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

by

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Quality Assurance Statement

This document has been reviewed by The Agricultural Group of Monsanto, New Products Division Quality Assurance Unit.

Individual study reports and/or summaries for the referenced safety assessment studies conducted under Good Laboratory Practices were used to verify the accuracy of results and information from those studies presented here. These study reports and summaries, as well as the supporting raw data, have previously been reviewed by Quality Assurance.

The information and results presented in this document were found to accurately reflect the raw data generated during the studies.

[redacted]
Quality Assurance Specialist

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Date

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I. List of Abbreviations

AOAC - Association of Official Analytical Chemists
AOCS - American Oil Chemists' Society
APHIS - Animal and Plant Health Inspection Service
ATP - adenosine triphosphate
BAPNA - benzoyl-D-arginine-p-nitroanilide
CP4 EPSPS - 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4
CTP - chloroplast transit peptide
DAHP - 3-deoxy-D-*arabino*-heptulosonate 7-phosphate
EPA - Environmental Protection Agency
ELISA - enzyme-linked immunosorbant assay
EPSP - 5-enolpyruvylshikimate-3-phosphate
EPSPS - 5-enolpyruvylshikimate-3-phosphate synthase
FCM - fat-corrected milk
FCR - feed conversion ratio
FDA - Food and Drug Administration
FFDCA - Federal Food, Drug, and Cosmetics Act
F/G - feed / gain
GLP - Good Laboratory Practices
GTS- glyphosate-tolerant soybeans
GUS - ß-glucuronidase
HPLC - high performance liquid chromatography
KB - kilobase
kD - kilodalton
mg - milligram
NA - not analyzed
ND - not detectable
NE - net energy
NMD - not materially different
ODS - octyldecyl (C18)
PAGE - polyacrylamide gel electrophoresis
PEP - phosphoenolpyruvate
PCR - polymerase chain reaction
pNPP - para-nitrophenyl phosphate
ppm - parts per million
P.R. - Puerto Rico
PVDF = polyvinylidene fluoride
RAL - Ralston Analytical Laboratories
S3P - shikimate-3-phosphate
SCN - soybean cyst nematode
SDS - sodium dodecyl sulfate
SGF - simulated gastric fluid
SIF - simulated intestinal fluid
µg - microgram
U.S. - United States
USDA - United States Department of Agriculture

II. Introduction

The Agricultural Group of Monsanto Company (Monsanto) is providing the Food and Drug Administration (FDA) with a summary of our studies demonstrating that soybeans modified by the addition of a glyphosate tolerance gene are not materially different in composition, safety, or any relevant parameter from soybeans currently on the market. This soybean line, carrying the added gene and its expressed protein, is tolerant to glyphosate, the active ingredient of Roundup® herbicide. Monsanto has performed analyses and studies to support this conclusion using glyphosate-tolerant soybean (GTS) line 40-3-2, derived from A5403, a commercial soybean variety. The glyphosate tolerance locus associated with GTS line 40-3-2 has been transferred to other soybean varieties through traditional breeding methods, and our seed company partners plan to commercialize progenies derived from these crosses. The safety assessment summarized below serves to establish the safety of line 40-3-2 and all progenies derived from crosses between line 40-3-2 and traditional soybean varieties.

These glyphosate-tolerant soybeans contain a small amount of an added protein (CP4 5-enolpyruvylshikimate-3-phosphate synthase, CP4 EPSPS) which confers the glyphosate tolerance phenotype. The data from our studies demonstrate that the presence of this gene,protein and the process used to produce these plants result in no material difference between these soybeans and soybeans grown commercially today.

In anticipation of the need for regulatory oversight of the science of biotechnology and its products, The Office of Science and Technology Policy issued the "Coordinated Framework for Regulation of Biotechnology" on June 26, 1986. Based on the belief that a new agency or new legislation was not required, this coordinated framework assigned regulatory responsibility of biotechnology to existing Federal Agencies. Therefore, before commercializing GTS line 40-3-2 and any progenies derived from crosses between line 40-3-2 and traditional soybean varieties, Monsanto will complete the applicable regulatory procedures:

1) Obtain a determination from the United States Department of Agriculture / Animal and Plant Health Inspection Service (USDA/APHIS) that GTS line 40-3-2 and all progenies derived from crosses between 40-3-2 and traditional soybean varieties pose no plant pest risk and should no longer be considered a regulated article under regulation in 7 CFR part 340. This determination has been made and published (Federal Register 59 (99), 26781, 5/24/94).

USDA/APHIS has the authority to regulate the movement and release of genetically modified plants under the Plant Pest and Quarantine Act. Regulations were issued on June 16, 1987 (7 CFR part 340) covering the testing and commercialization of these plants. USDA determined that prior to

the movement or release of these organisms for testing purposes, permits would be required. These permits include environmental release, limited interstate movement, limited importation, and courtesy. Before issuing any of these permits, USDA must be satisfied that the movement or release would not be expected to cause any harm to humans or the environment (including production agriculture). Prior to the commercial introduction of any genetically modified plant (defined as a regulated article in 7 CFR part 340.1), USDA will need to make a determination as to the plant pest status of the genetically modified crop. Once USDA reaches the determination that the genetically modified crop is no longer considered a plant pest, U.S. permits are no longer required for field testing, importation, or interstate movement of the genetically modified crop.

2) Obtain an amendment to our glyphosate label from the Environmental Protection Agency (EPA) to allow for in-season application of glyphosate on GTS. Monsanto already has a preharvest label which allows glyphosate to be applied to soybeans 7 days prior to harvest, as well as labels for several other types of uses in soybean, such as preplant, spot treatment, and selective equipment applications. In addition, the EPA has recently approved the use of Roundup herbicide applied over-the-top of GTS for seed production purposes.

EPA thoroughly regulates the safety of pesticides applied to food crops under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). A major part of the registration process involves tolerance setting under the Federal Food, Drug, and Cosmetic Act (FFDCA). Two sections of the FFDCA apply to the setting of tolerances. Section 408 governs tolerances for pesticide residues in or on raw agricultural commodities. Section 409 governs tolerances for pesticide residues that concentrate in processed foods.

Although FDA has primary responsibility for implementing Section 409 for other food additives, EPA has complete responsibility for evaluating under Section 408 the safety of pesticide residues and under Section 409 the safety of pesticide residues that are food additives. EPA was vested with this authority pursuant to the Reorganization Plan No. 3 of 1970. The core of the typical tolerance-setting process is the comparison by EPA of the quantity and nature of residues to which humans might be exposed through consumption of pesticide-treated food with the levels of such substances judged to be safe based on the available toxicological data. Thus, as a matter of law and practice, EPA comprehensively addresses and evaluates the safety of pesticides such as Roundup herbicide on food crops such as soybeans.

3) Complete the consultation process with FDA following the guidance outlined in FDA's Statement of Policy "Foods Derived From New Plant Varieties", published in the Federal Register May 29, 1992 ("Food Policy") (1). That policy discusses the safety and regulatory status of foods derived from new plant

varieties, including plants developed by genetic modification.

FDA's Food Policy (1) provides guidance for determining whether a new plant variety is as safe and nutritious as its parental variety. This guidance provides a mechanism for establishing the lack of a material difference between the modified product and its traditional counterpart. We have carefully followed this guidance (which is consistent with the criteria for "substantial equivalence" developed and currently used by the OECD [Organization for Economic Cooperation and Development]) in demonstrating that our GTS line 40-3-2 soybeans are not materially different from other varieties of soybeans. To assess as thoroughly as possible whether a material difference might exist, we have conducted numerous compositional studies and thoroughly reviewed all relevant data and information. To focus the analysis on any effects of the introduced gene and protein, the soybeans from which the tested and analyzed seed were derived were not treated with Roundup herbicide. Upon quantitatively and qualitatively evaluating all of the data available, we have been able to ensure that, in all but a few instances, for every parameter there are no statistically significant differences between the 40-3-2 line of soybeans and its parental variety. In those few instances where a difference was noted, and in the cases where the available data did not permit statistical analysis, we have been able to establish that the values or effects are well within established ranges (or are fully consistent with effects) documented and reported in the scientific literature for soybeans. On the basis of these evaluations, we confidently conclude, that except for the tolerance to glyphosate, the GTS 40-3-2 line of soybeans is not materially different from and is as safe and nutritious as its parental variety and other soybean varieties now being marketed.

We have held consultations with the FDA, starting in 1992, to define and discuss studies to assess the composition and safety of GTS. The concepts and approaches we have employed are derived from and consistent with the guidance presented in the flowcharts found in the FDA Food Policy (1). For each question, we have developed answers based on extensive studies or analyses. The thoroughness and detail of these studies are unprecedented for the typical introduction of foods or feeds from a new plant variety. Our data and findings in every case have led us to the conclusion of "no concern" as described in the relevant sections of the following summary. Under these circumstances, following the agency's Food Policy has provided us with a basis for concluding that the 40-3-2 GTS line is as safe and nutritious as its traditional counterparts.

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

summary is background information regarding the development of GTS, including a description of CP4 EPSPS, the protein which confers the glyphosate tolerance phenotype.

III. Background Information Regarding Glyphosate-tolerant Soybeans

A. Rationale for the Development of Glyphosate-tolerant Soybeans

Glyphosate (N-phosphonomethyl-glycine) (CAS Registry #'s 1071-83-6, 38641-94-0), the active ingredient in the non-selective, foliar-applied, broad-spectrum, post-emergent herbicide Roundup (2,3), is the world's most popular herbicide. This is primarily due to its excellent weed control capabilities and its well-known, favorable environmental and safety characteristics. However, the sensitivity of crop plants to glyphosate has prevented the in-season use of this herbicide over-the-top on crops. The extension of the use of Roundup herbicide to allow in-season application in major crops such as soybeans will provide new weed control options for farmers. Recent advances in plant biotechnology have made it possible to insert a gene into soybeans to provide crop tolerance specifically to the non-selective herbicide glyphosate, and bring the benefits of its use to weed management in soybeans (4-9).

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

The use of GTS will provide farmers new options for effective weed control. Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaved weeds. Glyphosate has excellent environmental features, such as rapid soil binding (resistance to leaching) and biodegradation (which decreases persistence), as well as extremely low toxicity to mammals, birds, and fish (3). Recently, glyphosate was classified by the EPA as Category E (evidence of non-carcinogenicity for humans) (57 FR 8739). Roundup herbicide is currently registered with the EPA for pre-harvest application on soybeans. Studies separate from those summarized herein have been provided to the EPA in a request to amend the Roundup herbicide label to include in-season application on GTS.

The use of GTS for soybean production would enable the farmer to utilize Roundup herbicide for effective control of weed pests and to take advantage of this herbicide's environmental and safety characteristics. GTS can positively impact current agronomic practices in soybean by 1) offering the farmer a new wide-spectrum weed control option; 2) allowing the use of an environmentally

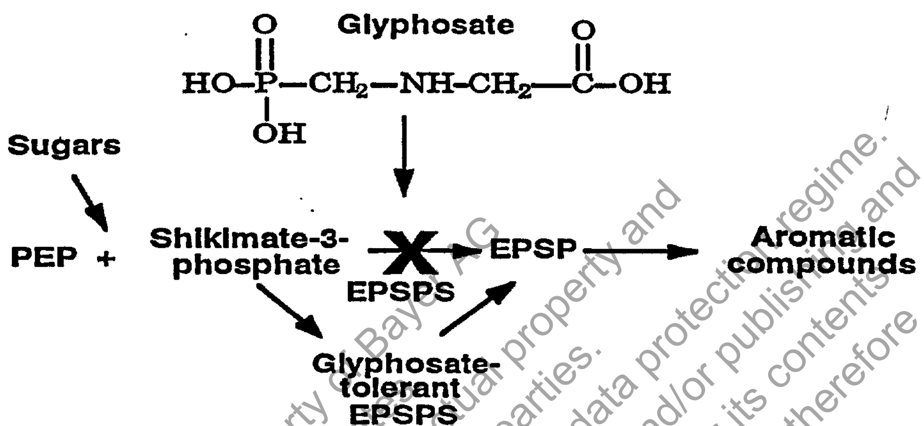
sound herbicide; 3) providing a new herbicidal mode of action for in-season soybean weed control with a product for which no weed resistance has developed in almost 20 years of use; 4) increasing flexibility to treat weeds on an "as needed" basis; 5) offering less dependence on herbicides used before planting; 6) providing an excellent fit with no-till systems, which results in increased soil moisture, while reducing soil erosion and fuel use; and 7) providing cost-effective weed control, not only because Roundup herbicide may be less expensive than most current options, but because the total number of herbicides used may be reduced compared to the farmer's current weed management program.

B. Development of Glyphosate-tolerant Soybeans

The development of glyphosate-tolerant crops has been ongoing since the early 1980's (4-9). The method of tolerance developed was the "target-site" approach, whereby a herbicide-insensitive target protein was identified and introduced into soybean by genetic modification techniques. Glyphosate specifically binds to and blocks the activity of its enzyme target, 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EPSPS) (E.C. 2.5.1.19) (10), an enzyme of the aromatic amino acid biosynthetic pathway (11). Glyphosate inhibition of EPSPS thus prevents the plant from synthesizing the aromatic amino acids essential for protein synthesis. EPSPS is the only physiological target of glyphosate in plants, and no other phosphoenolpyruvate (PEP)-utilizing enzymes are inhibited by glyphosate (12). EPSPS is present in all plants, bacteria, and fungi, but not animals; animals do not make their own aromatic amino acids, but receive them from plant, microbial, or animal foods. In plants, EPSPS is localized in the chloroplasts or plastids (13). Upon glyphosate treatment, the GTS plant remains unaffected because the continued action of the introduced glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic amino acids (Figure 1). This is in contrast to the death or severe yield reduction observed upon glyphosate treatment of non-glyphosate-tolerant soybeans and other plants.

Extensive research indicated that a crucial factor for obtaining high levels of glyphosate tolerance *in planta* depended on the expression of a glyphosate-tolerant EPSPS with a high catalytic efficiency in the presence of glyphosate (14). While several variant EPSPS enzymes fulfilled the requirement of exhibiting high levels of glyphosate tolerance, these EPSPSs also exhibited reduced binding of the natural EPSPS substrate phosphoenolpyruvate (PEP), measured as an elevated appK_m(PEP) kinetic constant. Extensive work was carried out on the G101A (glycine to alanine substitution at position 101) petunia EPSPS (15) and other variant EPSPSs (16), but no EPSPSs were identified which were both highly glyphosate-tolerant and bound the PEP substrate comparably to wild-type EPSPS.

Figure 1. Mechanism of glyphosate tolerance in GTS



C. CP4 EPSPS: The Protein Which Confers Glyphosate Tolerance to GTS Line 40-3-2

1. Kinetic characteristics

The EPSPS enzyme from *Agrobacterium* sp. strain CP4 (CP4 EPSPS) was identified from a screen of microorganism cell extracts as having very favorable glyphosate tolerance kinetic parameters, namely high glyphosate tolerance (K_i[glyphosate]=2.7 mM) and tight binding of PEP (appK_m[PEP]=12 µM) (14,17). EPSPSs from a number of bacteria have been described which exhibit tolerance to glyphosate (18). The appK_m(PEP) of CP4 EPSPS is only about 2-fold greater than the appK_m(PEP) of the wild-type petunia EPSPS (5 µM) (19). In fact, CP4 EPSPS exhibits the lowest appK_m(PEP) constant of any highly glyphosate-tolerant EPSPS identified to date. The constant k_{cat}•appK_i(glyphosate) / appK_m(PEP), which is a measure of the catalytic efficiency of the EPSPS in the presence of glyphosate, is approximately 10-fold higher for CP4 EPSPS than for the aforementioned petunia G101A EPSPS, and results from the 17-fold reduction in appK_m(PEP) for the CP4 enzyme relative to G101A petunia EPSPS. Based on these kinetic parameters, and thus the suitability for use in conferring glyphosate tolerance to crops, the gene for CP4 EPSPS was cloned from *Agrobacterium* sp. strain CP4, and expressed in *Escherichia coli* (*E. coli*) for further characterization (17).

2. Cloning of the CP4 EPSPS gene

The EPSPS gene from *Agrobacterium* sp. strain CP4 was identified and cloned by two parallel approaches: cloning based on the expected phenotype for a glyphosate-tolerant EPSPS, and purification of the enzyme to provide material to raise antibodies and to obtain amino acid sequences from the protein to facilitate the verification of clones (17). A cosmid bank was constructed using DNA from *Agrobacterium sp.* strain CP4, and the cosmids were transformed

into *E. coli* and selected for the EPSPS gene using inhibitory concentrations of glyphosate. The EPSPS gene was then cloned from one of the resulting cosmids imparting glyphosate tolerance, and the nucleotide sequence was determined (17). The deduced amino acid sequence from the resulting cloned EPSPS gene from *Agrobacterium* sp. strain CP4 is shown in Figure 2. CP4 EPSPS is a 47.6 kD protein consisting of a single polypeptide of 455 amino acids (17). The identification of codons in the gene encoding four peptide sequences obtained directly from the purified enzymatically-active CP4 EPSPS conclusively demonstrated that the gene cloned was the EPSPS gene from *Agrobacterium* sp. strain CP4 (Figure 2).

Figure 2. Deduced amino acid sequence of the *Agrobacterium sp.* strain CP4 EPSPS gene from pMON17081 (17). The underlined sequences represent amino acid sequences obtained from peptides of the purified enzyme.

```
  1  MSHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
 51  LEGEDVINTG KAMQAMGARI RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101  TGCRLTMGLV GVYDFDSTFI GDASLTKRPM GRVLNPLREM GVQVKSEDGD
151  RLPVTLRGPK TPTPITYRVP MASAQVKSAV LLAGLNTPGI TTVIEPIMTR
201  DHTEKMLQGF GANLTVETDA DGVRTIRLEG RGKLTGQVID VPGDPSSTAF
251  PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED
301  VADLRVRSST LKGVTVPEDR APSMIDEYPI LAVAAAFAEG ATVMNGLEEL
351  RVKESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401  HLDHRIAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451  DTKAA
```


3. Sequence homology of EPSPS proteins

There is considerable divergence in the amino acid sequences of EPSPSs which are typically present in foods. The divergence of the CP4 EPSPS sequence from typical food EPSPS sequences is of the same order as the divergence among the food EPSPSs themselves. All crops and common microbial food sources such as Baker's yeast (*Saccharomyces cerevisiae*) or *Bacillus subtilis* (20) contain EPSPS proteins. EPSPSs show a wide range of amino acid compositions as deduced from their DNA sequences. For instance, the soybean EPSPS is only 56% similar and 30% identical to *Bacillus subtilis* EPSPS. The CP4 EPSPS shows a comparable range of similarity to the EPSPS enzymes from foods. Paired comparisons of EPSPS sequences were performed with the program "Gap" (21). Comparing the deduced amino acid

sequences of CP4 EPSPS with EPSPS from soybean, corn, petunia, *E. coli, Bacillus subtilis,* and *Saccharomyces cerevisiae* (Baker's yeast) yields similarities of 51%, 49%, 50%, 52%, 59%, and 54%, respectively, and identities of 26%, 24%, 23%, 26%, 41%, and 30%, respectively (17).

To gain further insight into the relationship between CP4 EPSPS and other known EPSPSs, the amino acid sequence of CP4 EPSPS was aligned against a consensus sequence of previously-identified EPSPSs (17). Several residues which have been previously identified by Monsanto as important for EPSPS function are conserved in CP4 EPSPS (17). These residues include Lys^{28} of CP4 EPSPS, which corresponds to Lys^{22} in *E. coli* EPSPS and likely interacts with PEP (22); Arg^{33}, which corresponds to Arg^{27} of *E. coli* EPSPS and is involved in S3P binding (23); and Arg^{128}, which corresponds to Arg^{124} of *E. coli* EPSPS, and is believed to be involved in PEP binding (23). The identification of homology at catalytically critical sites between CP4 EPSPS and other known EPSPSs, including soybean EPSPS, demonstrates an overall relatedness between CP4 EPSPS and plant EPSPSs. In addition, recent results indicate that the 3-dimensional X-ray crystal structure of CP4 EPSPS exhibits the same overall folding pattern as the *E. coli* EPSPS enzyme ([redacted] Monsanto unpublished results).

We conclude that CP4 EPSPS is functionally similar to the EPSPS proteins typically present in food and feed derived from plant and microbial sources, based on the reaction catalyzed. The structural relationship between CP4 EPSPS and other food EPSPSs is demonstrated by 1) the amino acid sequence comparison; 2) the homology of active site residues; and 3) the 3-dimensional structure.

4. pH and temperature dependence of CP4 EPSPS

We evaluated the temperature stability of EPSPS activity in order to determine whether EPSPS activity in glyphosate-tolerant raw agricultural commodities expressing CP4 EPSPS may be inactivated upon heat processing. Virtually 100% of soybeans are heated prior to consumption as human food. Upon incubation of CP4 EPSPS at 55°C for 15 minutes, less than half of the 25°C incubation activity was present; enzymatic activity was completely abolished after 15 minutes incubation at 65°C (17). For comparison, soybean flakes are heated approximately 38 minutes at 66-107°C during the preparation of toasted meal for animal feed (24). These results indicate that it is very likely that the enzymatic activity of CP4 EPSPS will be lost or significantly decreased upon heat processing of GTS seeds. EPSPS enzymatic assays of GTS defatted, toasted meal were consistent with these initial results: no EPSPS activity was detectable in protein extracts from the toasted meal samples, while activity was detected in non-toasted seed protein extracts (25,26).

Similarly, it was of interest to determine whether CP4 EPSPS would be enzymatically active in the acidic environment of the human stomach, should the activity survive processing. The pH dependence of CP4 EPSPS was therefore measured from pH 4 to pH 11 (17). The maximal activity of CP4 EPSPS under these conditions was observed to be in the range of pH 9 to 9.5. No enzymatic activity was detectable at pH values less than pH 5. These results establish that CP4 EPSPS would not be enzymatically active in the acidic environment of the human stomach.

D. Petunia EPSPS chloroplast transit peptide (CTP)

The CP4 EPSPS gene was engineered for plant expression by fusing the 5'-end of the CP4 EPSPS gene to a chloroplast transit peptide (CTP) sequence derived from petunia EPSPS (13,27,28). As discussed below, the current literature on transit peptides supports a model whereby the CTP is degraded rapidly and completely by proteases after the targeting of the precursor protein has occurred. Thus, cleaved CTPs are not believed to have any measurable lifetime in the plant. This is the basis for our conclusion that the "mature" (not containing the CTP) CP4 EPSPS is the only introduced protein present in GTS line 40-3-2.

The petunia EPSPS CTP has been previously shown to deliver bacterial EPSPSs to the chloroplasts of higher plants, which is the site of the aromatic amino acid biosynthetic pathway and the organelle to which plant EPSPS is targeted (29). Previous results showed that it was critical to target glyphosate-tolerant EPSPSs to the chloroplast to obtain the highest levels of *in planta* glyphosate tolerance. *In vitro* chloroplast uptake assays verified that the petunia EPSPS CTP delivered CP4 EPSPS to the chloroplasts and was cleaved from the pre-protein (unpublished results). Thus, after the "pre-CP4 EPSPS" protein (containing the CTP amino-terminal extension) reaches the chloroplast or plastid stroma, the CTP is cleaved and degraded, as are the CTPs from other nuclear-encoded chloroplast-targeted proteins (30). This leaves the "mature" CP4 EPSPS, with no CTP sequences retained, as the CP4 EPSPS species present in soybean seeds (31).

Transport of proteins into chloroplasts, mitochondria, and microbodies has been relatively well-studied (32-34). In plants, most chloroplast-targeted proteins are synthesized on cytosolic ribosomes as larger precursors containing an amino terminal CTP extension (33). As of 1991, 260 different transit peptide amino acid sequences were known and available in a database of CTPs (35). Overall, although different types of membranes and energetics may be involved, the mechanisms for transport of proteins into different organelles appear to be similar. This is especially true for chloroplasts and mitochondria. The proteins are synthesized in a precursor form with amino-terminal signal (transit) sequences which are efficiently removed by signal peptidases during or after transport. The necessity of transit peptides has

been demonstrated by experiments showing that precursor proteins lacking transit peptides cannot be imported into chloroplasts (33). Extensive experimental studies do not support the hypothesis that specific transit peptide amino acid sequences have specific essential functions; i.e. the uptake function of the CTP does not appear to reside in the primary amino acid sequence. Rather, the results suggest that the essential feature of a transit peptide is some secondary or higher-order structure (33).

