the transgenic cotton was on average 7.3% lower than those fed either the commercial or isogenic, control varieties. However, there was no significant change in weight variables between chickens fed sprayed cotton line LL25 and those fed the other three diets. If unsprayed cotton line LL25 caused a consistent effect in breast and thigh muscle, similar effects would be expected from the treated transgenic crop that is the presence of LL25 in the diet was not deemed the cause for the weight decrease. Furthermore, there were no significant differences observed between sprayed and unsprayed cotton line LL25, indicating that the herbicide treatment did not have any effect on thigh weight, also, according to the compositional analyses, herbicide treatment did not produce any changes in the composition of the food.

Overall, the results from this study indicate that cotton line LL25 (regardless of herbicide treatment) had no relative influence on survival, feed consumption, total weight gain or muscle production in ROSS broiler chickens.

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