Although sequence similarities generally exist among CTPs of the same precursor protein derived from different plant species, few similarities are found among different precursors, even when the precursors are derived from the same plant species (33). This is indeed the case with the petunia EPSPS CTP, which is highly homologous with the CTP from tomato EPSPS (28). A search of the GenBank (36) and SwissProt (37) databases of protein sequences, as well as Monsanto sequences, reveals that the petunia EPSPS CTP is most homologous with the CTPs from other EPSPSs. Comparing the deduced amino acid sequences of the petunia EPSPS CTP with the CTPs from tomato, tobacco, soybean, brassica, arabidopsis, and maize EPSPSs, using the program "Bestfit", (21) yields similarities of 73%, 68%, 55%, 47%, 45%, and 36%, respectively, and identities of 62%, 54%, 33%, 29%, 25%, and 17%, respectively (17). There are no other meaningful homologies to any other protein sequences in these databases. The petunia EPSPS CTP also has no homology with any identified protein allergen or toxin, using the methodologies described in Section IV.B.1.d.i and Appendix C.

Chloroplast targeting of proteins is known to be composed of at least two steps: a specific binding of precursor proteins to the surface of the chloroplastic envelope, followed by translocation of the precursor protein across the envelope (38,39). Then, the signal sequence is removed by a signal peptidase, also called a transit peptidase (32). Removal of the amino-terminal targeting signal is catalyzed in the mitochondria of yeast by a matrix-localized metalloprotein (40). This protease has been purified to homogeneity and shown to cleave synthetic transit peptide sequences containing a cleavage site.

Degradation of signal peptides after transport has occurred is critical for cell survival. This is because it is known that cleaved signal peptides or synthetic peptides based on signal peptide sequences inhibit transport. Transit peptides without (and with) the cleavage site inhibited processing of a mitochondrial preprotein (40). It was hypothesized that *in vivo*, the inhibitory effect of the cleaved transit peptides is probably prevented by rapid degradation of the cleaved peptide by other proteases that remain to be identified (40). Synthetic peptides based on the petunia EPSPS CTP also inhibit chloroplast uptake of pre-petunia EPSPS (unpublished data). A similar conclusion has been reached from experiments with the *E. coli* B prolipoprotein signal peptidase: the peptidase activity is inhibited by incubation with an appropriate transit

peptide, and it was concluded that rapid removal and degradation of the cleaved signal peptide is necessary to maintain proper export (41). In that case, *E.coli* B prolipoprotein signal peptide prepared by incubation with purified peptidase from *E. coli* B was degraded to the amino acid level by cell-free extracts of *E. coli* more rapidly (at least 300-fold) than other *E. coli* proteins (41). The inability to detect the transit peptide released by rat liver mitochondrial processing protease until the processing protease is purified also suggested that the cleaved transit peptides are too short-lived within the mitochondria to be detected (42). Other authors studying preproinsulin processing in rat pancreatic islets have data indicating that the cleaved transit sequences are rapidly degraded (43).

Thus, it is generally accepted in the literature that the chloroplast transit peptides, like the other transit peptides discussed above, are rapidly degraded after cleavage *in vivo* by other cellular proteases. This conclusion is based mainly on analogy with the results obtained with other transport systems, which are similar to chloroplastic transport. We therefore conclude that the chloroplast transit peptide removed from CP4 EPSPS is degraded, and that the sole introduced protein present in line 40-3-2 is the "mature" CP4 EPSPS.

E. GTS Line 40-3-2: Plasmid Utilized, Transformation, DNA Insert Analysis, and Insert Stability

1. Plasmid PV-GMGT04

The plasmid PV-GMGT04 (Figure 3), used to transform the parental soybean line A5403 to generate line 40-3-2, contained three genes driven by plant promoters: two CP4 EPSPS genes and a gene encoding ß-glucuronidase (GUS) from *E. coli* (44). PV-GMGT04 is a pUC-Kan vector delivered to the donor organism using the particle acceleration transformation procedure. This vector is a derivative of the high copy *E. coli* plasmid pUC119 (45) and was constructed by fusing the 1.3 Kb FspI-DraI pUC119 fragment containing the origin of replication to the 1.3 Kb SmaI-HindIII Klenow-filled fragment from pKC7 (46), which contains the KAN gene (neomycin phosphotransferase type II gene, *nptII*). The *nptII* confers bacterial kanamycin resistance and replaces the ampicillin resistance gene of pUC119. This *nptII* gene is driven by a bacterial promoter and bacterial signals which are different from those found in plants, preventing expression in plant cells (47). Prior to their combination in a single vector, the CP4 EPSPS and GUS genes were assembled with promoters and 3' sequences in the following steps: the CTP4-CP4 EPSPS fusion was combined with the CMoVb promoter and the NOS 3' end (47) and the GUS gene already fused to the MAS promoter and 7S 3' into a vector (pMON13615) via a triple ligation utilizing the BglII, HindIII, SalI, and XhoI restrictions sites. The SalI and XhoI 5' overhanging sequences are complementary and can be cloned into one another with the elimination of both recognitions sequences. The CTP4-CP4 EPSPS fusion was combined with the E35S (CMoVa) promoter and the NOS 3' terminator in a plasmid (pMON13620) where the

entire fusion product was flanked by HindIII recognition sequences to facilitate further subcloning. These three elements were then combined in the pUC plasmid (pMON13639) by subcloning the E35S/CTP4-CP4 EPSPS/NOS 3' fusion product from pMON13620 on a HindIII fragment into the unique HindIII site in pMON13615. The NotI fragment of pMON13639, which has the CP4 EPSPS and GUS elements, was moved into a derivative of pUC119 (pMON10081) which contains the origin of replication and the *nptII* gene. The resulting vector was PV-GMGT04 (Figure 3).

Extensive restriction analysis of the plasmid PV-GMGT04 and its progenitor plasmids demonstrated that all of the genetic elements and restriction fragments were correctly assembled and produced the correctly sized DNA fragments when digested and separated on a 0.8% agarose gel (44). Shown in Table 1 is a summary of the sequences of each of the genetic elements used to assemble plasmid PV-GMGT04. All of the clonings performed to construct plasmid PV-GMGT04 were done in non-pathogenic *Escherichia coli* strains derived from *E. coli* K-12 that are commonly used in molecular biology research (48) (*E. coli* LE392, JM101, and MM294).

Figure 3. Plasmid PV-GMGT04 (49,50)

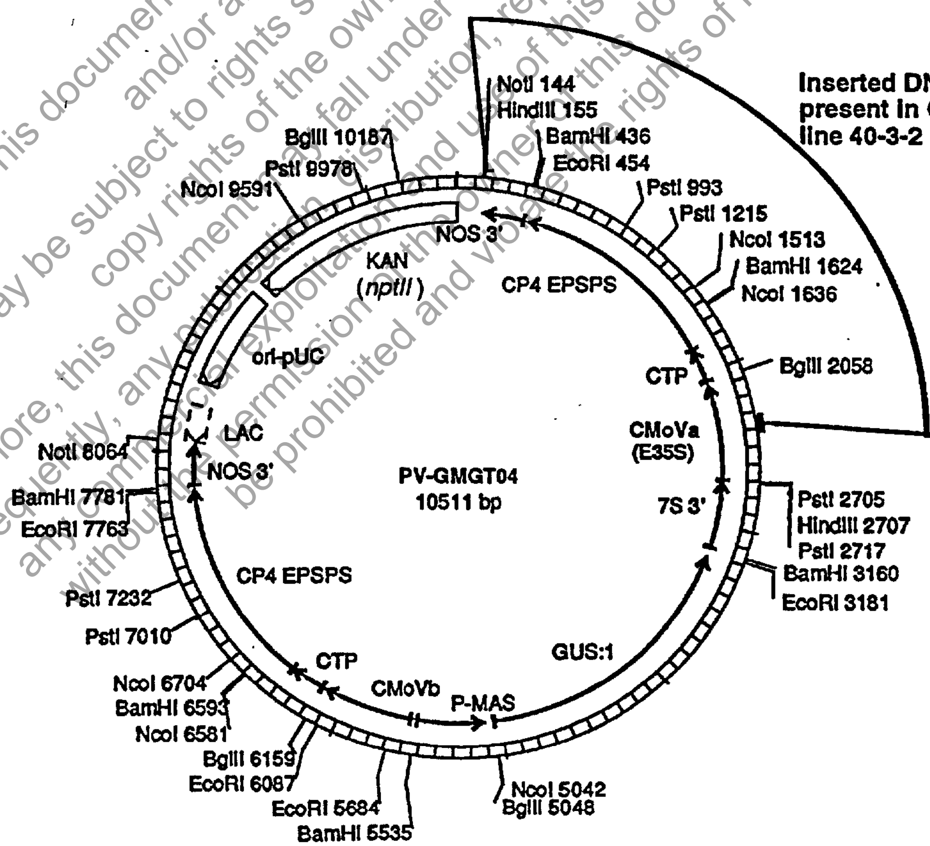


Table 1. Summary of sequences for PV-GMGT04

Genetic Element	Size, Kb	Function
P-E35S	0.61	The cauliflower mosaic virus (CaMV) 35S promoter (51) with the duplicated enhancer region (52).
CTP4	0.22	The N-terminal 0.22 Kb chloroplast transit peptide sequence from the *Petunia hybrida* EPSPS gene (27).
CP4 EPSPS	1.36	The C-terminal 1.36 Kb 5-enolpyruvylshikimate-3-phosphate synthase gene (CP4 EPSPS) from an *Agrobacterium* species (14,17).
NOS 3'	0.26	The 0.26 Kb 3' nontranslated region of the nopaline synthase gene (47).
KAN	1.32	The Tn5 neomycin phosphotransferase type II gene (*nptII*) from plasmid pKC7 (46). The *nptII* confers kanamycin resistance on the bacterial cloning host.
ori-pUC	0.65	The origin of replication from the high copy *E. coli* plasmid pUC119 (45).
LAC	0.24	A partial *E. coli* lacI coding sequence, the promoter Plac, and a partial coding sequence for ß-d-galactosidase or lacZ protein from pUC119 (53).
P-MAS	0.42	The 0.42 Kb TR 2' mannopine synthase promoter region (54).
GUS	1.81	The 1.81 Kb coding region of the *E. coli* ß-glucuronidase gene (55). The expression of the gene in plants is used as a scoreable marker for transformation.
7S 3'	0.43	The 0.43 Kb 3' nontranslated region of the soybean 7S seed storage protein alpha' subunit (56).
CMoVb	0.57	The 0.57 Kb figwort mosaic virus 35S promoter (57).

2. Recipient soybean variety, A5403

Glycine max L. cv. A5403 ("A5403") is the cultivar which was genetically modified to be tolerant to glyphosate, and is a commercial variety of Asgrow Seed Company. A5403 is a maturity group V cultivar which combines a consistently high yield potential with resistance to races 3 and 4 of the soybean cyst nematode (SCN). It also combines good standability, excellent emergence, and tolerance to many leaf and stem diseases. A5403 was one of the first group V cultivars with SCN resistance provided to farmers and has received protection under the Plant Variety Protection Act. The commercialization strategy for GTS is to use traditional backcrossing and breeding to transfer the glyphosate tolerance locus from this cultivar to a wide range of varieties and maturity groups of soybeans.

3. Particle acceleration transformation method

Introduction of PV-GMGT04 DNA into soybean tissue by the particle acceleration method (particle gun) has previously been described (58,59). During the transformation process, the plasmid DNA breaks at one or more locations and integrates into the plant DNA. The shoots which develop from the transformed cells express the phenotype encoded by the genes on the delivered DNA. The DNA utilized contained the chimeric plant expression genes and a marker gene encoding the GUS protein (55) (Figure 3). The

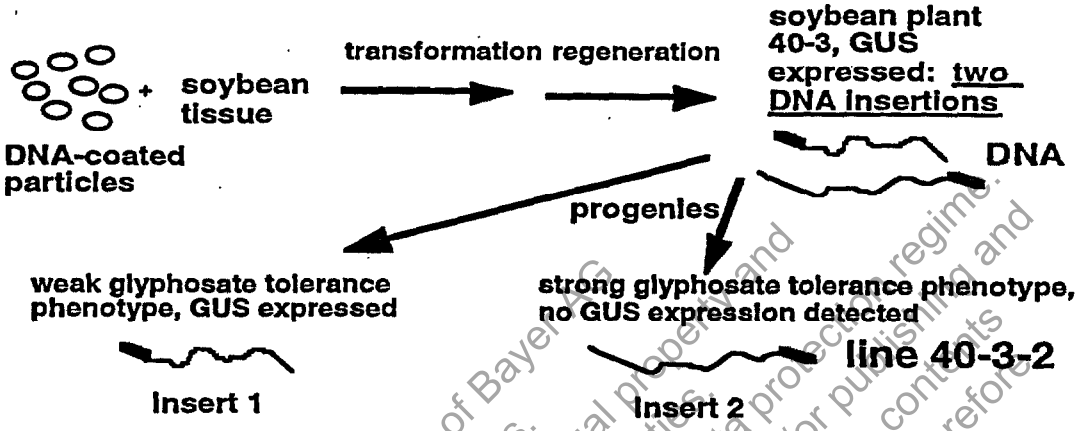
expression of the GUS protein was used as evidence of transformation. It was detected by a staining method in which the GUS enzyme converted a substrate (5-bromo-4-chloro-3-indolyl ß-D-glucuronide) into a blue precipitate. The majority of the shoots which are regenerated from the shoot tip cells do not contain any added genes, therefore GUS screening is necessary to identify the genetically modified tissue. The positive shoots were grown to maturity, and the resulting progeny plants were screened for glyphosate tolerance (by Roundup herbicide spray test) and gene expression.

4. Molecular description and stability of the DNA insert in GTS Line 40-3-2

GTS line 40-3-2 is an R_1 (first progeny generation) selection from an original R_0 transformant, 40-3, which was obtained by particle gun bombardment of the Asgrow variety A5403 with vector PV-GMGT04. This vector contains two genes encoding CP4 EPSPS and the gene encoding the GUS marker protein, as shown in Figure 3. Progenies from 40-3 R_1 seed were grown in a greenhouse during the winter of 1990-91 and evaluated for glyphosate tolerance by spray test. R_2 seeds from individual R_1 plants were planted in the field during the summer of 1991. The 40-3-2 R_2 progeny that were selected exhibited strong glyphosate tolerance and expressed CP4 EPSPS, but no GUS enzyme activity or GUS protein (by ELISA) were present (25,26).

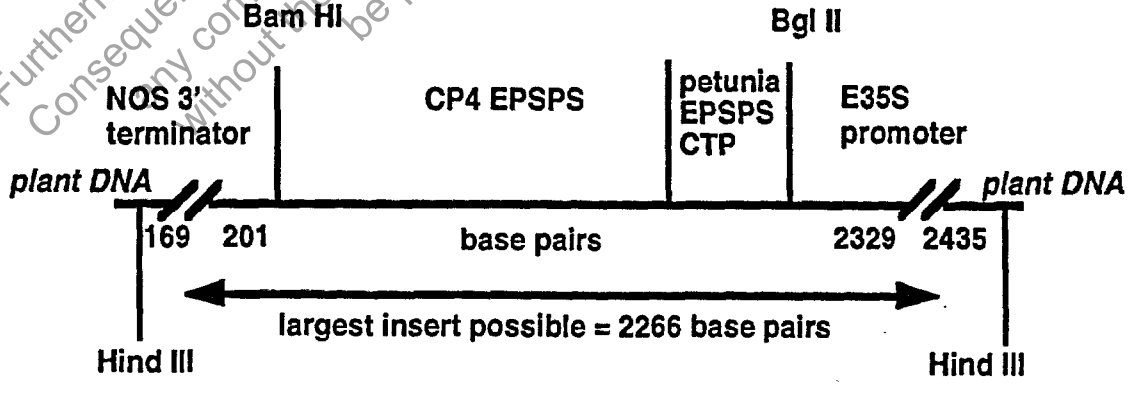
The absence of the GUS protein in the 40-3-2 GTS line seems contradictory to the fact that GUS was utilized as a scoreable marker in the transformation process, and requires explanation. The parental (R_0) line of GTS line 40-3-2 was denoted 40-3. Through the standard production and analysis of the R_2 progenies of line 40-3, it became apparent that the original 40-3 R_0 plant had received two DNA inserts located at different positions in the genome (Figure 4). Insert 1 was responsible for the expression of the GUS marker protein. Insert 2 had a strong expression of the glyphosate tolerance trait, but did not express the GUS protein. The 40-3-2 specific R_2 progeny containing only insert 2 exhibited strong glyphosate tolerance but no GUS enzyme activity. Thus insert 1 had been lost through normal genetic segregation. This was confirmed by the fact that none of the progenies from line 40-3-2 expressed GUS protein, based on leaf GUS enzyme assays. Extensive analyses of line 40-3-2 seed and leaves using a validated GUS ELISA confirms that no GUS protein is expressed in line 40-3-2 (25,26). The production of line 40-3-2 and the segregation of the inserted DNA regions is represented schematically in Figure 4.

Figure 4. Schematic diagram of segregation of DNA insertions in GTS line 40-3



Based on PCR and Southern blot data, GTS line 40-3-2 was shown to contain a single insert of introduced DNA. This insert contains a portion of the E35S promoter, the petunia EPSPS CTP, the CP4 EPSPS gene, and a portion of the NOS 3' terminator (49). Therefore, the only protein encoded by PV-GMGT04 DNA present in line 40-3-2 is the CP4 EPSPS protein (Figures 3 and 5). This conclusion is confirmed by the following molecular data (49): 1) the positive detection of E35S, NOS 3' and the CP4 EPSPS gene by Southern analysis; 2) the lack of ori-pUC and KAN signals by PCR analysis; and 3) the lack of CMoVb and GUS signals by Southern analysis. The ends of the inserted DNA of 40-3-2 have been mapped. Based on extensive restriction and PCR analysis of line 40-3-2 DNA (49,50), it was concluded that one end of PV-GMGT04 DNA incorporated into the line 40-3-2 soybean genome falls between nucleotide 169 and nucleotide 201 (Figures 3 and 5). The other end of the PV-GMGT04 DNA incorporated into the line 40-3-2 soybean genome falls between nucleotide 2329 and nucleotide 2435 (Figures 3 and 5). Based on these experiments, the maximal size of the PV-GMGT04 DNA contained in line 40-3-2 was calculated to be approximately 2.27 Kb.

Figure 5. Schematic diagram of the inserted DNA in GTS line 40-3-2(50)



F_2 progenies of crosses between other soybean lines and GTS line 40-3-2 consistently segregate 3 tolerant to 1 sensitive, establishing that the 40-3-2 insert behaves as a single dominant gene inherited in a Mendelian fashion (49). The genetic stability was further confirmed by DNA analyses. An identical 5.8 Kb HindIII band was evident when either R_3 generation or R_6 generation line 40-3-2 was probed with the plasmid used to transform line 40-3-2 (PV-GMGT04) (50). This HindIII band has been previously identified as containing the insert DNA from PV-GMGT04 (49); the HindIII sites are not derived from the introduced plasmid DNA. The fact that this same size band is present in both generations of 40-3-2 indicates that the insert is stable. The glyphosate tolerance phenotype and Mendelian transmission has been consistent for every generation of line 40-3-2 soybean tested to date, which spans more than seven generations. The soybeans generated for the majority of the analyses and feeding studies summarized herein were from the R_5 and R_6 generations (25,26).

F. Field Testing History and USDA Approval of Line 40-3-2

GTS line 40-3-2 has been field tested in the United States, Central and South America, Europe, and Canada since 1991 at approximately 300 locations. Data collected from these trials (including yield, agronomic characteristics, vigor, disease and insect susceptibility), literature references, and expert opinion letters demonstrate that the GTS line 40-3-2: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than the non-modified parental varieties; 3) has not revealed any potential to increase the weediness of any other cultivated plant or native wild species; 4) does not negatively impact processed agricultural commodities; and 5) has not revealed any potential to harm other organisms that are beneficial to agriculture. Therefore, the Agricultural Group of Monsanto Company requested a determination from USDA/APHIS that the GTS line 40-3-2 and all progenies derived from crosses between line 40-3-2 and traditional soybean varieties no longer be considered a regulated article (49). USDA/APHIS accepted the petition for determination of nonregulated status of GTS line 40-3-2 on September 15, 1993. On May 19, 1994, USDA/APHIS, on the basis of an environmental assessment in response to the petition (APHIS Number P93-258-01), reached a finding of no significant impact on the environment from the unconfined, agricultural use of GTS line 40-3-2 and its progeny. Therefore, U.S. permits are no longer required from APHIS for field testing, importation, or interstate movement of line 40-3-2 or its progeny.

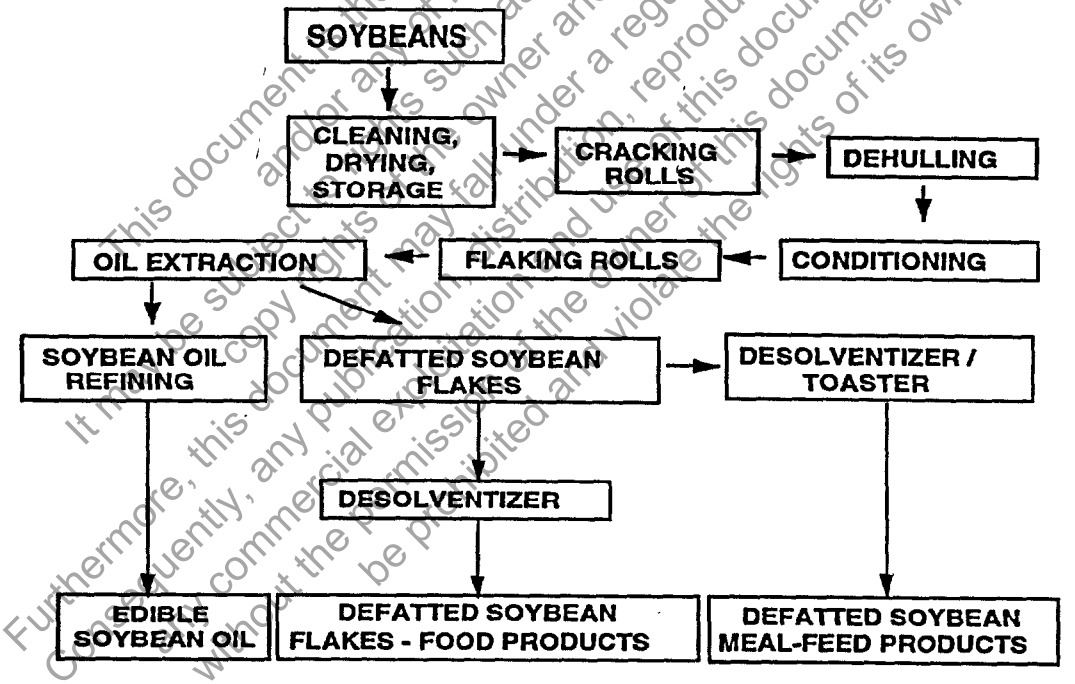
G. Soybean-based Products and Human/Animal Consumption

To design a relevant food/feed safety assessment program for GTS, it was crucial to understand the uses of soybeans. Summarized below are the key aspects of soybean food and feed utilization.

1. Soybean processing and export

There are three major soybean commodity products: beans, oil and meal (Figure 6). A 60-pound bushel of soybeans yields about 48 pounds of protein-rich meal and 11 pounds of oil (60). The primary use of the defatted toasted soybean meal is in animal feed (97%) (61). The various soybean protein fractions used in foods are derived from the processing of defatted soybean flakes (see Figure 8). The principle processed fraction used in the food industry, however, is soybean oil. There are no food uses of unprocessed soybeans, since they naturally contain certain factors, such as trypsin inhibitors, which may act as antinutrients if the soybeans are not properly heated during preparation (62). The United States has become the major exporter of soybeans to world markets. About 35% of the United States soybean crop has been exported as beans to be processed in the importing countries (63). More than one-half goes to countries in Western Europe, but the largest single importer continues to be Japan. The United States also exports the major processed products, meal and oil.

Figure 6. Processing of soybeans (64).

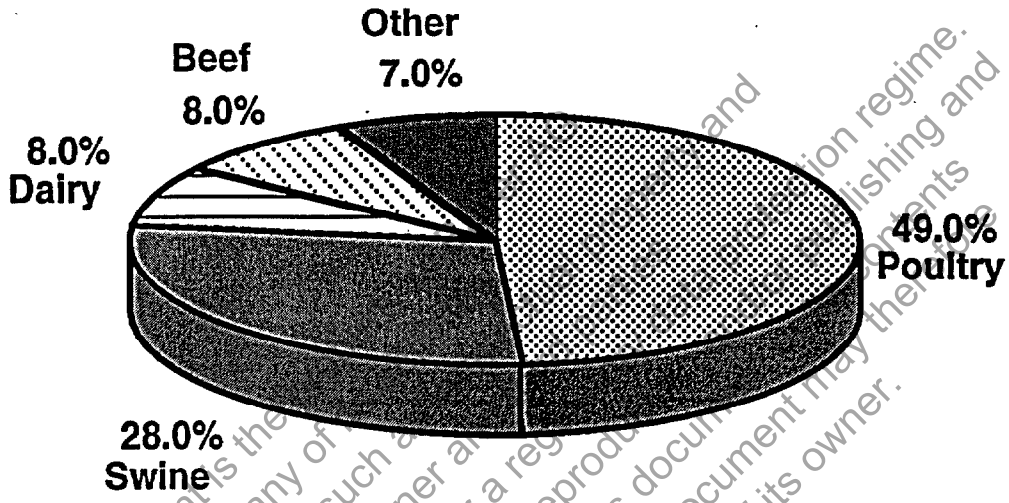


2. Animal consumption

The primary use of soybean is as a heat-processed meal for protein supplementation of animal feeds. The use of soybean meal in animal feeds accounts for about 97% of the soybean meal produced annually (61). Shown in Figure 7 is an estimate of the livestock feed distribution of soybean meal in the

U.S. (60).

Figure 7. U.S. soybean meal use by livestock, 1990. Data taken from 1992 Soy Stats, American Soybean Society (60).



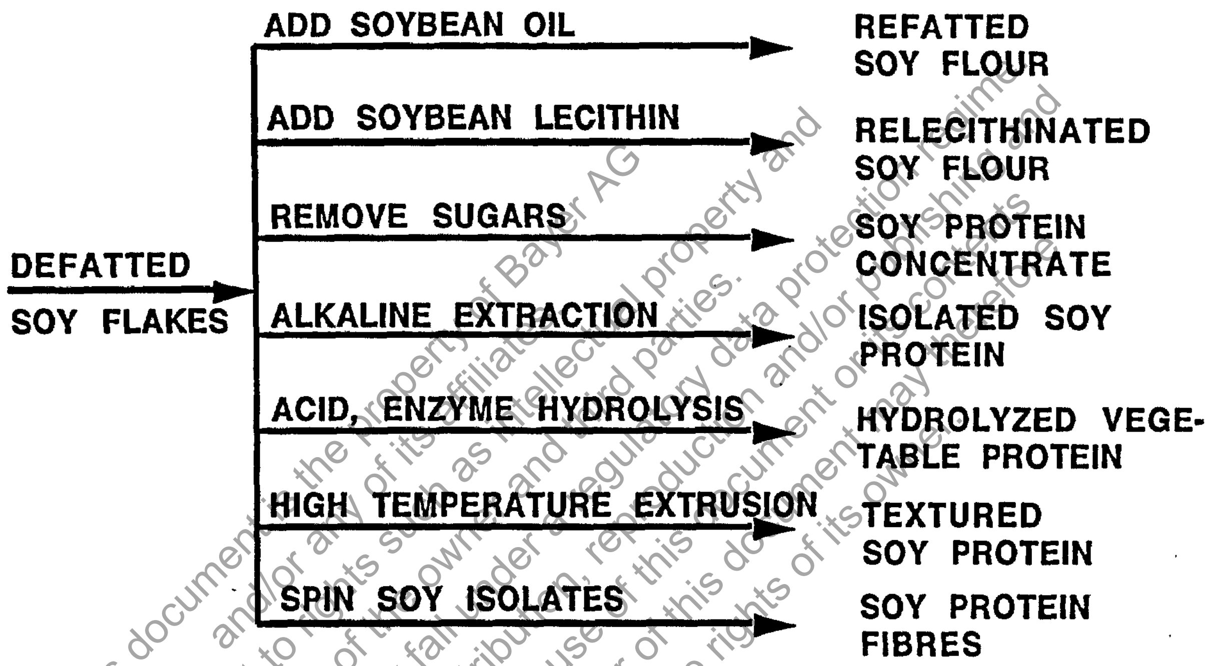
3. Human consumption

During the last several decades, the soybean has been developed as a major source of protein. Soybeans are capable of producing the greatest amount of protein per unit of land of any major plant or animal source used as food by people today (65). The amino acid profile of soy protein is unusually well-rounded for a plant protein (66). Especially important is the soybean's high content of essential amino acids, particularly lysine, leucine, and isoleucine (67). Extensive literature information exists on the nutritional value of soy protein (68-70). Soybeans naturally contain certain factors, such as trypsin inhibitors, which may act as antinutrients if the soybeans are not properly heated during preparation (62). This is the main reason that virtually 100% of soybean protein products are heated prior to human and most animal consumption.

As mentioned above, in terms of domestic usage of the U.S. soybean crop, only about 3% of the total protein derived from soybean is used in food (61,71). Foods containing soybean protein include bakery products, confections, meat products, textured foods, and nutritional supplements (60,64). The soybean is the highest natural source of dietary fiber, and soy hulls are processed into fiber bran breads, cereal, and snacks. Soybean flour products, either full fat or defatted, are added to many baked goods. Soybean protein isolate, which is manufactured from defatted soy flakes, is the protein source for soy-based infant formula. Defatted dehulled soybean flakes (minimally heat-treated to

retain solubility) are used as a starting material for a wide variety of soy food products, as shown in Figure 8 (72)

Figure 8. Food uses of soybean protein (72)



Soybean oil, which contains negligible quantities of protein (73), is extensively used in the food industry in products such as cooking and salad oils, salad dressings, shortening, and oleo margarine. Soybean oil is currently the major edible oil used in the U.S. (74).

The food industry uses products derived from soybeans as ingredients to manufacture food products. They are used to influence the physical structure, stability, or texture of products. Soy protein concentrates are incorporated in some meat products as an extender, but also in a textured form to simulate meat. Lecithin, a phosphatide removed from crude soybean oil, is used as a natural emulsifier, lubricant, and stabilizing agent (75,76).

IV. Safety Assessment of New Varieties

Detailed below is a summary of the safety assessment studies performed on GTS line 40-3-2. The safety assessment program was designed to address the specific questions detailed in the applicable FDA Food Policy flowcharts (1), and the data will be discussed accordingly. The pathway leading to "no concern" for GTS line 40-3-2 is highlighted with bold arrows in the flowcharts reproduced below.

A. Absence of Unexpected or Unintended Effects

1. Safety assessment of the host plant, soybean

Shown in Figure 9 is the flowchart from the FDA Food Policy concerning the safety assessment of the host plant.

a. Soybean

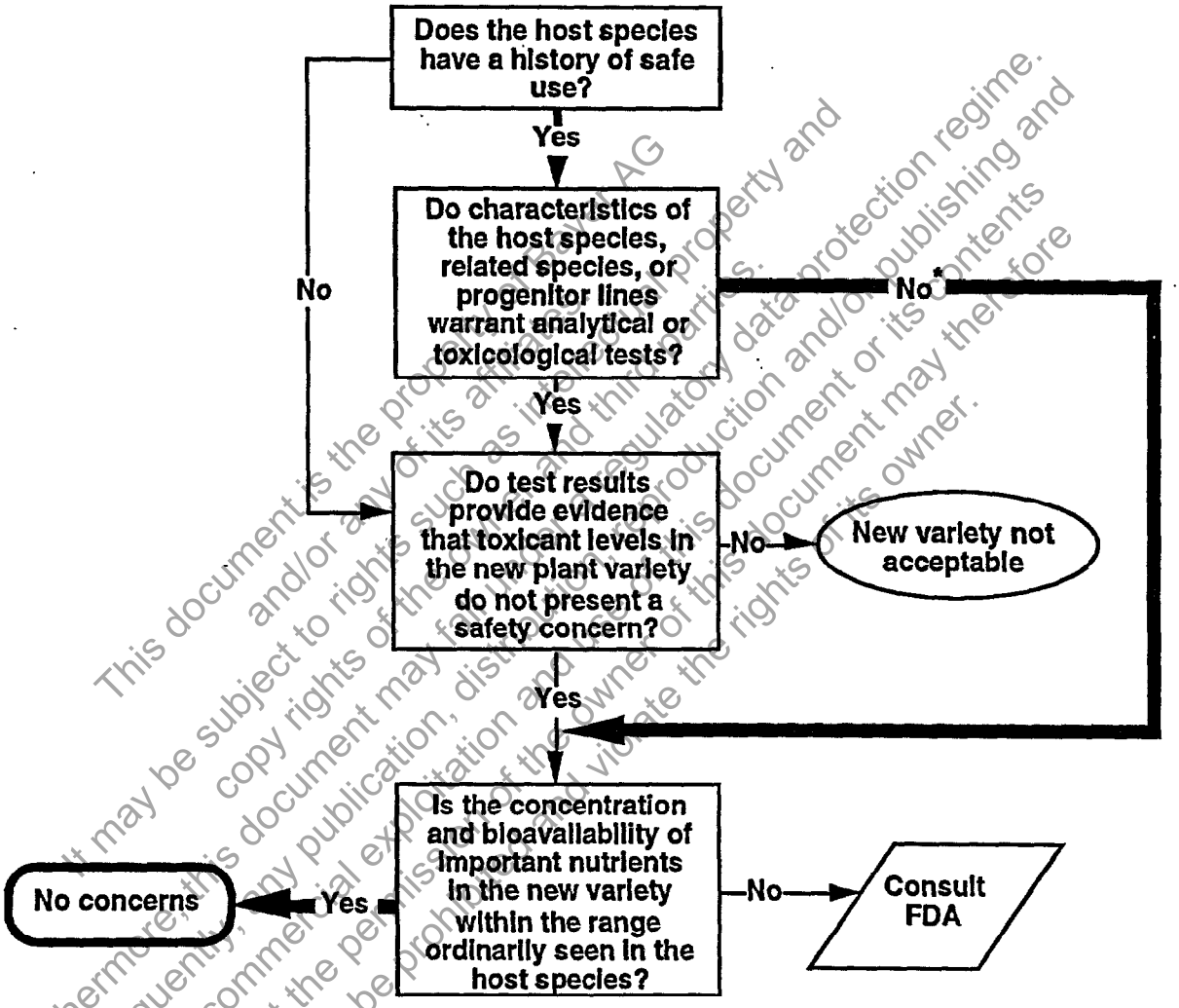
Soybean, the host plant, has a history of safe use; soybeans or processed fractions are consumed in many human food products or animal feeds. Soybean, *Glycine max*, is one of the world's largest sources of plant protein and oil. The soybean plant is a bushy, green legume which farmers plant in late spring, with pods developing in late summer. In 1829, U.S. farmers grew soybeans for soy sauce and until the mid-1940's soybean was used mainly as a forage crop. The establishment of soybean as a major crop in the United States started in 1940 with incentives due to World War II (75). Today, more soybeans are grown in the United States than anywhere else in the world. Farmers in over 29 states grow soybeans, making soybeans the third largest U.S. cash crop (76). The technology and yield of soybean production in the United States has steadily advanced. According to the American Soybean Association, 57.7 million acres of soybeans were planted in 1990 in the United States, yielding an average of 34 bushels/acre (76). Yields have increased due to the development of new cultivars, the availability of better field equipment, and the use of herbicides which have greatly reduced weed competition to crop growth.

The safe use of soybeans has been well characterized. The characteristics of soybean, or the specific progenitor line, therefore do not warrant analytical or toxicological tests. Typically, soybean breeders make genetic crosses to generate new cultivars with enhanced commercial value, and they evaluate new varieties primarily based on yield, as well as protein and oil content. However, the FDA Food Policy (1) recommends that key compositional components of genetically-modified plant varieties be assessed prior to commercial introduction. Monsanto has therefore performed extensive analytical studies to compare the compositional quality of soybeans from GTS line 40-3-2 to the parental line, A5403 (25,26).

b. Compositional analyses of GTS line 40-3-2 seeds

Analyses show that GTS seeds are not materially different from other soybeans in essential nutrients or antinutrients (25,26). The strategy taken for measurement of compositional parameters was to focus as much as possible on the raw agricultural commodity, the soybean seed. It is reasonable to infer that if the GTS soybean seeds are not materially different from control soybean seeds, then products derived from the GTS seeds will also not be materially different from the products derived from control seeds.

Figure 9. Safety assessment of new varieties: the host plant (taken from Food Policy, Figure 2) (1). The pathway leading to "no concern" for GTS is highlighted with bold arrows.



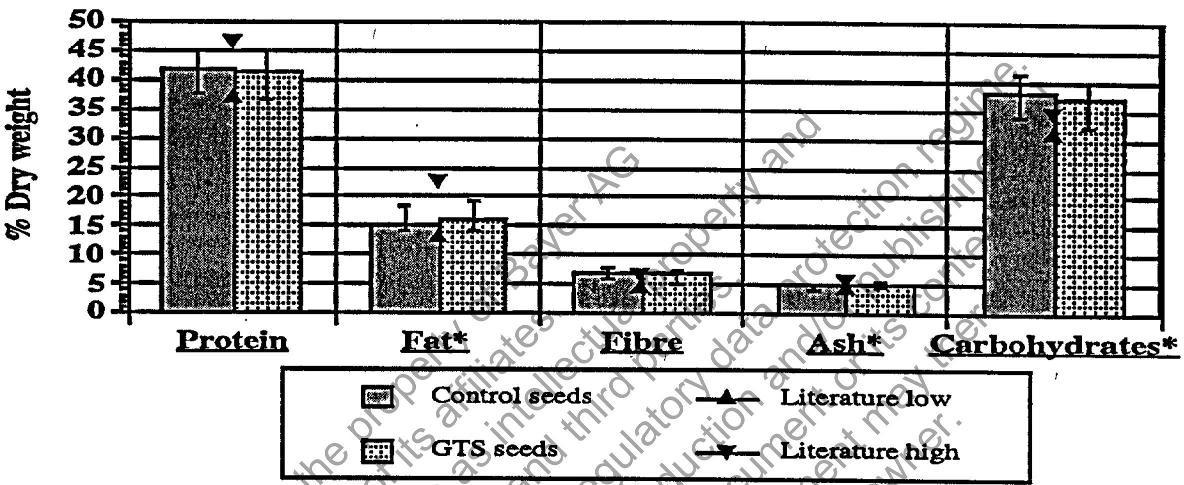
* New soybean varieties are not typically subjected to extensive analytical or feeding tests. However, compositional analyses to verify levels of nutrients and antinutrients, as well as feeding studies to ensure the wholesomeness of GTS line 40-3-2, were performed as discussed in the Food Policy.

In order to provide test material for these analyses, GTS line 40-3-2 and the parental variety A5403 were grown at nine field locations in 1992 under Good Laboratory Practices (GLP) guidelines (25,77). Seed grown from each of the nine sites were analyzed, and statistical analysis of the data comparing GTS to the control soybeans was performed (25). It should be noted that the majority of these studies were performed on two GTS lines, line 40-3-2 and line 61-67-1. Based on the data obtained from these studies, neither line was materially different from the parental control line, A5403. Due to a commercial prioritization, Monsanto is only planning to introduce GTS line 40-3-2 and its progeny into commerce. Therefore, the following data summaries focus exclusively on GTS line 40-3-2. Note that data from line 61-67-1 was utilized in the statistical analyses, in addition to the line 40-3-2 data, in order to obtain a more precise estimate of error in the experiment. Compositional data on seed from a single site in Puerto Rico (26), which was similar to that obtained in the U.S. study, will not be summarized in detail below. This is because the Puerto Rico study was a statistically separate study at one site, while the U.S. study covered nine sites. The results using material from the U.S. sites were therefore more representative of the wide geographical area in which soybeans are grown. In addition, a four-site field test with limited analytical evaluations was performed in 1993 (78). To focus the analysis on any effects of the introduced gene and protein, the soybeans from which the tested and analyzed seed were derived were not treated with Roundup herbicide.

Although we focused our analyses on the seed, we also manufactured several selected, important soy protein products for additional analysis. Toasted meal was chosen because it is the main soybean protein product used in animal feed. Defatted meal (flour) was prepared because this is the starting material for a large number of soybean protein products used in food. Protein isolate and protein concentrate were manufactured from the defatted meal due to the food use of these two fractions. In addition, crude lecithin and refined, bleached deodorized oil were manufactured. Therefore, while we focused on the seeds, we also obtained data on several of the most important processed commercial soybean products. The data from line 40-3-2 seed, along with data from the processing studies using a composite of that seed, are graphically summarized below. The soybean seeds and processing fractions were analyzed for compositional quality characteristics under GLP guidelines by Ralston Analytical Laboratories (RAL), St. Louis, MO.

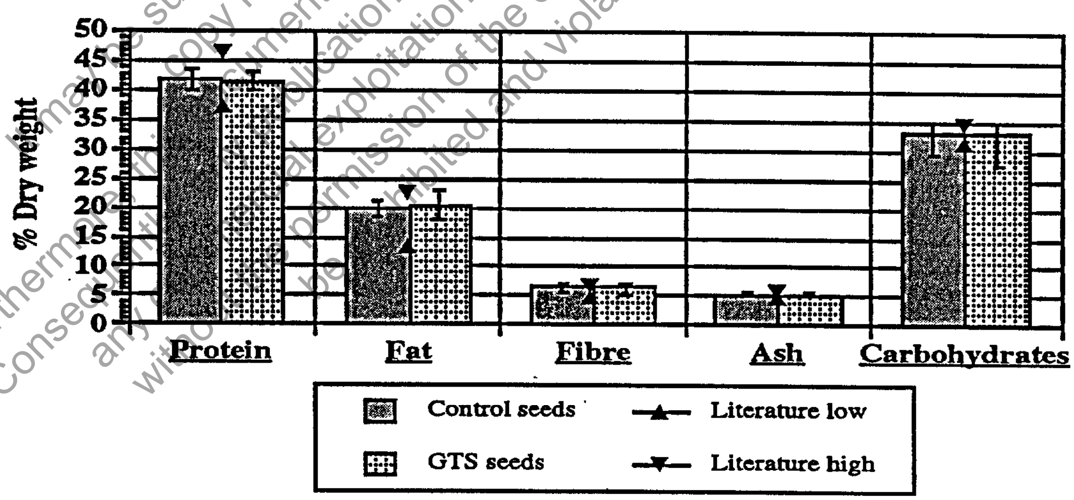
i. Proximate analysis:
Compositional (proximate) analyses were performed on the soybean seeds from GTS line 40-3-2 and the control soybeans. Appendix A provides additional information on the analytical methods utilized. The results, expressed on a dry-weight basis, are shown in Figure 10. Components measured were protein, fat, moisture, fiber, and ash. The carbohydrate content was derived by calculation. The data summarized in Figure 10 show that the levels of these

Figure 10. Proximate analysis of soybean seeds (25). Bars represent the means of seeds from nine field sites, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the specific product components (71,79-81).



*Significantly different from control line at 5% level (protected LSD).

Figure 11. Proximate analysis of soybean seeds from second year field tests (78). Bars represent the means of seeds from four field sites, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the specific product components (71,79-81).



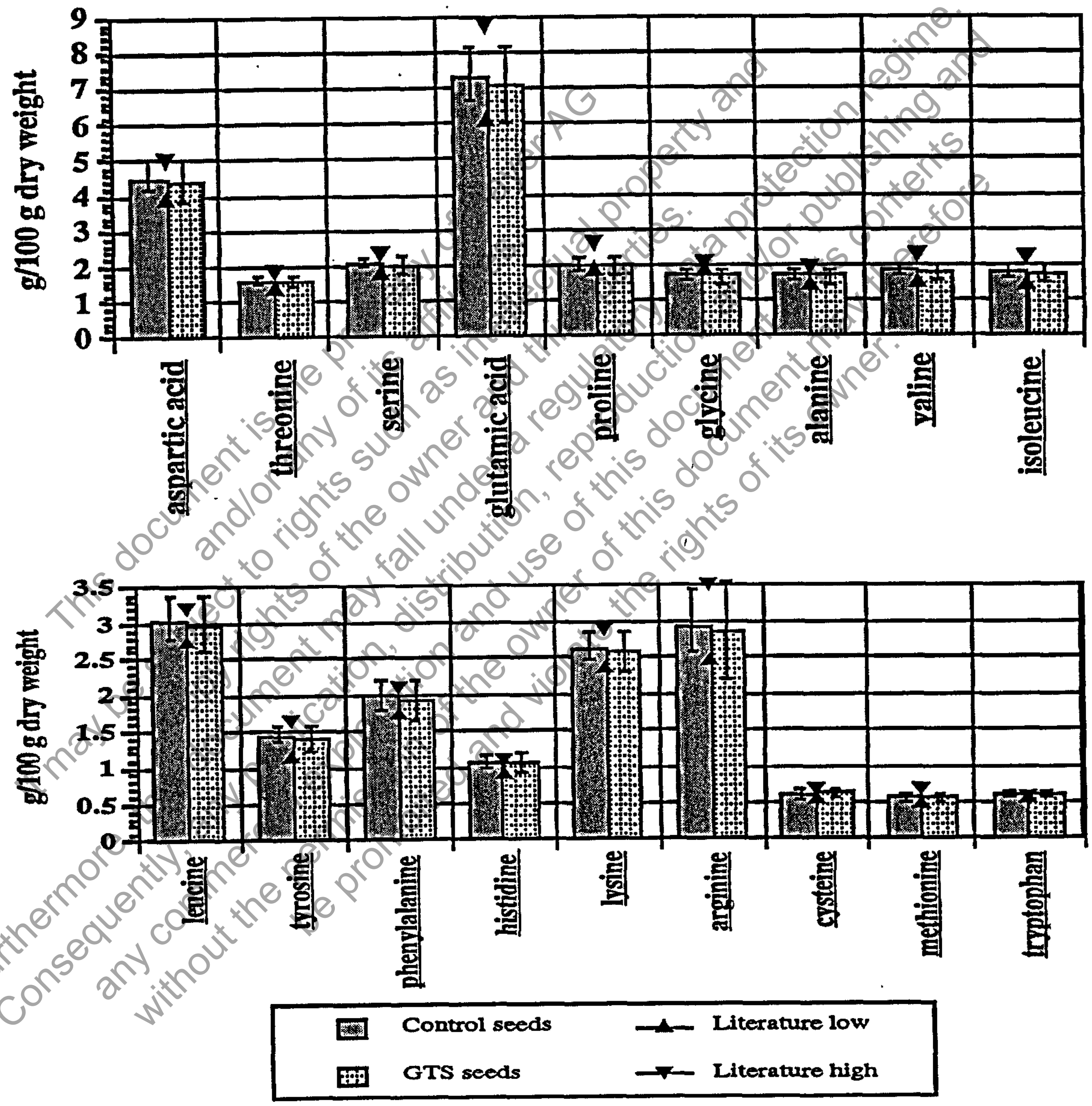
*No significant differences from the control line were observed at the 5% level (protected LSD).

components in GTS were not materially different than the levels in the parental control. It should be pointed out that the mean seed carbohydrate level in this nine-site experiment was 37.1% dry weight (25), which is greater than the literature ranges obtained (literature high was 34%). However, since the mean commercial soybean variety control carbohydrate level in the experiment was 38.1% dry weight, the GTS is indeed within the known range for carbohydrates in soybean seeds. In addition, for a one-site Puerto Rico study, there was no significant difference observed in the carbohydrate levels between the GTS line and the control (26). Additional proximate data was also obtained from a second-year GTS field test utilizing four field sites (Figure 11). No statistically significant differences in protein, fat, fiber, ash, or carbohydrate levels were observed for GTS line 40-3-2 relative to the control line in that second year study, and all means obtained were within literature ranges. The data obtained therefore support the conclusion of no material difference with respect to the proximate composition of soybean seeds from GTS.

ii. Amino acid analysis:
The mean amino acid composition of the soybean seeds, and the range of values observed, is given in Figure 12. Of the 18 amino acids measured, there were no statistically significant differences in the levels of any of the amino acids, including aromatic amino acids, between the GTS seeds and the control soybean seeds (25). This is in accord with our expectation that EPSPS is not the rate limiting step in aromatic amino acid biosynthesis in bacteria and plants (82).

The shikimate pathway is of central importance in metabolism (11,83,84) because it is estimated that one-fifth of all of the carbon fixed by plants is subsequently channelled through this pathway (11). Extensive studies have been performed on the regulation of the shikimate pathway (85). Data supports the conclusion that the carbon flow through the shikimate pathway is controlled at the first step of the pathway by modulation of the activity of DAHP synthase (82,85). The next steps of the pathway from DAHP to the synthesis of chorismic acid are not expected to be under feedback control. In fact, enzymes of this part of the pathway have often been found to be only slightly inhibited or repressed by later intermediates or endproducts (85,82). Therefore, all evidence available suggests that EPSPS is not a regulatory enzyme. Increased EPSPS activity would not be expected to increase the level of aromatic compounds in plants. In fact, it is known that plant cells that produce 40-times more EPSPS than wild-type cultures do not over-produce aromatic amino acids (86). The data presented in Figure 12 on GTS line

Figure 12. Amino acid analysis of soybean seeds (25). Bars represent the means of seeds from nine field sites, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the specific product components (81,87). Several literature values were calculated by converting g amino acid / 100 protein to g amino acid / 100 g sample by using the mean protein concentration of the seeds analyzed, 41.5%.

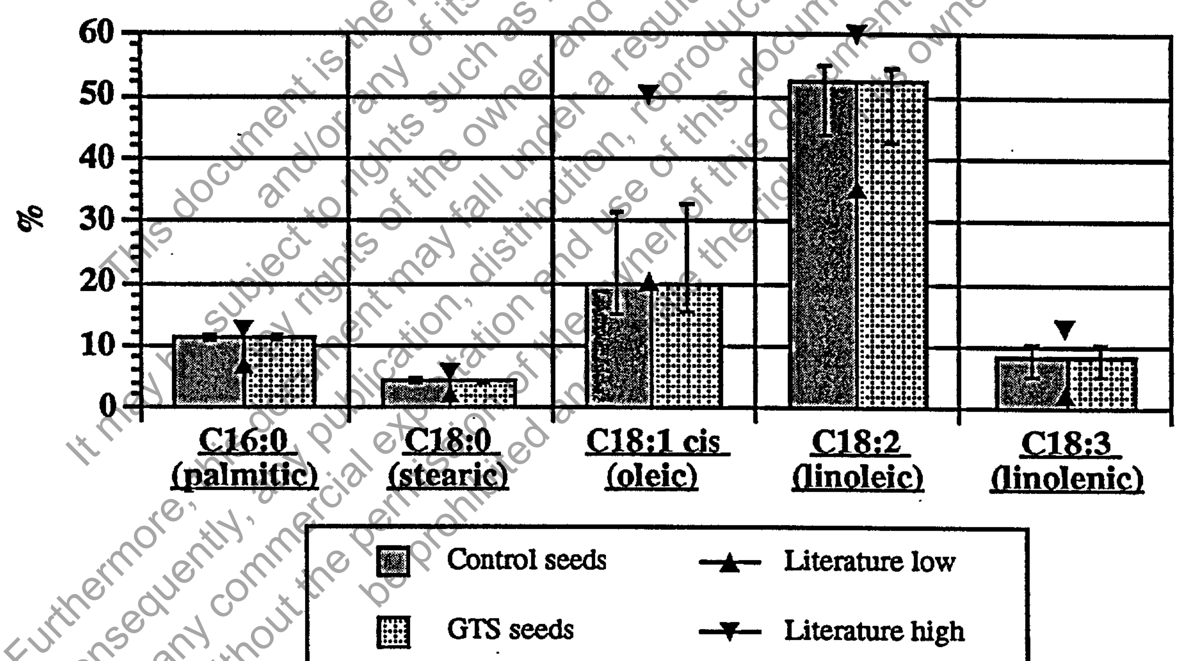


*No significant differences from the control line were observed at the 5% level (protected LSD).

40-3-2 is thus in accordance with literature expectations: no statistically significant increase in the aromatic amino acids tyrosine, phenylalanine, or tryptophan (or any other amino acid) accompanies the presence of the CP4 EPSPS gene and enzyme in GTS line 40-3-2.

iii. Fatty acid analysis:
The fatty acids of the soybean seeds were measured, and the means and ranges of the most abundant fatty acids are given in Figure 13. There was only one statistically significant difference in seed fatty acid composition between GTS and the control soybeans; this was for C22:0 fatty acids (data not shown), which represents a relatively minor proportion (less than 0.6%) of the fatty acid fraction. The values, however, were within the ranges reported in the literature for soybeans.

Figure 13. Fatty acid analysis of soybean seeds (25). Bars represent the means of seeds from nine field sites, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the specific product components (88).

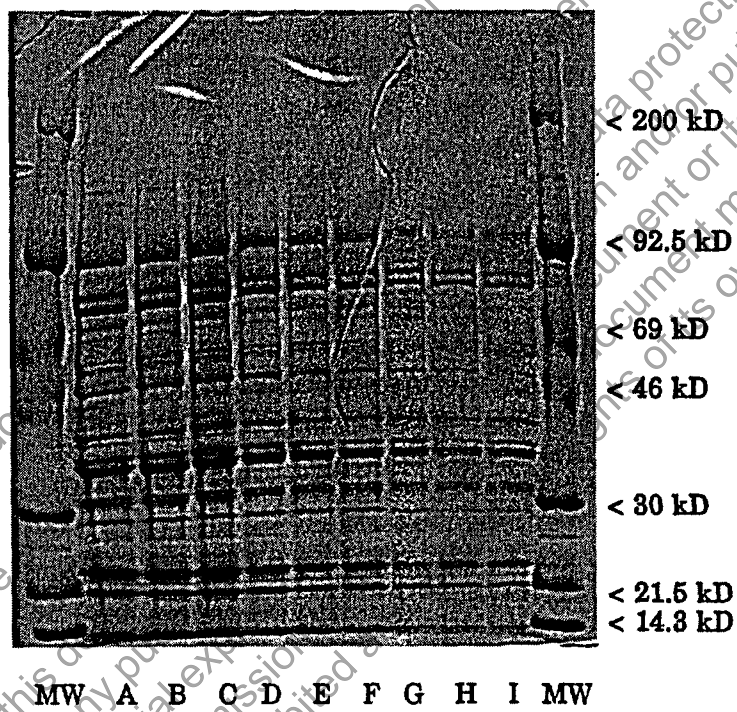


*No significant differences from the control line were observed at the 5% level (protected LSD).

iv. Soybean seed proteins:
It was of interest to compare the soybean seed storage protein profile of GTS with the control soybeans. This was accomplished by extracting protein from pools of seeds from GTS and control soybeans as described for the ELISA assays (89), followed by analysis using SDS-PAGE with Coomassie blue

staining. As shown in Figure 14, there were no discernable differences in the seed protein profiles between the GTS and the control soybeans. These results indicate that the protein compositions of the seeds of GTS are not materially different from that of the control soybeans. The line 61-67-1 referenced in Figure 14 is a second GTS line included in most safety assessment studies but which is not being pursued commercially.

Figure 14. Coomassie stained SDS-PAGE of soybean seed proteins (25). Pools of seeds from lines A5403, 40-3-2, and 61-67-1 were extracted, digested in SDS / ß-mercaptoethanol, and separated by SDS-PAGE on 4-20% gradient gels). The gel was stained with Coomassie Blue, destained, and dried.



Lane	Identity
MW	Molecular weight standards
A	Line A5403 seed extracts, 25 µg
B	Line 40-3-2 seed extracts, 25 µg
C	Line 61-67-1 seed extracts, 25 µg
D	Line A5403 seed extracts, 12 µg
E	Line 40-3-2 seed extracts, 12 µg
F	Line 61-67-1 seed extracts, 12 µg
G	Line A5403 seed extracts, 6.2 µg
H	Line 40-3-2 seed extracts, 6.3 µg
I	Line 61-67-1 seed extracts, 6.2 µg
MW	Molecular weight standards

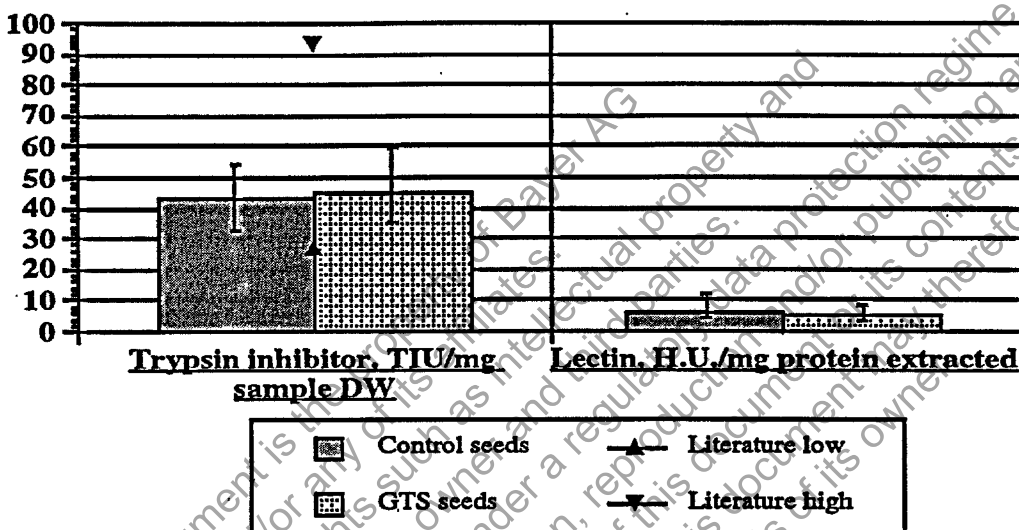
v. Trypsin inhibitor:
Intensive research has been performed on soybean trypsin inhibitors (TI), due to the contribution of trypsin inhibitor to the anti-nutritive activity of unheated soybean products (62,90). Anderson *et al.* (91) has stated that "both the conversion of raw soybeans into products with excellent protein quality and the elimination of the hypertrophic pancreas effect result from the simultaneous destruction of trypsin inhibitors and the transformation of raw protein into a more readily digestible form". Therefore, it was of interest to determine whether the glyphosate-tolerant soybeans had comparable trypsin inhibitor activity to the control soybeans.

Shown in Figure 15 is the mean trypsin inhibitor levels determined for the glyphosate-tolerant and control soybean seeds. There were no statistically significant differences in trypsin inhibitor between GTS and the control soybeans.

vi. Lectin analysis:
Proteins which agglutinate red blood cells are distributed widely in plants (62). An early report indicated that soybean hemagglutinin, sometimes called soybean lectin, accounted for about 25% of the growth inhibition seen for raw soybean meal in the rat (92). Although it has since been concluded by some that soybean hemagglutinin does not play any major role as a determinant of the nutritional quality of soybean protein (93), other authors still believe that circumstantial evidence exists that soybean lectin may make an appreciable contribution to the growth inhibition of rats caused by dietary proteins in uncooked soybean meal (94). However, there is no evidence of agglutination of red cells upon ingestion of hemagglutinins, presumably due to inactivation by pepsin in the stomach (81). Since the role of soybean hemagglutinin in nutrition is still somewhat open to speculation (62), it was determined whether GTS had comparable activities of soybean hemagglutinin relative to the control soybeans when measured in a rabbit red blood cell assay.

There were no statistically significant differences in the lectin activity between GTS and the control soybeans (Figure 15). Levels of lectin activity were found to be very low in the soybean seeds (less than 7 hemagglutinating units [H.U.] / mg extracted protein). Due to the variability in red blood cell lots, it is very difficult to compare these results to literature values for hemagglutinating proteins in soybean. However, since a positive control of soybean lectin yielded readings of 461-541 H.U./mg total protein (26), there is no reason to believe that the assay was not able to successfully detect lectin activity. Clearly, however, the assay results obtained in this experiment were significantly lower than previously reported for soybean (60-426 H.U./mg protein) (95). The main point to make regarding the lectin seed data is that the GTS had a similar quantity of lectin activity to the control soybeans.

Figure 15. Trypsin inhibitor and lectin analysis of soybean seeds (25). Bars represent the means of seeds from nine field sites, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the seed trypsin inhibitor, calculated from reference (95) using an estimate of 40% protein in soybean seed. As mentioned in the text, lectin values obtained in this study were not comparable to literature values; hence no literature ranges are shown.



*No significant differences from the control line were observed at the 5% level (protected LSD).

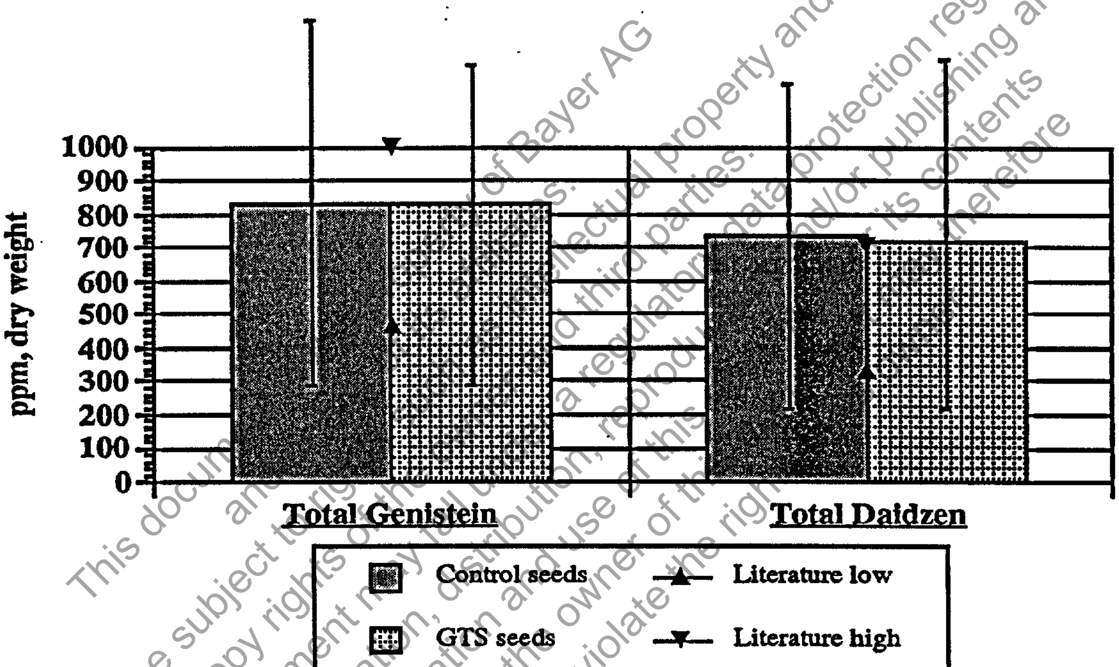
vii. Isoflavone analysis:

The isoflavones genistein, daidzein, and coumestrol are naturally present in soybeans, and have been reported to possess a number of biochemical activities in mammalian species, including estrogenic and hypocholesterolemic activities (96,97). It has been postulated that these compounds may contribute to deleterious health effects of animals fed soybean meal (98). In light of these reports, we determined whether GTS had comparable levels of genistein, daidzein, and coumestrol, relative to the control soybeans.

The isoflavones genistein and daidzein were determined in soybean seeds for both the total and free forms. Analyses were also performed for bound coumestrol and biochanin A. However, only minute quantities of biochanin A were detected, and the bound coumestrol was lower than the confidence limit of the assay (10 ppm) (26). No statistical differences in any of the isoflavones measured were detected between GTS and the control soybeans. Data for total genistein and daidzein are presented in Figure 16. It can be seen that there is an extremely large range of experimental values obtained for genistein and daidzein; this large site-to-site variability can be attributed to the effect of environmental influences on the formation of these compounds. Note that it has been concluded by one author that the absence of reports on estrogenic

responses in humans consuming soybean protein products suggests that there is no problem in humans [regarding estrogen activity] when practical levels of soy are ingested (70).

Figure 16. Genistein and daidzein analysis of soybean seeds (25). Bars represent the means of seeds from nine field sites, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the compounds (96,99).



*No significant differences from the control line were observed at the 5% level (protected LSD).

c. Compositional analyses of GTS line 40-3-2 processing fractions

Several important soybean processing fractions were manufactured from GTS and the control (parental) soybeans. The compositions of the processed fractions (including % protein, % fat, % fiber, % ash, % carbohydrates in the protein fractions; fatty acid analysis of oil; and crude lecithin composition) were investigated. The protein samples prepared were: toasted meal (defatted), defatted meal (non-toasted), protein isolate, and protein concentrate. In addition, refined bleached deodorized soybean oil and crude lecithin were also prepared. Toasted meal was chosen because it is the main soybean protein product used in animal feed. Defatted meal (flour) was made because that is the starting material for a large number of soybean protein products used in food (Figure 8). Protein isolate and protein concentrate were manufactured from the defatted meal due to the food use of these two fractions.

GTS line 40-3-2 and the control variety A5403 were manufactured into processed fractions under GLP guidelines at the Engineering and Biosciences Research Center of Texas A & M University in the United States, in three separate experiments. The processes used were designed to mimic commercial processes as much as possible, although the scale was much smaller than in commercial processing plants, which dictated that non-commercial equipment was utilized. Nevertheless, during this small-scale operation, the processibility of GTS was comparable to that of the control soybean variety. This was confirmed by fraction yields (25,26,77) and composition (see below) (25,26).

In some cases the values reported for certain components do not fall within ranges cited in the literature for soybean products. These results are to be expected, considering that the processing was conducted on an experimental scale (40-730 Kg), rather than on a commercial scale (> 900 tons / day for toasted soybean meal [(71)]). Therefore, for these small scale GTS processing fractions, the most important comparison is to the fractions from the parental soybean controls.

i. Nutrient analysis of protein processing fractions

As can be seen from the data presented in Figures 17 through 25, the composition of the processing fractions derived from GTS was not materially different from comparable fractions derived from the control soybeans. The levels of macronutrients (protein, ash, fat, fiber, carbohydrate) in toasted meal (Figure 17), non-toasted meal (Figure 18), protein isolate (Figure 19), and protein concentrate (Figure 20) in GTS were comparable to the levels in the parental soybean controls. Likewise, the levels of the major fatty acids (Figure 21) and crude lecithin components (Figure 22) in refined, bleached, deodorized soybean oil were comparable in GTS and control soybeans.

ii. Analysis of anti-nutrients in toasted soybean meal

Toasted soybean meal was prepared to confirm that: 1) GTS behaved similarly to the control soybeans in the dehulling, defatting, and toasting process (25,77); 2) to confirm that the trypsin inhibitor (and urease) levels were reduced in the GTS comparably to the control soybeans, at equal processing levels; and 3) to measure additional anti-nutritional components in soybean meal, namely phytate (100), stachyose, raffinose, lectins, and isoflavones. For the lectin and trypsin inhibitor data summarized below, data from one processing study (Puerto Rico) (26) was not considered, since that batch of toasted meal was incompletely cooked (26).

Figure 17. Proximate analysis of toasted meal (25,26,77). Bars represent the means of three processing studies, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the specific product components (71,79,81,87,101,102).

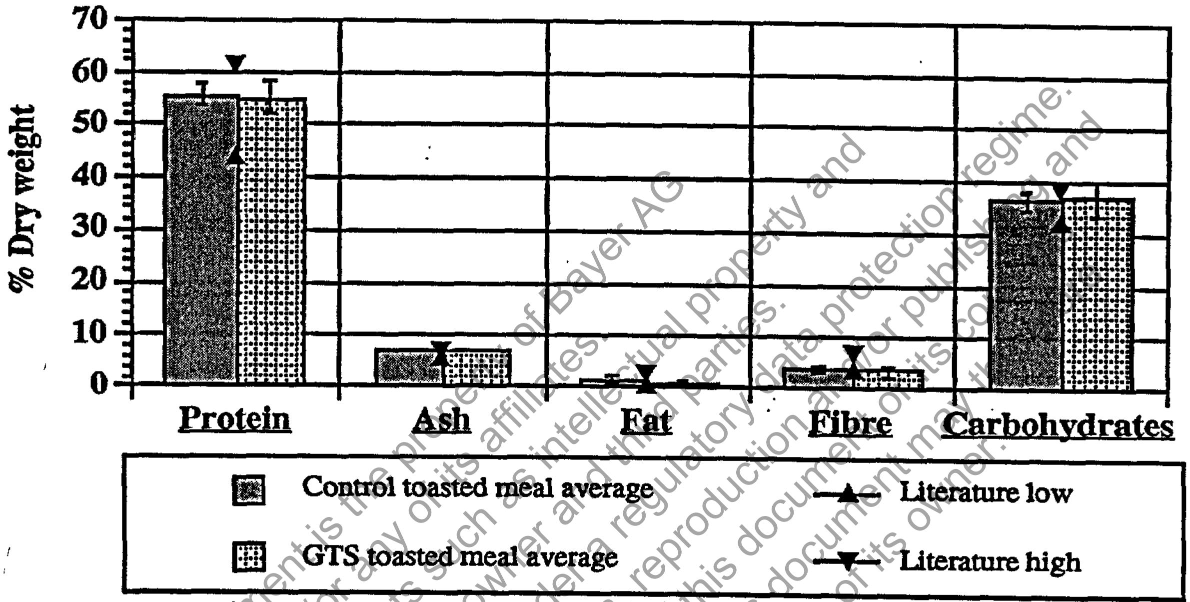


Figure 18. Proximate analysis of non-toasted meal (25). Triangles represent high and low literature values for the specific product components (61,70,72,101,103).

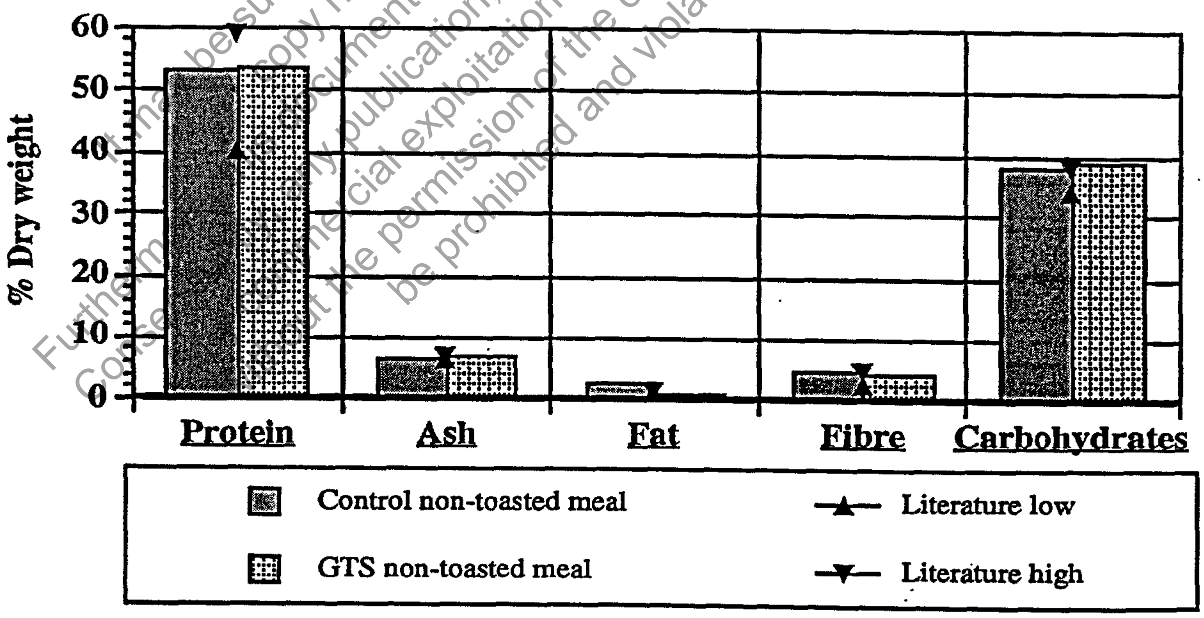


Figure 19. Proximate analysis of protein isolate (25). Triangles represent high and low literature values for the specific product components tested (61,64,70,103,104). Note that fiber values were reported as <0.2 %

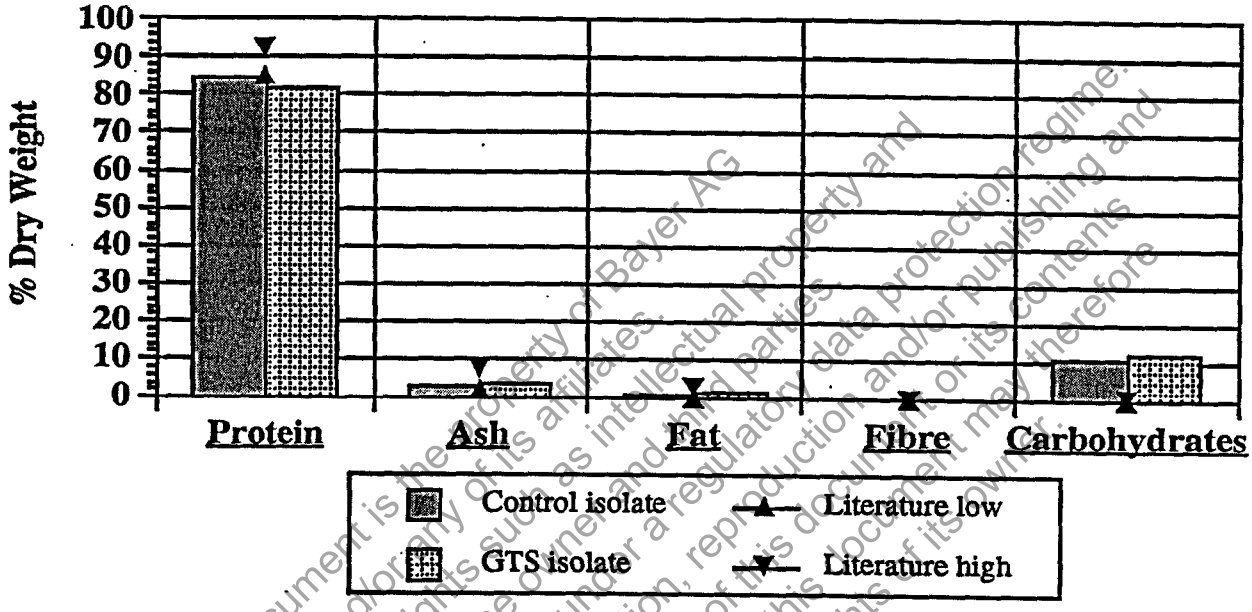


Figure 20. Proximate analysis of protein concentrate (25). Triangles represent high and low literature values for the specific product components (70,103,105-108).

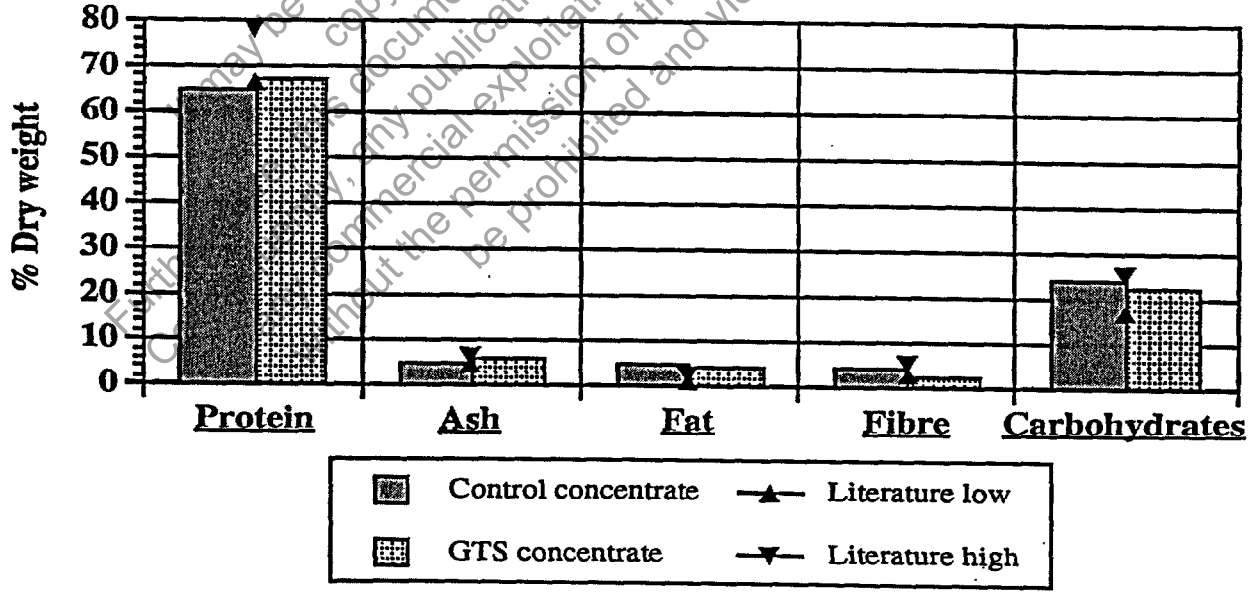


Figure 21. Fatty acid analysis of refined, bleached, deodorized soybean oil (25,26). Bars represent the means of two processing studies, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the specific product components (88).

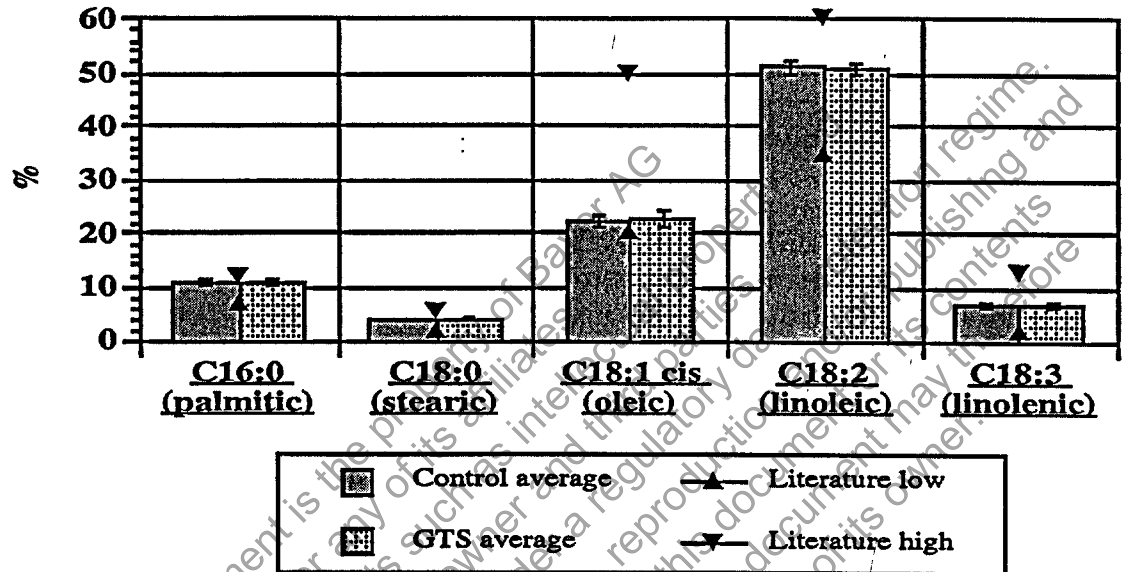
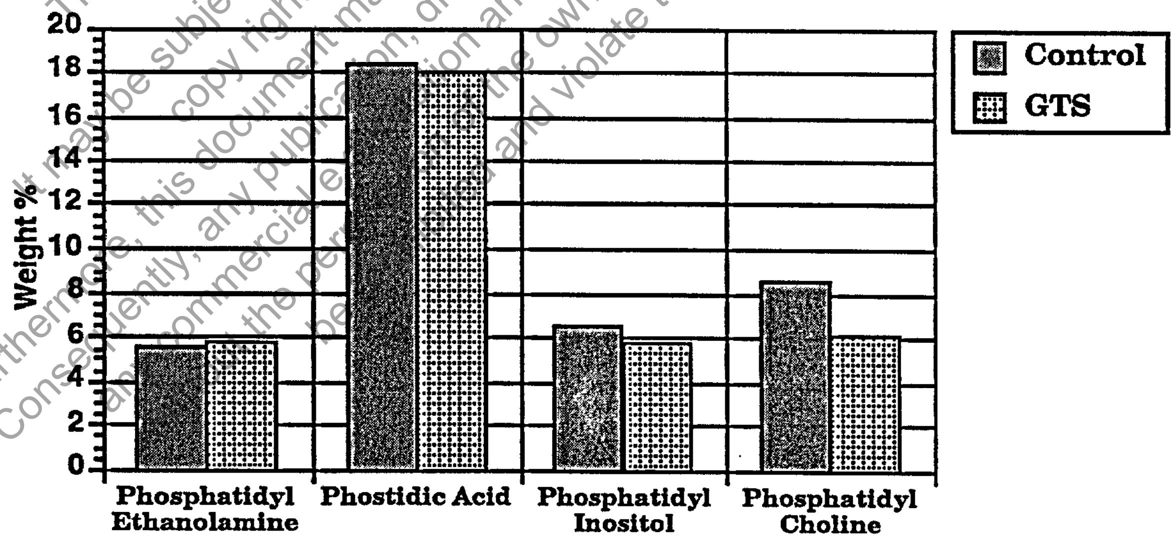


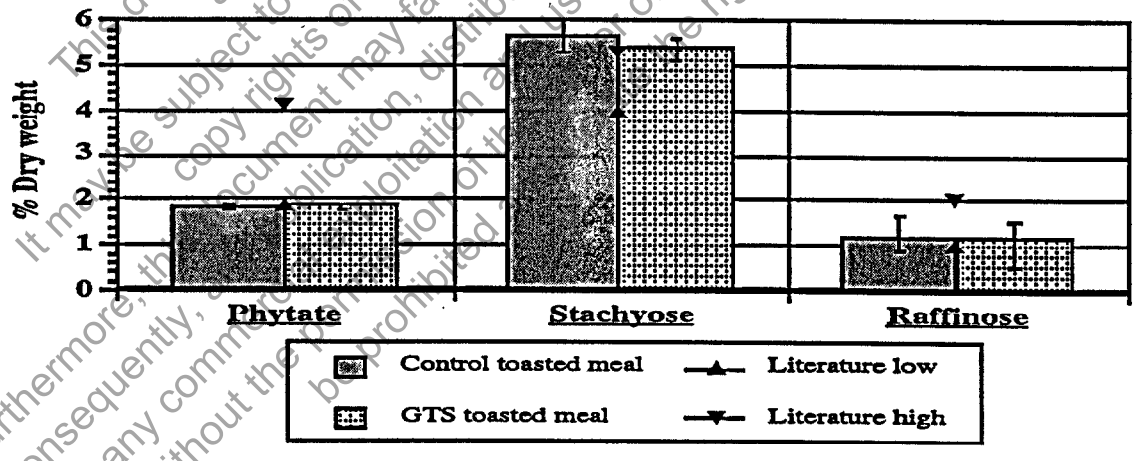
Figure 22. Crude lecithin analysis of refined, bleached, deodorized soybean oil (25). Literature values were not available for the components of this crude lecithin fraction.



iii. Stachyose and raffinose in toasted meal:
Flatus activity is a well-known characteristic of soybean products. The gas-producing factors reside mainly in the low molecular weight carbohydrates, which exist primarily as raffinose and stachyose (62,109). The flatus activity of stachyose in rats is much greater than with raffinose (62). We therefore assessed whether the glyphosate-tolerant soybean meal had comparable levels of stachyose and raffinose relative to the control soybean meal. As shown in Figure 23, the levels of stachyose and raffinose in GTS and control soybeans are not materially different.

iv. Phytate in toasted meal:
Phytic acid (phytate) is the hexaphosphoric acid derivative of inositol, and exists mainly in soybeans as an insoluble, non-nutritionally available calcium-magnesium-potassium complex (81,100). Since phytate is involved in mineral availability, it was of interest to compare phytate levels in the glyphosate-tolerant soybean meal with the control soybean meal. As shown in Figure 23, the phytate concentration in toasted GTS meal is not materially different than control soybean toasted meal.

Figure 23. Phytate, stachyose, and raffinose analysis of toasted meal (25,26). Bars represent the means of three processing studies, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the specific product components (62,100,106).

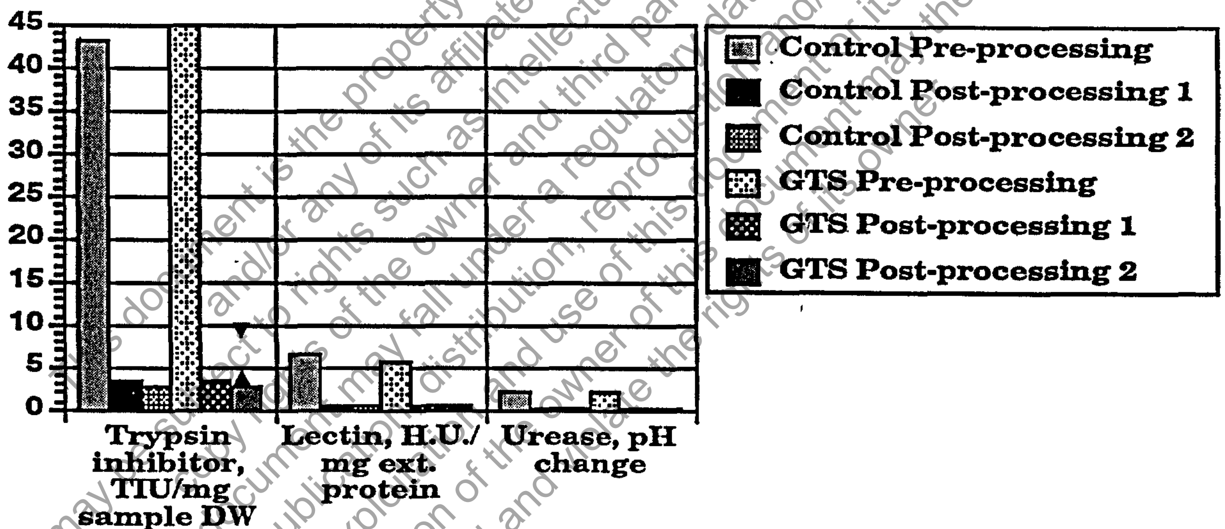


v. Trypsin inhibitor (TI) and urease in toasted meal:
As described above, trypsin inhibitors are known to be antinutrients in unheated soybean products (62,90). Processing soybean protein significantly inactivates trypsin inhibitor, so the level of trypsin inhibitor in the toasted soybean meal made from GTS and control soybeans was measured. Urease activity is commonly measured to assess the effectiveness of the toasting process, since the urease activity reduction parallels the inactivation of trypsin inhibitor upon moist heating (110). Therefore, urease was also

measured in the toasted meal fractions, and the activity compared to the seed urease levels.

As shown in Figure 24, the toasted meal batches from GTS and control soybeans were fully processed, based on the significant reduction in both trypsin inhibitor and urease activity of the toasted meal relative to the seed. The trypsin inhibitor levels in the toasted meal lots analyzed in this study are all comparable to or lower than the values reported in the literature (24,62,87,90).

Figure 24. Trypsin inhibitor, urease, and lectin analysis of toasted meal (25). Bars represent the results of two processing studies. Triangles represent high and low literature values for the specific product component (62), where available. Lectin levels after processing were < 0.5 H.U. / mg extracted protein.

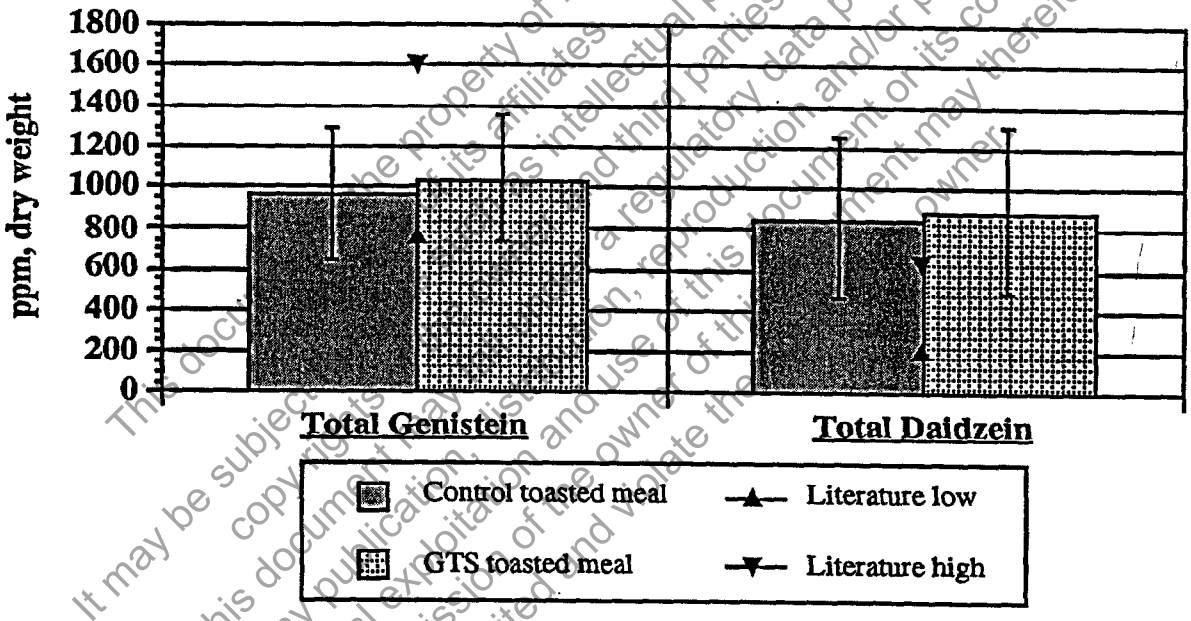


The results present herein clearly show that the GTS is not materially different from the control soybeans and that commercially acceptable toasted meal can be prepared from the GTS.

vi. Lectins in toasted meal:
The levels of lectins in the toasted meal samples were below the detectable limits in the toasted meal samples. Although as described previously, the seed lectin values measured were lower than reported in the literature, these results do show that toasting does significantly inactivate lectin activity, in both the GTS and control lines (Figure 24). This result parallels literature reports that lectin activity is substantially reduced upon moist heating (109).

vii. Isoflavones in toasted meal:
As shown in Figure 25, the isoflavones measured for soybean seeds were also measured in the toasted soybean meal batches. The GTS toasted meal batches were not materially different than the control soybean toasted meal. It has been previously noted (70) that isoflavones remain active in commercially processed soybean meal, and thus are stable to moist heat. The results presented here support this observation.

Figure 25. Genistein and daidzein analysis of toasted meal (25,26,77). Bars represent the means of three processing studies, and the thin lines represent the ranges of experimental values obtained. Triangles represent high and low literature values for the specific product component (96,97,99).



d. Summary of compositional analysis
A summary of the results of the compositional analyses performed with seeds and toasted meal is given in Table 2. The results of all analyses (> 1400 individual assays) show that GTS seeds and the processed fractions (toasted meal, defatted meal, protein isolate, and protein concentrate) are not materially different from the control soybean seeds or fractions. It is also important to point out that processing (to toasted meal) inactivated trypsin inhibitors and lectin in GTS, as expected. The levels of these antinutrients in GTS toasted meal were not materially different from the levels in the control soybean toasted meal. Based upon this compositional information alone and the criteria provided in the Food Policy (1), we believe that we have established the identity and safety of GTS line 40-3-2.

Table 2. Summary of compositional analyses performed on GTS line 40-3-2 (25)

Component	GTS line 40-3-2 seeds	GTS line 40-3-2 toasted meal
Proximate analysis	NMD	NMD
Amino acid composition	NMD	NA
Fatty acid composition	NMD	NA
Trypsin inhibitors	NMD	NMD
Lectins	NMD	NMD
Isoflavones	NMD	NMD
Stachyose, raffinose	NA	NMD
Phytate	NA	NMD

NMD = not materially different from the control
NA = not analyzed

e. Confirmatory animal feeding studies and comparison of feed efficiencies

Insertion of the CP4 EPSPS gene into soybeans was not expected to affect wholesomeness (ability to support typical growth and well-being). However, to provide additional data to establish that GTS line 40-3-2 is not materially different from the parental soybean variety as an animal feed, several animal feeding studies were performed under GLP guidelines. These studies, which were not designed as toxicology tests, were undertaken to determine whether there were any material differences in the wholesomeness of GTS line 40-3-2 compared to the parental line, A5403.

The animal studies included a four week rat feeding study with processed soybean meal, since rats serve as an indicator for the safety of consumed products. Processed soybean meal was utilized in this study since the majority of soybeans used for human food and animal feed is processed by heat treatment. Rats were also fed unprocessed soybean meal for four weeks; here rats serve as a surrogate for wild mammals that may eat soybeans in the field. This would be a worst-case test of wholesomeness, as antinutrients naturally present in soybeans would not have been removed by processing. As a rigorous test of wholesomeness, broiler chickens were fed processed soybeans for 6 weeks as a growth study. Note that chickens consume approximately 49% of soybeans fed to farm animals (Figure 7). Raw (cracked) soybeans were also fed to dairy cattle for 4 weeks, since raw soybeans are normally fed to ruminants as a source of protein. Catfish were fed processed soybean meal for 10 weeks using a test designed to assess the wholesomeness of new fish diets, since soybean meal is used in diets for commercial aquaculture. Lastly, unprocessed soybean meal was fed for 5 days to bobwhite quail, since birds may feed on soybeans left in the field after harvest.

Since the purpose of the feeding studies was to determine if there were material differences in the wholesomeness of GTS line 40-3-2 compared to its parental control (A5403), the feed efficiencies (feed consumed/weight gain) for both lines of soybeans were summarized and compared across studies (Table 3). The quail (bobwhite) study is not included in this comparison due to the short duration of the study (5 days). No statistically significant differences in feed efficiencies were observed when GTS line 40-3-2 was used as a feed source compared to the parental variety, A5403 (Table 3). These results are consistent with the extensive compositional analyses which demonstrated that line 40-3-2 soybeans are not materially different from the control soybeans.

Presented below are summaries of the individual animal feeding studies performed to assess the wholesomeness of GTS line 40-3-2. Additional information on the animal feeding studies is supplied as Appendix B.

i. Rat 4 week feeding study with processed soybean meal
The wholesomeness of processed soybean meal from glyphosate-tolerant line 40-3-2 was not materially different from that of the parental line A5403 when fed in the diet of rats at 24.8% (w/w) for 4 weeks (111).

Soybean meal is included in commercial rodent chow as a source of protein at an incorporation rate of approximately 25% w/w. Since the body weight of an 8 week old male rat increases approximately 70% in 4 weeks, rodent chow provides a sufficient amount of nutrients, including protein, to support rapid growth. To assess the wholesomeness of GTS, processed meal was completely substituted for commercially derived processed soybean meal in the rodent diet and fed to rats.

There was no mortality in the study and test animals appeared healthy. No statistically significant differences were observed in body weight, cumulative body weight gain, organ weights, or food consumption between rats fed diets containing processed soybean meal from the parental line and the glyphosate-tolerant line. In summary, the wholesomeness of processed soybean meal from glyphosate-tolerant line 40-3-2 was not materially different from that of the parental line A5403 when fed in the diet of rats at 24.8% (w/w) for 4 weeks.

ii. Rat 4 week feeding study with unprocessed soybean meal
Based on the results of feeding high levels of unprocessed soybean meal to rats, it was concluded that the wholesomeness of unprocessed soybean meal from the glyphosate-tolerant line 40-3-2 was not materially different from that of the parental line A5403 (112).

Table 3. Comparison of feed efficiencies across feeding studies

Line	Mean Feed Consumption (gm/animal)	Mean Feed Efficiency	Mean Wt. gain (gm)	Mean Final Wt. (gm)
Rat Feeding Study (4 weeks) Processed soybeans* (111)				
Males				
Negative Control	811	4.58	177a	426a
Control	764	4.63	165a,b	415a,b
GTS	749	4.86	154b	403b
Females				
Negative Control	549	8.23	66.7	256
Control	538	7.87	68.4	259
GTS	538	8.78	61.3	252
a,b Means with different letters are statistically different, p<.05				
Rat Feeding Study (4 weeks) Unprocessed Soybeans* (112)				
Males				
Negative Control	753	6.55	115	431
Control 5%	755	7.26	104	421
Control 10%	769	7.25	106	424
GTS 5%	750	7.35	102	420
GTS 10%	768	6.86	112	430
Females				
Negative Control	510	12.6	40.6	241
Control 5%	493	16.3	30.2	231
Control 10%	513	13.9	36.8	238
GTS 5%	502	13.9	36.2	237
GTS 10%	491	14.2	34.6	236

* Feed efficiencies were calculated by dividing the total food consumed over the study/group by the total body weight gained/group. Feed efficiencies were calculated each week/group and analyzed statistically. There were no statistically significant differences observed between groups (data not shown).

Line	Mean Feed Consumption (gm/animal)	Mean Feed Efficiency	Mean Wt. gain (gm)	Mean Final Wt. (gm)
Chicken Battery Study (6 weeks) Processed soybeans (113)				
Combined Sex - No statistically significant differences were observed, P<.05				
Control	3893	1.816	2147	2193
GTS	3844	1.832	2099	2144
Catfish Study (10 week) - Processed soybeans (114)				
Mixed Sex -No statistically significant differences were observed, P<.05				
Control	22.1	1.12	19.7	22.6
GTS	21.8	1.17	18.8	21.8

Four Week Dairy Cow Feeding Study - least squares means (115)

Line	Milk, kg/day	Fat, %	3.5% Fat-corrected milk (FCM), kg/day	Net energy intake, mcal NE_L/day	FCM/NE_L, kg/mcal
Control	34.9	3.37	34.1a	40.1	0.81
GTS	36.2	3.59	36.8b	42.9	0.88

a,b Values with unlike superscripts are statistically significant (P<.05)

This rat feeding study was undertaken with raw, unprocessed soybeans to assess the wholesomeness of soybeans that may potentially be eaten in the field by wild mammals. Prior to initiating the study with the GTS line, a pilot study with commercially available parental line soybeans was performed to investigate the feasibility of feeding high levels of unprocessed soybeans to rats (116). There were no adverse findings in this pilot study.

Diet incorporations of 5% and 10% unprocessed soybean meal were used for the GTS study (112). A negative control of commercial rat chow containing processed soybean meal was also included. There was no mortality in the study, and all animals appeared healthy. No statistically significant differences in body weight, cumulative body weight gain, absolute and relative organ weights, or food consumption were observed between rats fed the parental line and glyphosate-tolerant line 40-3-2 unprocessed soybean meal. There were no gross pathologic findings at necropsy that were considered adverse. Dark livers were observed in some animals in all groups at necropsy, but this effect was not considered adverse because: 1) there was no difference between the incidence of dark livers in rats fed the parental and 40-3-2 lines at the highest dose; 2) no dose response was observed for the 40-3-2 line; and 3) there was no effect on absolute or relative liver weights. Additionally, the occurrence of dark livers was not reproducible because they were not observed in the pilot study with the parental line (116).

In summary, the wholesomeness of unprocessed soybean meal from glyphosate-tolerant line 40-3-2 was not materially different from that of the parental line A5403 when fed in the diet of rats for 4 weeks.

iii. Broiler chicken 6 week feeding study with processed soybean meal

The data from a 6 week grower study demonstrated that there were no material differences in the ability of glyphosate-tolerant processed soybean meal to support growth and livability of broiler chickens when compared to meal made from the parental line of soybeans (113).

Rapidly growing broiler chickens were used to compare the wholesomeness of GTS with control (parental line) soybeans. Commercial broilers, as a consequence of genetic selection, reach a market weight of around 2 kg in 6 weeks (from hatch). Considering that the birds weigh around 30 gm when hatched, this amounts to a 66-fold increase in body weight over 6 weeks. To accommodate this rapid growth, there are considerable nutritional requirements, including protein, which must be provided by the diet. Any deficiencies in the protein source would be manifested by growth retardation in the growing broiler. This test was considered to be rigorous, as small differences in body weight gain (3.5%) or feed/gain (2%) could be detected between soybean lines (113). Thus, the growing broiler is a very sensitive test species to detect differences in nutrient quality and was used to assess the

wholesomeness of processed meal from glyphosate-tolerant soybeans.

For the entire 6 week study period, there were no statistically significant differences between birds fed soybean meal from 40-3-2 or A5403 with respect to bodyweight, bodyweight gain, feed intake, feed/gain, or livability. As expected, males were heavier, consumed more feed, and had better F/G than females but had lower livability than females. There were no statistically significant differences between groups in breast muscle and fat pad weights either as total weights or as a percentage of body weights.

In summary, the data from this 6 week grower study demonstrated that there was no material difference in the ability of GTS processed soybean meal to support the growth and livability of broiler chickens, when compared to the parental A5403 line processed soybean meal.

iv. Dairy cow 4 week feeding study with raw soybeans
For nearly all of the parameters measured in the dairy cow feeding study, there were no material differences between the group fed the GTS line and the control (parental) line of soybeans (115). Fat-corrected milk production (FCM) was slightly higher for cows fed the GTS line which may be due to a higher but non-significant increase in net energy (NE_L) intake. The combination of these factors resulted in similar FCM/NE_L ratios for both groups. The lack of differences in apparent dry matter digestibility and nitrogen balances as well as ruminal fermentation products indicate that the nutritive value of GTS is not materially different from the control (parental) line.

Soybean meal and/or whole raw soybeans are normally fed to ruminants as a source of protein. Since dairy cows require adequate nutrition to support lactation, they were considered an appropriate test species to assess the wholesomeness of GTS. Ruminants, unlike monogastrics, can tolerate antinutrients found in raw soybeans.

Animal health was good throughout the study. Least square means for milk production (kg/day) were not statistically different for any of the groups. There was a small (2.5-2.7 kg/day) but statistically significant increase in 3.5% FCM for cows fed the glyphosate-tolerant line compared to cows fed the control (parental) line soybeans. Fat-corrected milk is calculated from milk production and milk-fat percentage, neither of which were different among groups in the study. Similarly, fat-corrected milk per unit energy intake was not affected by diet. Milk composition (protein, fat, lactose) was comparable for all groups. Dry matter and net energy intakes were similar for all groups and body weight changes were also not different. Apparent dry matter digestibility and indices of nitrogen balance were similar among the two groups. The source of soybeans had no apparent effects on rumen fermentation since the molar proportions of volatile fatty acids in rumen and rumen nitrogen were

comparable for all groups.

In summary, the lack of differences in apparent dry matter digestibility and nitrogen balances as well as ruminal fermentation products establish that the nutritive value of GTS line 40-3-2 is not materially different from the control (parental) line.

v. Catfish 10 week feeding study with processed soybeans
There were no material differences in the wholesomeness of catfish food containing GTS line 40-3-2 processed soybean meal and the parental line (A5403) meal (114). GTS meal from line 40-3-2 was considered acceptable for use in commercial catfish diets.

Channel catfish are raised commercially in large ponds and are fed diets containing processed soybean meal as a source of protein. During a 10 week period, fingerling catfish experience a 700% increase in growth. Since growth is a function of the nutrient profile of catfish diets, the [redacted], has used fingerling catfish maintained for 10 weeks in aquaria to test the nutrient quality of experimental catfish diets. Therefore, it was considered appropriate to assess the wholesomeness of processed soybean meal from GTS in this test system.

Feed efficiency (conversion) ratios were not significantly different between fish fed the control (line A5403) diet compared to those fed GTS line 40-3-2. Percentage weight gain and survival of fish fed the GTS line was not significantly different from fish fed the control diet. Based on three quantitative feedings (weeks 2,6,10), fish fed GTS line 40-3-2 consumed slightly less feed (2.85%) (expressed as a percentage of mean body weight) than fish fed the control diet (3.63%). Since there were no differences observed in the other parameters measured, the biological significance of this finding is doubtful and could be related to differences between meal or diet batches. Body composition data were not different when expressed on a wet weight basis. There were no differences in moisture, protein, fat, or ash in fillets generated from these fish regardless of dietary treatment. In summary, there were no material differences in the wholesomeness of catfish food containing GTS line 40-3-2 processed soybean meal compared to the parental line (A5403) meal.

vi. Quail (bobwhite) 5 day feeding study with raw soybean meal
Based on the results of the quail feeding study, it was concluded that the wholesomeness of raw meal from GTS line 40-3-2 was not materially different from that of the parental line A5403 when fed in the diet to quail (117).

Since birds such as quail may feed on soybean seeds left in the field after harvest, a test of the wholesomeness of glyphosate-tolerant varieties of soybean seed for quail was undertaken. Because young quail are traditionally

used in feeding studies, it was considered more feasible to feed quail raw soybean meal rather than intact seeds which are relatively large and difficult to ingest by small birds. Prior to initiating the study with the GTS line, a pilot study with commercially available soybeans was performed to assess the dose ranges appropriate for the GTS experiment (118). Based on the pilot study, a dose of 20% was used to compare the wholesomeness of the GTS line 40-3-2 and the A5403 control.

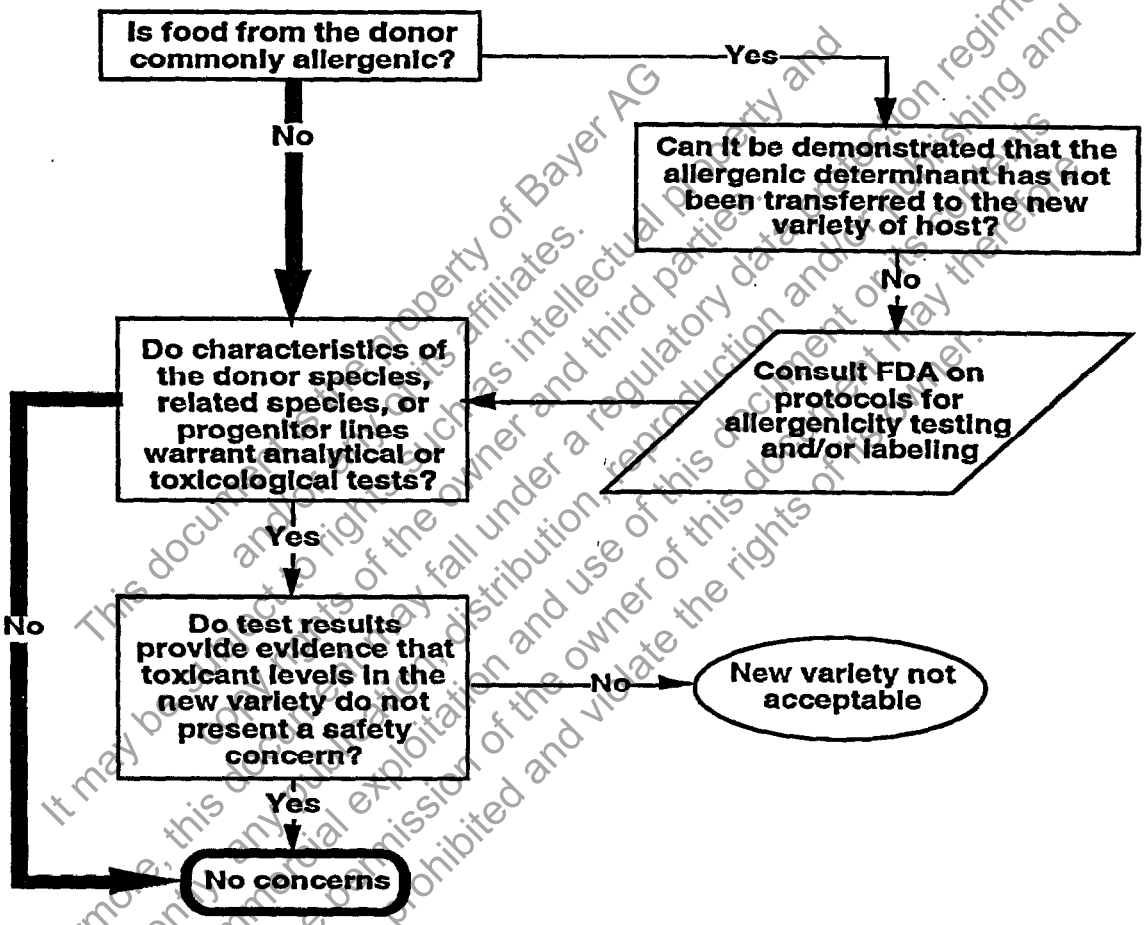
There was no mortality observed during the study in any of the groups tested. Body weight gain and food consumption were comparable for quail fed soybean meal from GTS line 40-3-2 and those fed the control (parental line) and basal diet. Consumption of a diet containing 200,000 ppm (20% of the diet w/w) of raw soybean meal is equivalent to eating 356 soybean seeds/kg body weight bird per day. In summary, the wholesomeness of raw meal from GTS line 40-3-2 was not materially different from that of the parental line A5403 when fed in the diet to quail.

2. Safety assessment of the donor organism, *Agrobacterium*
Shown in Figure 26 below is the flowchart from the FDA Food Policy concerning the safety assessment of the donor organism.

The safety of the donor organism, *Agrobacterium* sp. strain CP4, was considered. *Agrobacterium* sp. strain CP4 is not a food source but is related to microbes commonly present in the soil and in the rhizosphere of plants. Only one gene, the CP4 EPSPS gene, was transferred to produce GTS line 40-3-2. The sequence of the DNA transferred and of the protein produced are completely known. All of our plant, microbial, and fungal food sources contain EPSPS's; therefore, this enzyme and its activity are not novel to the food supply. Characteristics of the donor species, *Agrobacterium*, do not warrant analytical or toxicological tests since only the specific, sequenced gene encoding EPSPS was transferred to the host organism, soybean (49). These points, taken with the properties and safety of the CP4 EPSPS protein discussed below (including lack of typical profile for protein allergens), led us to a conclusion of "no concern" for the source of the donor gene.

3. Summary of the assessment of unintended effects
The absence of unexpected or unintended effects due to the CP4 EPSPS gene in GTS line 40-3-2 was demonstrated by: 1) classification of the donor organism as the common soil microorganism *Agrobacterium*; 2) establishing that the recipient organism, soybean, has a history of safe use; 3) extensive compositional analysis of GTS line 40-3-2, focusing on both nutrients and antinutrient compounds; and, 4) animal feeding wholesomeness studies in rat, broiler chicken, dairy cow, catfish, and quail. These data led to the conclusion of "no concern" using the flowcharts shown in Figures 9 and 26, via the pathways highlighted in bold type.

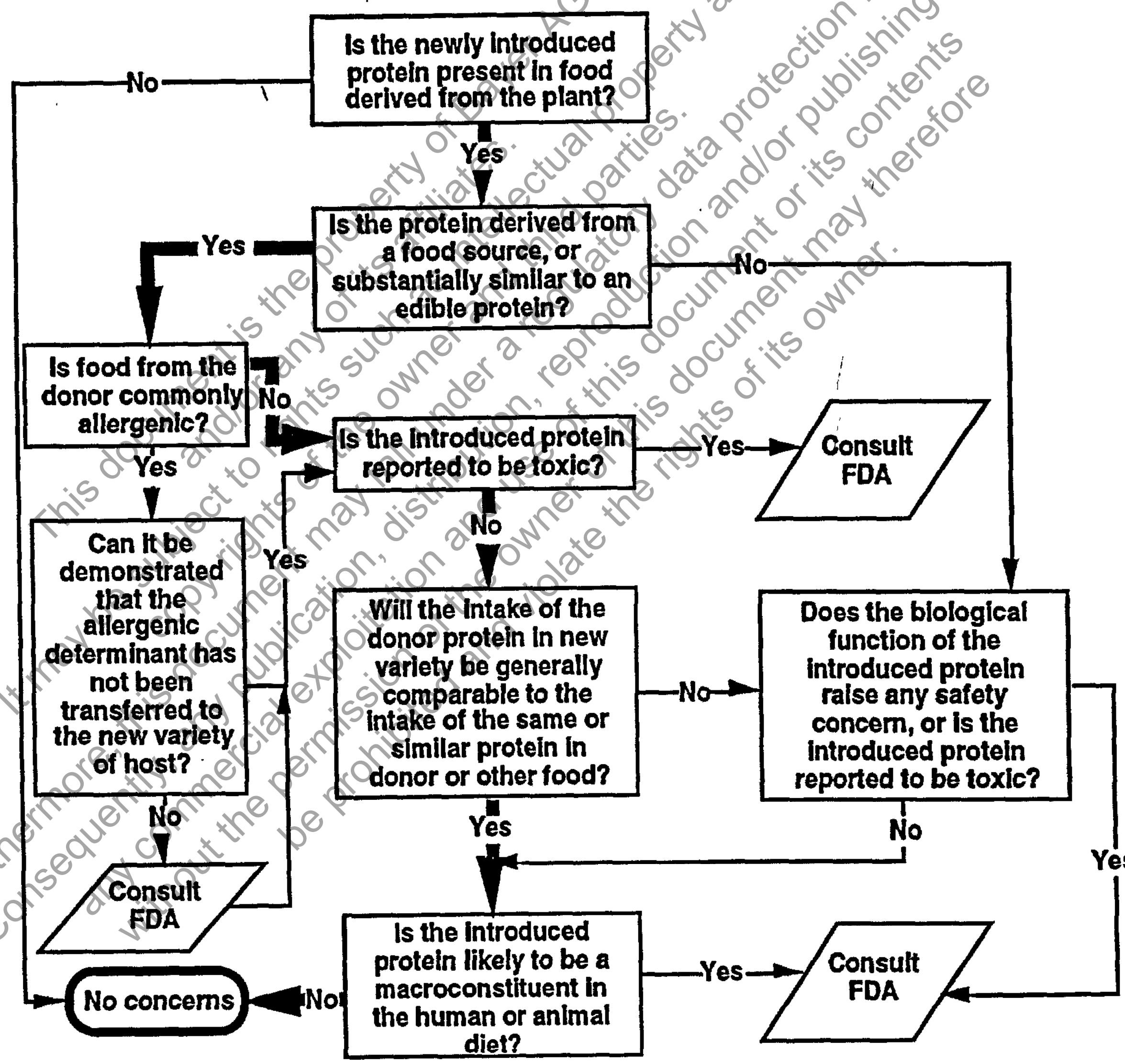
Figure 26. Safety assessment of new varieties: the donor (taken from Food Policy Figure 3) (1). The pathway leading to "no concern" for GTS is highlighted with bold arrows.



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

Shown in Figure 27 is the flowchart from the FDA Food Policy concerning the safety assessment of the protein introduced from the donor organism.

Figure 27. Safety assessment of new varieties: proteins introduced from donor (taken from Food Policy Figure 4) (1). The pathway leading to "no concern" for GTS is highlighted with bold arrows.



1. Protein introduced from the donor

a. Expression levels of CP4 EPSPS in GTS line 40-3-2

To assess the expected or intended effects in food and feed of the added CP4 EPSPS protein, levels of the protein were determined under GLP guidelines in whole seed, toasted meal (feed ingredient), defatted meal, protein isolate and protein concentrate (25,26). The mean expression of CP4 EPSPS in line 40-3-2, based on ELISA analysis of seeds from 9 field sites, was 0.288 µg/mg tissue fresh weight (0.08% of the total protein) (25). In the protein processing fractions manufactured from line 40-3-2 soybeans, the level of CP4 EPSPS was no greater than 0.1% of the total protein in any of the fractions. CP4 EPSPS enzymatic activity was not detectable in toasted meal, protein isolate nor protein concentrate due to processing, but was measurable in the defatted, non-toasted meal. Based on these results (and recognizing that virtually all soybean protein-derived food and the majority of feed is heated prior to consumption), the catalytically inactive form of CP4 EPSPS is expected to be present at low levels in food and feed derived from GTS line 40-3-2.

b. Relationship between CP4 EPSPS and EPSPS enzymes found in food

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

c. Assessment of the allergenic potential of GTS

We conclude that the allergenic potential of GTS and the products derived from these soybeans is not significantly different than other soybean varieties. EPSPS proteins are a diverse set of related proteins typically present in foods and feeds derived from plants and microbes and are not known to be commonly allergenic. The lack of allergenic potential of GTS was further supported from two perspectives. First, the endogenous protein allergens and their levels present in commercially available soybeans were compared to those present in GTS to assess whether the composition and relative quantities of these allergens are comparable. Secondly, the biochemical properties of known allergenic proteins were compared with the CP4 EPSP synthase protein as an approach to assess the allergenic potential for the CP4 EPSP synthase.

The endogenous allergenic proteins in GTS are qualitatively and quantitatively not materially different from those present in non-engineered, commercially available soybeans (119) as determined by a well-established immuno-blot assay that is routinely used for the assessment of soybean allergens (120). Protein extracts were prepared from non-toasted, defatted soy flour derived from GTS line 40-3-2, the A5403 parental variety, and three commercially available soy flour preparations. Proteins were separated by SDS-PAGE and

transferred to a membrane for specific detection of the allergenic proteins using IgE antibodies. These antibodies were obtained from pooled serum from several individuals shown to be sensitive to soybean products by direct food challenges (120). As controls, IgE antibodies from pooled serum from normal and peanut-sensitive individuals were used to assure the specificity of the IgE antibodies. Both the presence and the relative levels of the endogenous allergenic proteins in all of these soybean preparations were comparable, demonstrating that the endogenous allergenic proteins were not altered during the production of GTS.

Although large quantities of a vast variety of proteins are consumed in diets each day, rarely do any of these tens of thousands of proteins elicit an allergenic response (121). Although there are no predictive assays available to assess the allergenic potential of proteins (1), the biochemical profile of the CP4 EPSPS protein provides a basis for allergenic assessment when compared with known protein allergens. Allergenic proteins are often, though not always, glycosylated proteins between 10,000 and 70,000 daltons in size. Moreover, protein allergens must be stable to the peptic and tryptic digestion and the acid conditions of the digestive system if they are to reach and pass through the intestinal mucosa. Another significant factor contributing to the allergenicity of proteins is their high concentrations in foods that elicit an allergenic response (121-123).

CP4 EPSPS at 47,600 daltons (17) fits the mass criteria of 10,000 to 70,000 daltons, as do most proteins. CP4 EPSPS possesses none of the other characteristics common to protein allergens. CP4 EPSPS is not heat stable, and all detectable functional activity and ELISA reactivity are lost after the processing / toasting procedure (25). However, a portion of the protein mass is still present as detected by immunoblot analysis of the processed material. These data establish that the tertiary structure was altered, and the protein was converted to a non-functional, denatured molecule after heat treatment. The instability of the CP4 EPSPS protein during processing was expected since the purified CP4 EPSPS protein loses its activity rapidly upon heat treatment. All CP4 EPSPS activity was lost in solution upon incubation at 65°C for 15 minutes (25). More importantly, CP4 EPSPS was shown to be extremely labile to digestion by the proteases present in the mammalian digestive system (124) (as will be discussed below), which supports the prediction that the CP4 EPSPS protein will not survive the peptic and tryptic conditions of the mammalian digestive system.

Since most protein allergens are glycosylated, the CP4 EPSPS, as purified from GTS seed, was examined for glycosylation and shown not to be glycosylated (125,126). This result was expected since enzymatic glycosylation in plants requires passage through the rough endoplasmic reticulum and Golgi bodies (127). This transport requires specific targeting

sequences on the protein, which were not engineered into the CP4 EPSPS gene and hence the resulting CP4 EPSPS protein. In addition, CP4 EPSPS protein is targeted to the chloroplast, the site of aromatic amino acid synthesis. This targeting does not require or enable glycosylation. Because there is no glycosylation of the CP4 EPSPS, it does not fit the profile of some common allergenic proteins.

CP4 EPSPS shows no significant homology to any known protein allergen (128). CP4 EPSPS and endogenous soybean EPSPSs show 51% similarity and 26% identity in amino acid sequence. In contrast, CP4 EPSPS showed no significant homology to any of the 121 amino acid sequences reported for the allergens in the three current protein data bases (Genpept, Pir protein and Swissprot databases). There was no greater homology of the native CP4 EPSPS to any of the 121 amino acid sequences for the allergenic proteins than for a scrambled sequence of the same amino acids that comprise the CP4 EPSPS (see section IV.B.1.d.i. below).

Taylor and coworkers (121,123) discussed the correlation of the "degree of foreignness" of a protein and its likelihood of being an allergen. The more "foreign" a protein is for the host, the more likely it is to be recognized as "foreign" and elicit an allergic host response. As discussed in section III.C.3, CP4 EPSPS is related to the EPSPSs typically found in food. This relationshp supports the conclusion that CP4 EPSPS is unlikely to become a food allergen.

Finally, most allergens are present as major protein components in the specific food. This is true for the allergens in milk (122,129-131), soybean (120,132,133), and peanuts (134-137). In contrast to this generality for common allergenic proteins, CP4 EPSPS is present in soybean seed at low levels, approximately 0.03% of fresh weight of the soybean seed and approximately 0.08% of the total protein (25,26). The low levels of CP4 EPSPS in soybeans, combined with the thermal and digestive lability of this protein relative to that for known food allergens, suggest a very low probability of CP4 EPSPS surviving to be absorbed via the intestinal mucosa during consumption and thus potentially triggering production of antibodies including the IgE antibodies responsible for allergenicity.

Several studies using direct food challenges with oil derived from several different crops including soybean, peanut, and sunflower did not report an allergic reaction in patients shown to be sensitive to these foods (138-140). Bush *et al.* (138) concluded that "soybean oil is not allergenic to soybean-sensitive individuals." The level of protein in the oil derived from both the control soybean variety and the glyphosate-tolerant variety was shown to be at or below the level of detection (<1 ppm) (25,26). These data are consistent with the extremely low or negligible level of protein in soybean oil reported by Tattrie and Yaguchi (73).

Table 4. Characteristics of known allergenic proteins[a]

Characteristic	Allergens	CP4 EPSPS
Molecular wt 10-70 kdal	yes	yes
Glycosylated	yes[b]	no
Stable to digestion	yes	no
Stable to processing	yes	no
Similar to known allergens	-[c]	no
Similar to soybean proteins	-	yes
Prevalent protein in food	yes	no

[a] As described in Taylor (121) and Taylor *et al.* (122,123)
[b] Typically but not absolutely
[c] Implicit for allergenic proteins from soybeans

In summary, the data and analyses described above and summarized in Table 4 support the conclusion that CP4 EPSPS does not possess the characteristics of known protein allergens, that CP4 EPSPS shows considerable homology to the endogenous soybean EPSPS (as well as other plant and microbial [food/feed] EPSPSs), and that CP4 EPSPS shows no significant homology to allergenic proteins that have been characterized.

This data, coupled with the rapid digestion of CP4 EPSPS under *in vitro* digestive conditions that mimic human digestion (as discussed below), establishes that, using the best methodology available today, there is no reason to believe that the CP4 EPSPS protein should pose any significant allergenic risks from consumption of products generated containing this protein.

d. Studies demonstrating lack of toxicity associated with CP4 EPSPS protein
i. Lack of homology of CP4 EPSPS to known protein toxins and allergens
CP4 EPSPS does not show meaningful amino acid sequence homology when compared to 1,935 known protein toxins present in the Pir protein, Swissprot (37), and Genpept (36) protein databases (128). Patterns of amino acid sequence or regions of strong homology shared between two or more proteins may provide insight to the biological significance of a protein. For instance, amino acid sequences may provide information about structural properties, hydrophilicity/hydrophobicity, immunogenicity, stability, evolution, and the possible function or role of the identified protein. The use of protein databases has proven to be a useful tool for predicting biological function of an unknown protein. CP4 EPSPS was compared to peptide sequences identified as "allergens" and "toxins" from all available protein databases, to identify if CP4 EPSPS has any meaningful sequence homology with known allergens or toxins.

The FASTa-type algorithm, which is the standard method for database searching, was used to conduct the amino acid homology comparison between the CP4 EPSPS protein and all available sequenced allergen and toxin proteins from all available electronic databases of protein sequences (141-144). The results show that no meaningful homologies exist between known allergens or toxins and the CP4 EPSPS protein sequence. The lack of significance between the alignments was assessed by randomizing the CP4 EPSPS protein sequence, keeping the amino acid content identical, and comparing the randomized CP4 EPSPS amino acid sequence to the identical database of known allergens and toxins. The output comparisons generated from the randomized CP4 EPSPS amino acid sequence closely resembles the output comparisons generated with the native, unrandomized CP4 EPSPS sequence. The evidence indicates, using the best methods available today, that CP4 EPSPS protein does not share any sequence similarity with the database of known sequenced protein allergens and toxins. This conclusion that CP4 EPSPS is not expected to be toxic is supported by both the wholesomeness studies described above and the digestion and acute mouse gavage studies discussed below. Further information on the homology searches are provided in Appendix C.

ii. Acute mouse gavage study with CP4 EPSPS protein

An acute mouse gavage study with mature (lacking the chloroplast transit peptide) CP4 EPSPS as the test material was performed in order to directly assess any potential toxicity associated with the protein (145). Results from this study demonstrated that the CP4 EPSPS protein is not toxic. The mature (lacking the chloroplast transit peptide) CP4 EPSPS protein was over-produced (146) and purified (147) from *E. coli*, demonstrated to be equivalent to the GTS soybean seed-produced CP4 EPSPS (125), and administered by gavage to mice in an acute toxicity test. The criteria assessed for equivalence are shown in Table 5.

There were no adverse effects in mice administered CP4 EPSPS protein by oral gavage at dosages up to 572 mg/kg (145). This dose represents an approximate 1300-fold safety margin relative to the highest potential human consumption of CP4 EPSPS if the protein were expressed in soybean, corn, tomato, and potato (assuming no loss of CP4 EPSPS due to processing) (148). Acute administration was considered sufficient to assess the safety of CP4 EPSPS, since proteins that are toxic act via acute mechanisms (149-151).

No treatment-related adverse effects were observed in animals dosed with CP4 EPSPS protein. There were no statistically significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and CP4 EPSPS protein-treated groups. In summary, there were no treatment-related adverse effects in mice administered CP4 EPSPS protein by oral gavage at dosages up to 572 mg/kg.

This is not surprising since the CP4 EPSPS was demonstrated to be digested readily in gastric and intestinal fluid *in vitro*, as summarized below.

Table 5. Summary of equivalence analyses: GTS vs. *E. coli* CP4 EPSPS proteins (125)

Analytical Method	Criteria	Results
SDS-PAGE	Similar electrophoretic mobility	Similar apparent MW
Western blot	Similar electrophoretic mobility and immunological response	Similar apparent MW and immunological response
Glycosylation	Comparable response with glycosylation detection	No CP4 EPSPS specific carbohydrate moieties detected
Amino Acid Sequence	Corresponds through 10 amino acid positions	Correct N-terminus through 15 positions (N-terminal methionine present on *E. coli*-produced CP4 EPSPS)
CP4 EPSPS enzymatic activity	Specific activities (SA) will not differ by more than a factor of 2	GTS 3.9 U/mg *E. coli* 3.0 U/mg
ELISA	Comparable dose response	Dose response curves comparable

iii. Digestion of CP4 EPSPS in simulated gastric and intestinal fluids
In vitro, simulated mammalian gastric and intestinal digestive mixtures were established and used to assess the susceptibility of mature (lacking the chloroplast transit peptide) CP4 EPSPS to proteolytic digestion. The method of preparation of the simulated digestion solutions used is described in the United States Pharmacopeia (152), a frequently cited reference for *in vitro* digestion. *In vitro* studies with simulated digestive solutions are widely used as models of animal digestion. They have been used to investigate the digestibility of plant proteins (153,154), animal proteins (155) and food additives (156); to assess protein quality (157); to study digestion in pigs and poultry (158); to measure tablet dissolution rates to monitor biodegradation for pharmaceutical applications (159); and to investigate the controlled-release of experimental pharmaceuticals (160).

CP4 EPSPS was shown to be rapidly degraded by the components of the mammalian digestive system (124), greatly minimizing any potential for this protein to be absorbed by the intestinal mucosa. The data demonstrated a

half-life for CP4 EPSPS of less than 15 seconds in the gastric system and less than 10 minutes in the intestinal system, based on Western blot analysis. To put the rapid degradation of the CP4 EPSPS protein in the simulated gastric system into perspective, solid food has been estimated to empty from the human stomach by about 50% in two hours, while liquid empties 50% in approximately 25 minutes (161). If some of the CP4 EPSPS protein did survive the gastric system, it would be rapidly degraded in the intestine. Greater than 50% of CP4 EPSPS protein was degraded in the simulated intestinal system in less than 10 minutes (Western blot analysis). This compares with transit times through the intestine (for radiolabelled chromate, which is not absorbed) of 4 to 10 hours for the first products to appear in the feces and 68 to 165 hours for the last to be detected (162). Thus, using both simulated gastric fluids (SGF) and simulated intestinal fluid (SIF) model systems, CP4 EPSPS is predicted, as expected, to be readily digested in the mammalian digestive tract.

e. Consumption of the donor protein and macroconstituent status
Expression studies on GTS seed show that the CP4 EPSPS protein is expressed at approximately 0.03% (0.288 µg/mg) of the fresh weight of soybean seed (25). This is higher than the level of endogenous EPSPS, and thus the consumption of total EPSPS will increase in the GTS varieties. The magnitude of this increase has not been directly determined because although the endogenous soybean EPSPS can be detected by Western blots in extracts from soybean seed (using antibodies raised to petunia EPSPS), no reliable and accurate assay exists to quantitate the soybean EPSPS. However, the CP4 EPSPS protein in soybeans represents 0.1% or less of the total soybean seed protein. In comparison, the main soybean seed storage protein, glycinin (11S fraction) comprises 31 to 42% of the total soybean seed protein (81,163). ß-Conglycinin, the major component of the 7S fraction, comprises approximately 15% of the total soluble soybean seed protein (163). Because CP4 EPSPS is present in soybean seed at levels over a hundred times lower than that of these seed storage proteins, the CP4 EPSPS should not be considered a macronutrient. In addition, the lack of macronutrient status of CP4 EPSPS has been confirmed by measurements of total seed protein (Figures 10,11, and 14), which demonstrate that the introduction of CP4 EPSPS protein has not increased total protein levels in GTS soybean seeds compared with the control. There is no reason to expect that the addition of CP4 EPSPS to soybean would lead to an increased intake of soy proteins in the human or animal diet.

2. Lack of effects on carbohydrates, fats or oils
The addition of the CP4 EPSPS gene is not expected to alter the carbohydrate, fat, or oil composition, structure, or levels in GTS line 40-3-2. The proximate and other analyses confirmed that there has been no alteration in the level of carbohydrates or level and composition of fats in oil from line 40-3-2 (section IV.A.1.b).

3. Summary of expected or intended effects due to the CP4 EPSPS gene and expressed protein in GTS line 40-3-2

We have concluded that there are no deleterious effects due to the insertion of the CP4 EPSPS gene and the expression of the protein in GTS line 40-3-2. This was demonstrated by: 1) the relationship between CP4 EPSPS and EPSPS enzymes found in food and microbes; 2) the lack of allergenic potential of CP4 EPSPS; 3) the lack of homology of CP4 EPSPS with known protein toxins; 4) the lack of acute toxicity of CP4 EPSPS as determined by a mouse gavage study; 5) the rapid digestion of CP4 EPSPS in simulated gastric and intestinal fluids; 6) the lack of macroconstituent status of CP4 EPSPS; and 7) the lack of expected alterations in carbohydrates, fats, or oils in line 40-3-2. This data allowed us to reach the conclusion of "no concern" using the flowchart shown in Figure 27, via the pathway highlighted in bold type.

V. Conclusion for GTS Line 40-3-2 Safety Assessment

Glyphosate-tolerant soybeans (GTS) are not materially different from the parental soybean line or soybeans now being sold, in any meaningful way except for the ability to tolerate glyphosate. Over 1400 individual analytical measurements were compared. The results demonstrated that the levels of nutrients (protein, oil, fiber, ash, carbohydrates, calories, amino acids, and fatty acids) in GTS line 40-3-2 are comparable to the parental variety or are within established ranges for soybeans. Natural soybean antinutrients (trypsin inhibitor, lectins, phytosterols, stachyose, raffinose, and phytate) were also measured, and comparisons again showed no material difference when compared to the parental control.

The single new protein found in line GTS line 40-3-2, CP4 EPSPS, is related to EPSPSs already found in foods derived from plants, microbes, and fungi. CP4 EPSPS is inactivated by the heat processing required prior to consumption of soybeans by humans and most farm animals. The CP4 EPSPS is rapidly degraded in simulated digestive fluids and lacks other properties associated with allergenic proteins. The wholesomeness of the soybean meal and seed was confirmed in feeding studies with rats, chickens, dairy cows, catfish, and quail. No differences in feed efficiencies were observed when GTS line 40-3-2 was used as a feed source compared to the parental variety.

These data led to a conclusion of "no concern" for every criterion in the flowcharts outlined in the Food Policy. Soybeans modified to be tolerant to glyphosate are not materially different in composition, safety, wholesomeness or any relevant parameter from soybeans now grown, marketed, and consumed. Sales and consumption of these soybeans and all progenies derived from crosses between GTS line 40-3-2 and traditional soybeans would be fully consistent with the agency's Food Policy, the Federal Food Drug and Cosmetic

Act, and current practices for the development and introduction of new soybean varieties.

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Appendix A. Summary of analytical methods:

Following are brief descriptions of the primary assays used in the analytical studies for GTS safety assessments (25,26).

1. CP4 EPSPS ELISA: An enzyme-linked immunosorbent assay (ELISA) was developed and validated (89) for the detection of CP4 EPSPS. Quantitation of CP4 EPSPS levels was accomplished by extrapolation from the logistic curve fit of the purified mature CP4 EPSPS standard curve. The ELISA utilizes two antibodies from two different animal species raised against the non-denatured CP4 EPSPS protein. The double antibody sandwich is detected with donkey anti-rabbit alkaline phosphatase conjugate followed by development with pNPP. The assay recognizes the non-denatured CP4 EPSPS. The standard extraction buffer utilized was 100 mM Tris-HCl, pH 7.8, 10 mM sodium borate, 5 mM magnesium chloride, 0.05% v/v Tween 200, and 0.2% sodium ascorbate.

2. CP4 EPSPS enzymatic assay: The procedure utilized for determining the amount of functionally active EPSPS entailed the use of an HPLC with radioactivity detector, which has been previously described (19,164). Labelled substrate ^{14}C-phosphoenolpyruvate (^{14}C-PEP) is converted to ^{14}C-5-enolpyruvylshikimate-3-phosphate (^{14}C-EPSP) in the presence of shikimate-3-phosphate (S3P) by EPSPS, and the resultant ^{14}C-EPSP is detected using HPLC and radioactive flow detection. The final reagent concentrations in the assay were 50 mM Hepes, 0.1 mM ammonium molybdate, 5 mM potassium flouride, 1 mM ^{14}C-PEP, and 2 mM S3P, pH 7.0. Reactions were run at approximately 25°C. The reactions were quenched after 2 to 5 minutes with an equal volume of 9:1 ethanol: 0.1 M acetic acid, pH 4.5. The samples were then centrifuged and chromatographed by HPLC anion exchange using approximately 0.25 M potassium phosphate eluent, pH 6.5, at 1 ml/min flow rate. The percent turnover of ^{14}C-PEP to ^{14}C-EPSP was determined by peak integration. For EPSPS, 1 unit (U) is defined to be 1 µmol EPSP produced/ min. at approximately 25°C, under the assay conditions described.

3. Western blot assays for CP4 EPSPS: Detection of CP4 EPSPS at low levels from a variety of samples was accomplished by separating protein samples on polyacrylamide gels and electrophoretically blotting the proteins in the gels onto nitrocellulose paper or PVDF membrane (89). For visualization of CP4 EPSPS, specific antibodies were hybridized to the blots, then reacted with ^{125}I-Protein G. Quantitation of results was either by autoradiography followed by densitometry (LKB Ultrascan) of the autoradiograms, or by imaging the blot directly using a luminescence detection system and quantitative image analysis (BioMolecular Dynamics Phosphoimager).

4. Protein assays for extracts (Bio-Rad assay): Protein levels in extracts

used for ELISA and Western blot analyses, except for extracts containing SDS, were determined using the Bio-Rad protein assay in a 96-well plate format (165).

5. Protein / amino acids in oil assays: In order to investigate whether any appreciable amount of protein (or amino acids) is found in soybean oil, a new method was developed at Monsanto. This method involves hydrolyzing one-half milliliter samples of oil with equal volumes of trifluoroacetic acid: HCl: propionic acid (50:25:25) for approximately 24 h at approximately 145°C. After cooling, the released amino acids are extracted from the oil phase with 2 x 0.5 ml of 30% methanol/0.1 N HCl. The combined extracts are dried down, reconstituted in 200 µl Na-S citrate buffer and analyzed on a Beckman 6300 amino acid analyzer.

6. Compositional quality assays: Soybean compositional assays were performed at Ralston Analytical Laboratories (RAL), St. Louis, MO. A list of the assays and their primary literature references is shown below:

Analyte	Reference
urease	(166)
sugars	(167)
nitrogen solubility	(168)
tryptophan	(169,170)
moisture	(171,172)
cysteine and methionine	(173-175)
amino acids	(176-178)
trypsin inhibitor	(179,180)
ash	(181)
fatty acids	(182,183)
fiber	(184)
fat, ether extration	(185-187)
total kjeldahl nitrogen - protein	(188)
phytic acid	(189-191)
carbohydrates	(by difference)
free and bound isoflavones	(26,99,192)
lectins	(26,193,194)

Following are brief descriptions of the RAL standard methods utilized in this study.

a. urease: Urease was assayed using an AOCS (American Oil Chemists' Society) method (166). The assay is based on treatment of the sample with phosphate buffer containing urea. Urea reacts with any urease present, liberating ammonia, which raises the pH of the solution. The lowest confidence level of this assay was 0.01 Δ pH increase using a 0.2 g sample.

b. sugar analysis by HPLC: Sugars were measured by HPLC using a

Waters μ-Bond-a-pak carbohydrate column, or equivalent, according to a modification of a published procedure (167).

c. nitrogen solubility analysis: Nitrogen solubility was assayed using an official A.O.C.S. method (168). The nitrogen solubility index is defined as [(% water soluble protein)/ (% total protein)] x 100. The lowest confidence level of this assay was 0.5%, using a 5 g sample.

d. tryptophan-alkaline hydrolysis: For tryptophan determination, a portion of sample was mixed with sodium hydroxide solution, and precautions were taken to prevent oxidation of the amino acids as they were hydrolyzed. Samples were prepared and analyzed by HPLC, based on a modification of several methods (169,170).

e. moisture: The sample was placed in a force-draft oven, set at approximately 133°C, for two hours. The loss in weight was quantitated and calculated to percent moisture, as previously described (171,172).

f. cysteine and methionine (CYPA method): Cystine and cysteine in samples were oxidized to cysteic acid and methionine oxidized to methionine sulfone by treatment with performic acid solution for 16 h at approximately 0°C. After acid hydrolysis, the sample was separated on an anion exchange column, and detected by ninhydrin reaction. This assay has been previously described (173-175). Note that this method, denoted "CYPA" in the data, was used exclusively for the cysteine and methionine quality analysis comparisons in this report, due to the higher recoveries for this method. Results for methionine determined by the "AAHV" method, which were also performed but not listed in this report or statistically analyzed, may be found in the raw data file.

g. amino acids (AAHV method): Samples were hydrolyzed with hydrochloric acid; after acid hydrolysis, the sample was separated on an anion exchange column, and detected by ninhydrin reaction. This assay is based on previously published references (176-178).

h. trypsin inhibition: Components that inhibit trypsin activity were extracted at a pH of 9.5 to 9.8, using a sodium hydroxide solution. An aliquot of the sample suspension was mixed with a known volume of trypsin solution and incubated several minutes to allow the trypsin inhibiting factors to react with the added trypsin. An aliquot of benzoyl-D-arginine-p-nitroanilide (BAPNA) was added to the suspension. Uninhibited trypsin catalyzes the hydrolysis of BAPNA, forming yellow-colored p-nitroanaline. After 10 minutes of reaction, the hydrolysis was halted by lowering the solution pH with acetic acid, denaturing the enzyme. The solutions were evaluated spectrophotometrically and trypsin inhibition is evaluated from the difference in degree of BAPNA hydrolysis between the sample solution and the uninhibited trypsin solution. One trypsin unit is defined as an increase equal to 0.01 absorbance units at 410 nm after 10 minutes of reaction per 10 mls of final reaction volume, read in 1/2" tubes. The lowest confidence level of this method was 1 trypsin inhibitor unit (TIU) / mg sample, using 1 g sample. This method is based on published procedures (179,180). Note that the unit TIU is used interchangably

with the unit TUI.

i. ash: The sample was charred on a hot plate, and ashed to a constant weight at approximately 550°C in a muffle furnace. The residue was quantitated and calculated to percent ash. Using a 3 g sample, the lowest confidence level of this method was 0.2%. This method has been previously published (181).

j. fatty acid profile: For foods, feed, or ingredients, lipids were extracted with chloroform/methanol. Extracted lipid, fat, or oil was saponified with alcoholic potassium hydroxide. Free fatty acids were extracted with hexane, washed with water and dried with sodium sulfate. The fatty acids were then esterified with methanol, with boron trifluoride as a catalyst, taken up in heptane, and injected on a gas chromatograph with a flame ionization detector. The percents of individual fatty acid methyl esters were calculated relative to the total amount of fatty acid methyl esters present. The lowest confidence level of this method was 0.1% of an individual fatty acid methyl ester, relative to the total fatty acid methyl esters from the lipid. This assay has been previously described (182,183).

k. fiber: The sample was dried, if necessary, to remove excessive moisture, ground to pass a 1.0 mm screen and extracted in refluxing ether to remove excessive fat. It was then digested in refluxing 1.25% H_2SO_4, filtered, digested in refluxing 1.25% NaOH, and filtered. The residue was washed, dried, weighed, ignited, and reweighed. Crude fiber was calculated from the loss on ignition of the residue. Using a 2 g sample, the lowest confidence level of this method was 0.2%. This method has been published (184).

l. fat, ether extraction: Ether-soluble material (primarily "free" fats and oils) was extracted from the sample with petroleum ether. The ether was volatilized, and the residue was dried, quantitated gravimetrically, and calculated as percent fat. Using a 2 g sample, the lowest confidence level of this method was 0.1% fat. The method is based on published procedures (185-187).

m. total kjeldahl nitrogen - protein: Nitrogenous compounds in the sample were reduced, in the presence of boiling sulfuric acid, catalyzed by a potassium sulfate/titanium dioxide/cupric sulfate mixture, to form ammonium sulfate. The resultant solution was cooled, diluted, and made alkaline with a sodium hydroxide-thiosulfate solution. Ammonia was liberated and distilled into a known amount of standard acid. The distillate was titrated, and nitrogen or protein (N x 6.25) was calculated from the known amount of reacting acid. Using a 1 g sample, the lowest confidence level of this method was 0.1% protein (0.02% nitrogen). The method is based on a published procedure (188).

n. phytic acid, by ion-exchange: Phytic acid was extracted with dilute hydrochloric acid solution, and separated from inorganic phosphates on an anion exchange column. Phytate was eluted with a sodium chloride solution. The eluate was digested with sulfuric/nitric acid, freeing phosphorus, which was reacted with ammonium molybdate and sulfonic acid solutions, forming a blue color complex which was measured spectrophotometrically. Values were converted to phytic acid based on molecular weight equivalence. Using a 2 g

sample, the lowest confidence level of this method was 0.028% phytic acid. The method is based on published procedures (189-191).

o. carbohydrates: Carbohydrates were calculated by difference using the fresh weight-derived data using the following equation:

% carbohydrates = 100 % - (% protein + % fat + % ash + % moisture)

p. free and bound isoflavones: Daidzein, genistein, coumestrol, and biochanin A were determined by HPLC (ODS column) with U.V. detection after sample extraction and cleanup. Experimentally, free isoflavones and total isoflavones were determined; samples analyzed for total isoflavones were subjected to refluxing HCl conditions. Bound isoflavones were calculated by subtracting the free from the total isoflavones for a given sample. This assay was based on previously published methods (99,192). For coumestrol, values less than 10 ppm were considered unreliable due to poor peak shape. Additional methods development for the isoflavone determination is included in a separate study (26).

q. lectins: Lectins were determined according to a modification of literature procedures, by measuring the agglutinating properties of soybean sample extract on rabbit red blood cells (193,194). Additional methods development for the lectin determination have been performed (26).

r. lecithin: The main components of lecithin - phosphatidyl ethanolamine, phostidic acid, phosphatidyl inositol, phosphatidyl choline - were analyzed as previously reported (195).

Appendix B. Additional information on confirmatory animal studies supporting the wholesomeness and safety of GTS when used in animal feeds

The majority of the safety assessment and wholesomeness studies were performed on two GTS lines, line 40-3-2 and line 61-67-1. Based on the data obtained from these studies, neither line was materially different than the parental control variety A5403. Due to a commercial prioritization, Monsanto is only planning to introduce GTS line 40-3-2 and its progeny into commerce. Therefore, the following data summaries focus exclusively on GTS line 40-3-2. Note that data from line 61-67-1 was utilized in the statistical analyses, in addition to the line 40-3-2 data, in order to obtain a more precise estimate of error in the experiment.

1. Broiler chicken 6 week feeding study with processed soybean meal (113)

PURPOSE

Soybean meal is a major component of commercial broiler chicken diets. This study was undertaken to compare the wholesomeness of processed glyphosate tolerant soybeans to processed parental line soybeans.

Commercial broilers, as a consequence of genetic selection, reach a market weight of around 2 kg in 6 weeks from hatch. Considering that the birds weigh around 30 gm when hatched, this amounts to a 66-fold increase in body weight over 6 weeks. To accommodate this rapid growth, there are considerable nutritional requirements, including protein, which must be provided by the diet. Any deficiencies in the protein source would be manifested by growth retardation in the growing broiler. This test was considered to be very rigorous, as small differences in body weight gain (3.5%) or feed/gain (2%) could be detected between soybean lines (113). Thus, the growing broiler is a very sensitive test species to detect differences in nutrient quality and was used to assess the wholesomeness of processed meal from GTS.

METHODS

Soybeans from parental (line A5403) and glyphosate-tolerant lines (61-67-1, 40-3-2) were grown at the same time in the same field test plots. Soybeans were processed (dehulled, defatted, toasted) into meal at the Food Protein Research and Development Center (Texas A&M University System, College Station, Texas). The only other source of dietary protein was corn. Supplemental methionine activity and lysine were added to all test diets. Corn and the processed soybean meal from each line were analyzed for proximates, total amino acids, minerals etc. to assist formulation of the diet blends. All test diets were formulated to contain approximately equal amounts of the following dietary essential amino acids (methionine, cystine, lysine, arginine, tryptophan, and threonine) and minerals (calcium, phosphorous, sodium, and chloride). All diets met or exceeded NRC requirements for poultry. No medications or feed additive growth promotants were added to the diets. Diets were blended and pelleted at [redacted], MO). Starter diet (crumbled pellets) was fed from day 0 to 21 and pelleted diet was fed from day 22 to 42 (study termination).

A commercial strain of broiler chickens (White Plymouth Rock X White Cornish; Cobb 500 cockerel X Cobb 500 pullet) were purchased for the study. The eggs were delivered by air freight and transferred into an incubator. Hatching was completed in 18-22 days. Chicks were feather sexed and vaccinated for Marek's disease. Healthy birds were randomly assigned to each group and birds were individually identified by wingband.
The study was conducted in 2 environmentally controlled battery rooms. Each

room contained a total of 48 cages in 3 tiers. Birds were randomly assigned to each treatment group according to sex, 5 per cage. The experiment was blocked according to the cage tier within each room (1, 2 or 3). The trial was designed as a 3 x 2 x 3 factorial experiment. There were 3 sources of soybean meal (A5403 parental line; 61-67-1 and 40-3-2 glyphosate tolerant lines), and 2 sexes (cockerels and pullets) and 3 cage tiers (top, middle, bottom). The tier effects were nested within room, and the residual error mean square was used to test for significance. Each battery room contained 3 tiers of 16 pens for a total of 48 pens. For the study, each tier had 12 pens filled; 2 replicate pens of 3 lines and 2 sexes. There were 4 pens left empty on each tier and the study included a total of 72 pens of 5 birds each. This allowed 12 replicate pens/sex (60 birds/sex) for each soybean line tested (a total of 360 birds/study).

Birds were checked daily for mortality, and any that died on test were removed, weighed and necropsied to determine the probable cause of death. Body weights and food consumption were measured weekly for each pen. After 6 weeks, all birds were sacrificed and major and minor pectoralis muscles (breast muscles) from the right side of the chicken were dissected and weighed by pen. Abdominal fat pads were also removed and weighed.

RESULTS

Starter and grower diets for each soybean line tested were analyzed during the study for proximates, complete amino acids, and minerals. Results of these analyses compared favorably with predicted values.

For the starter period (days 0-21), there were no differences in body weight and liveweight gain or livability for any of the groups. Chickens fed glyphosate tolerant line 61-67-1 consumed slightly (3.5%) more feed and thus had a slightly lower feed/gain when compared to the other groups. As expected, females consumed less feed and grew slower with a lower feed/gain than males, independent of the source of soybean meal tested. For the 22-42 day period and the cumulative study period (days 0-42), there were no statistically significant differences between groups for bodyweight, liveweight gain, feed intake, feed/gain (F/G) or livability. As expected, males were heavier, consumed more feed, and had better F/G but lower livability than females, regardless of the source of soybean meal tested.

There were no statistically significant differences between groups in breast muscle and fat pad weights either as absolute weights or as a percentage of body weights. Males had heavier breast muscle weights and lighter fat pad weights than females. Breast muscle and fat pad weights as a percentage of body weights were higher for females than males.

There was good survival in all groups and livability was similar across all treatments. A total of 34 birds were lost to the study, of which 15 were culled

and 19 died of apparent natural causes. The three major causes of death or culling were ascites, sudden death syndrome and twisted leg. None of these deaths were associated with infectious causes and these syndromes are commonly observed in broilers. The incidence of these syndromes was similar across all groups.

CONCLUSIONS

For the cumulative study period (days 0-42), there were no substantive differences in broiler performance across the 3 groups. The test was considered to be very robust as small differences in body weight gain (3.5%) or F/G (2%) could be detected between soybean lines. Since the differences observed were less than those that could be detected as statistically significant, it was concluded that the nutritive value of meal derived from GTS is not materially different than that of the parental line.

2. Dairy cow 4 week feeding study with raw soybeans (115)

PURPOSE

Raw soybeans are fed to dairy cattle in the United States at levels up to 10% (dry matter basis). Ruminants are relatively resistant to antinutritive factors such as trypsin inhibitors in raw soybeans which are degraded by rumen flora during digestion. This study was undertaken to compare the wholesomeness of raw glyphosate tolerant soybeans (GTS) to raw soybeans (parental line).

METHODS

Soybeans from parental (line A5403) and glyphosate-tolerant lines (61-67-1, 40-3-2) were grown at the same time in the same field test plots. Raw soybeans were incorporated in the mixed diet ration (35% alfalfa hay, 17% corn silage, 37% commercial grain mix) for cows at a concentration of 10% (w/w dry matter basis). This dietary level represents an upper limit for incorporation of raw soybeans into mixed cow diets as fed by dairy farmers. Soybeans were cracked daily, prior to blending with other dietary components. Two weeks prior to study initiation, cows were placed on diets supplemented (10% w/w dry matter basis) with a commercial variety (Asgrow A5403) of raw soybeans. This pretreatment period allowed dairy cows to adapt to high soybean diets.

Soybeans from each line and total mixed rations (TMRs) containing soybeans from each line were analyzed for proximates (dry matter, crude protein, acid and neutral detergent fiber, fat, and ash). TMR's for mixed diets for each group were formulated to meet or exceed nutrient recommendations of the 1989 National Research Council (NRC) and provided similar quantities of crude protein, net energy, lipid, fiber, major minerals and vitamins while maintaining equal proportions of soybeans on a dry matter basis. Feed was offered twice daily *ad libitum* so that fresh feed was available after each milking. Feed refusals were collected and weighed prior to the morning milking. Diet samples were collected each day for proximate analyses.

Thirty-six multiparous Holstein dairy cows between 93 and 196 days of lactation were assigned to one of two blocks based on availability. The days of lactation for cows in the first block ranged from 122 to 196 days and for the second block, 93 to 135 days. Within blocks, cows were randomly assigned to groups (5-6 cows) fed either the control (parental line A5403) or glyphosate tolerant lines 61-67-1 or 40-3-2 of raw soybeans. Cows were individually identified and housed in a tie-stall barn. They were released into an exercise lot prior to each milking in the parlor. Observations for overall health were recorded twice daily. Cows were weighed on treatment days -1, 0, 28 and 29.

During the last pretreatment week and for the first 3 weeks of the study, A.M. and P.M. milk samples were collected daily. Selected samples were analyzed for lactose, fat, protein, and somatic cells. Total urine and fecal output was

collected daily for each cow during the fourth week of treatment to determine dry matter digestibility and nitrogen balance. Cows remained in tie-stalls throughout this period and were milked with portable milkers. Bladders were catheterized for total urine collection. Samples of diets refused each day (orts) were also collected for each animal during this period. At the end of the 7 day collection period, samples were composited for each cow. Composite samples of milk and urine were analyzed for nitrogen; samples of diets, orts, and feces were analyzed for proximates. On the last day of the study (day 29) rumen fluid samples were collected via stomach tube from each cow at approximately 1.5, 3 and 6 hrs after the A.M. feeding. Samples were analyzed for volatile fatty acids and ruminal ammonia. Data were analyzed by Analysis of Variance using a randomized block design. A covariate was used in the model when appropriate. Deviations from block means during the 2 week pretreatment period were used as the covariate. Differences were declared significant at $\partial = 0.05$. The model used included covariate, design block, and treatment by block interactions. The model for all variables from the total collection period contained deviation from block mean for pretreatment milk production as a covariate.

RESULTS

Animal health was good throughout the study. One control animal was removed from the study as she developed mastitis. There were no statistically significant differences in least-squares means for milk production, milk fat, protein, lactose, somatic cell count, dry matter intake, net energy intake, FCM/NE_L intake, body weight change. There was a small (2.5-2.7 kg/day) but statistically significant increase in 3.5% fat corrected milk for cows fed both GTS lines. This higher production is consistent with a non significant increase in net energy intake of GTS lines resulting in similar ratios of FCM production to NE_L intake. There were no statistically significant differences in least-squares means for dry matter intake, nitrogen intake, dry matter digestibility, and milk, urine, fecal, absorbed, retained or productive nitrogen. There were no statistically significant differences in least-square means for ruminal fatty acids (acetate, propionate, isobutyrate, isovalerate, valerate, acetate/propionate) or ruminal ammonia nitrogen.

CONCLUSIONS

There were no important differences in feed intake, milk production, and milk composition between groups fed GTS lines and the parental line. The lack of differences in apparent dry matter digestibility and nitrogen balances as well as ruminal fermentation products indicate that the nutritive value of GTS is not materially different than that of the parental line.

3. Catfish 10 week feeding study with processed soybeans (114)

PURPOSE

Soybean meal is a major component of diets used at commercial catfish rearing facilities. The design of this study was to compare the wholesomeness of individual glyphosate tolerant soybean (GTS) lines to a parental (non-transformed) soybean line when fed in the diet of catfish for approximately 10 weeks.

The catfish feeding study was a particularly sensitive method for the evaluation of the wholesomeness of feedstuffs containing soybean and is relevant for the nutritional value of GTS in other animal diets. During a 10 week period, fingerling catfish experience a 700% increase in growth. Since growth is a function of the nutrient profile of catfish diets, the [redacted], has used fingerling catfish maintained for 10 weeks in aquaria to test the nutrient quality of experimental catfish diets. Therefore, it was considered appropriate to assess the wholesomeness of processed soybean meal from GTS in this test system.

METHODS

Processed meal from glyphosate tolerant and parental line soybeans was administered in the diet to catfish for 10 weeks. Soybeans from parental and glyphosate-tolerant lines were grown at the same time and in the same field test plots. Soybeans were processed (dehulled, defatted, toasted) and made into meal at the Food Protein Research and Development Center (Texas A&M University System, College Station, Texas). Processed soybean meal was shipped to [redacted] for formulation into catfish diets. A proximate analysis (fat, protein, fiber, ash, moisture etc.) was completed on the processed soybean lines prior to formulation of the diets. Processed soybean meal from each line was incorporated into the catfish diets at the same substitution levels used commercially (45-47% w/w or 450,000 - 470,000 ppm). All diets were prepared so that they were isonitrogenous; the final concentration of total protein in the catfish diets was 32%.

Fingerling channel catfish, *Ictalurus punctatus*, Mississippi Select strain were used for this study. These fish were raised from eggs at the [redacted] which carried out the test. Three hundred fish of mixed sex weighing approximately 3 grams per fish were used. The flow-through system provided fresh well water at a rate of approximately one liter per minute. Water temperature, dissolved oxygen, and pH were monitored at regular intervals during the study. Twenty fish were housed in a 120 L glass aquarium. There were 5 aquaria (replicates) per treatment. Each dietary treatment had a total of 100 fish.

Fish were fed diets at an initial rate of approximately 4% of body weight. The feeding rate was adjusted weekly based on observations of consumption during the previous week through the end of the test. In this manner feeding rates that approximated satiation were maintained.

Fish were weighed at study initiation, week 2, week 6, and week 10. This was accomplished by transferring the fish from the test tank into a pre-tared bucket containing enough water to hold the fish for a short time. On weeks 2, 6, and 10 feed consumption was quantified by feeding a precise quantity of feed, and feed consumption was calculated by subtracting the uneaten pellets removed from the tanks from those offered at initiation of feeding. Cumulative feed conversion ratio (FCR) was estimated at weeks 2, 6, and 10 by dividing the sum of the feed offered to that point by corresponding total gain. FCR was adjusted for mortalities.

At the end of the ten week feeding period, several fish were randomly selected from each tank and the edible tissue (fillets) was composited and subjected to proximate analysis. Percent moisture, protein, fat, and ash were determined using AOAC (Association Official Analytical Chemists) methods.

Analysis of variance and Duncan's Multiple Range Test were used to statistically compare results from all measured parameters.

RESULTS

Overall survival of fish (range of 95-99%) in the study was very good and comparable across all groups. Feed conversion ratios (FCR) were not significantly different between fish fed the control diet and those fed GTS lines 61-67-1 and 40-3-2. The percentage weight gain of fish fed the GTS lines was not significantly different from fish fed the control diet. The percentage weight gain of fish fed GTS line 61-67-1 was higher than that of fish fed GTS line 40-3-2. This difference was due to the fact that fish fed GTS line 61-67-1 consumed more feed (expressed as g of feed/fish) and converted the feed slightly better than fish fed GTS line 40-3-2.

Based on the three quantitative feedings (weeks 2,6,10) fish fed GTS line 40-3-2 consumed slightly less feed (2.85%) (expressed as a percentage of mean body weight), than fish fed the control diet (3.63%). Feed consumption (as % mean body weight) of fish fed GTS line 61-67-1 (3.23%) was not significantly different than that of fish fed GTS line 40-3-2 or the control diet.

Body composition data, expressed on a wet weight basis, were not different. There were no differences in moisture, protein, fat, or ash among fish regardless of dietary treatment. On a dry weight basis, percentage protein was slightly higher in fish fed GTS line 61-67-1 as compared to the control diet (84% versus 82.6%).

CONCLUSION

Survival, weight gain, feed conversion ratios, and body composition were similar across all groups. GTS lines 61-67-1 and 40-3-2 were considered to be suitable for use in catfish feeds. It was concluded that the nutritive value of meal derived from GTS is not materially different than that of the parental line.

4. Rat 4 week feeding study with processed soybean meal (111)

PURPOSE

This study, conducted with rats, was part of a series of animal feeding studies undertaken to compare the wholesomeness of processed (dehulled, defatted, toasted) meal from GTS to the parental soybean line. Processed soybean meal was administered to rats *ad libitum* in the diet for one month.

METHODS

Soybeans from parental and glyphosate-tolerant lines were grown at the same time and in the same field test plots. Soybeans were processed (dehulled, defatted, toasted) and made into meal at the Food Protein Research and Development Center (Texas A&M University System, College Station, Texas) and shipped to Purina Test Diets (Richmond Indiana) for formulation into rodent diets. A proximate analysis (fat, protein, fiber, moisture, ash, trypsin inhibitor, urease) was completed on the processed soybean lines prior to formulation of the diets. Based on the proximate analysis, the rodent diets were formulated to be isonitrogenous and as similar as possible to the nutrient profile for [redacted] Processed soybean meal from each line was incorporated into the rodent diets at the same substitution levels used commercially (24.8 % w/w or 248,000 ppm, nominal). The formulated rodent diets were shipped from [redacted] Diets to [redacted] for conduct of the rat feeding study.

Male and female Charles River CD® rats (approximately 8 weeks of age) were fed rodent chow containing processed soybean meal *ad libitum*. Rat chow consumption was measured for each rat on a weekly basis. During the course of the study, test animals were observed twice daily for mortality and adverse clinical signs. Body weights were recorded prior to randomization and weekly for each animal. There were 3 lines of processed soybeans tested; line A5403 (parental line) and two glyphosate tolerant lines, 40-3-2 and 61-67-1. Diets containing processed meal from each of these soybean lines was fed to 10 male and 10 female rats randomly assigned to each group. Negative control animals were fed [redacted] that was not substituted with processed soybeans from Monsanto test plots. At the end of the study, all test animals were sacrificed and necropsied. Liver, testes, and kidneys were weighed and approximately 40 tissues were collected and saved for each animal.

Dunnett's Multiple Comparison Test (two-tailed) was used to compare inlife body weights, cumulative body weight gain and food consumption for test and control groups. Terminal body weights, absolute organ weights, and organ/body weight ratios were evaluated by decision-tree statistical analysis procedures, which depending on the results for tests for normality and homogeneity of variances, used either parametric (Dunnett's Test and Linear Regression) or

nonparametric (Kruskal-Wallis, Jonckheere's and/or Mann-Whitney Tests) routines to detect group differences and analyze for trends.

RESULTS

There was no mortality in the study, and test animals appeared healthy. No statistically significant differences in body weight, cumulative body weight gain, or food consumption occurred between rats fed diets containing processed soybean meal from the parental line and glyphosate-tolerant lines. Body weights and cumulative body weight gains were slightly decreased for males fed meal from line 40-3-2 when compared to males fed the negative control diet. Food consumption was also slightly decreased for males fed meal from glyphosate-tolerant line 40-3-2 and the parental line when compared to negative control males. This may be due to differences in palatibility of the diets. Since there were no statistically significant differences in body weight, cumulative body weight gain and food consumption between males fed meal from the parental line (A5403) and glyphosate tolerant line 40-3-2, the aforementioned minor differences from the negative control group were considered unrelated to treatment. There were no statistically significant differences in absolute or relative organ weights for treated and control animals.

The few pathologic findings observed at necropsy were randomly distributed among all groups and are commonly observed in control animals in the testing facility.

CONCLUSIONS

No adverse effects were observed in rats fed up to 24.8% w/w (248,000 ppm) processed GTS meal in the diet. There were no statistically significant differences in measured parameters between rats fed processed soybean meal from the parental line and the glyphosate tolerant lines. Therefore, it was concluded that the wholesomeness of processed meal from GTS lines is not materially different than that of the parental line.

5. Rat 4 week feeding study with unprocessed soybean meal (112)

PURPOSE

This study with rats was one of a series of animal feeding studies undertaken to compare the wholesomeness of unprocessed glyphosate-tolerant soybean meal to unprocessed meal (from unmodified, parental line soybeans) when fed in the diet to rats for 4 weeks. In practice, unprocessed soybeans are not fed to monogastric animals due to the presence of antinutritive factors (*e.g.* trypsin inhibitors) in raw soybeans. Ruminants are the only animals that can tolerate raw soybeans in the diet since trypsin inhibitors are degraded by rumen flora.

METHODS

Soybeans from the parental (line A5403) and glyphosate-tolerant lines were grown at the same time and in the same field test plots for the rat feeding study. The raw soybeans were ground into meal at the Food Protein Research and Development Center, Texas A&M University System, College Station, Texas and shipped to [redacted] Test Diets (Richmond, Indiana) for formulation into rodent diets. A proximate analysis (fat, protein, fiber etc.) was completed on the unprocessed soybean meal from each line prior to formulation of the diets. The unprocessed soybean meal replaced processed (defatted, dehulled, toasted) soybean meal that is normally added to rodent chow. Based on the proximate analysis, the rodent diets were formulated so they were isonitrogenous and as similar as possible to the nutrient profile for Purina brand Rodent Laboratory Chow. Unprocessed soybean meal from each line was incorporated in the rodent diets at nominal concentrations of either 0, 5, or 10% w/w (50,000 or 100,000 ppm). The formulated rodent diets were shipped from [redacted] Test Diets to [redacted] for conduct of the rat feeding study.

Male and female Charles River CD® rats (approximately 8 weeks of age) were fed *ad libitum* rodent chow containing unprocessed soybean meal from either glyphosate tolerant lines or the parental control for approximately 4 weeks. Rat chow consumption was measured for each rat on a weekly basis. During the course of the study, test animals were observed twice daily for mortality and adverse clinical signs. Body weights were recorded prior to randomization and weekly for each animal. There were 3 lines of soybeans tested; line A5403 (parental line) and glyphosate tolerant lines 40-3-2 and 61-67-1. Each diet was fed to 10 male and 10 female rats randomly assigned to each group. Negative control animals were fed commercial rodent chow. At the end of the study, all test animals were sacrificed and necropsied. Liver, testes, and kidneys were weighed and approximately 40 tissues were collected and saved for each animal. Since unprocessed soybean meal contains trypsin inhibitors that can cause hypertrophy of the pancreas (Leiner, I.E. and Kakade, M.L. " Protease Inhibitors" in Toxic Constituents of Plant FoodStuffs, 2nd Edition, Academic Press, 1980) this organ was examined histologically for all animals on the

study.

Dunnett's Multiple Comparison Test (two-tailed) was used to compare inlife body weights, cumulative body weight gain, and food consumption for test and control groups. Terminal body weights, absolute organ weights, and organ/body weight ratios were evaluated by decision-tree statistical analysis procedures, which depending on the results for tests for normality and homogeneity of variances, used either parametric (Dunnett's Test and Linear Regression) or nonparametric (Kruskal-Wallis, Jonckheere's and/or Mann-Whitney Tests) routines to detect group differences and analyze for trends.

RESULTS

There was no mortality in the study and all animals appeared healthy. No statistically significant differences in body weight, cumulative body weight gain, or food consumption were observed between control and treated groups.

There were no statistically significant differences observed between any of the groups in absolute organ weights. Relative kidney weight was slightly increased for both GTS lines (5% dietary level) when compared to the negative control. However, there was no corresponding increase in relative kidney weights at the 10% dietary level. When relative kidney weights for the parental line and the GTS groups were compared, there were no statistically significant differences at either the 5% or 10% dietary levels. Since the differences in relative kidney weights were not dose related and were limited to comparisons to the negative control and not the parental line, they were not considered related to the glyphosate tolerance trait. A few pathologic findings were observed at necropsy. Dark livers were found in several animals (males predominately) fed glyphosate tolerant lines 40-3-2 and 61-67-1. Livers normally appear as tan or light brown at necropsy; these livers appeared a darker brown color. A similar number of males fed the parental line (10% level) also exhibited dark livers at necropsy. Since the incidence of dark livers at the 10% dietary level was similar for all groups fed unprocessed soybeans (negative controls were fed commercial chow containing processed soybeans meal) and in the absence of any changes in absolute or relative liver weights, the finding of dark livers was not considered to be related to the glyphosate tolerance trait. The occurrence of dark livers in rats fed unprocessed soybeans was not reproducible since this finding was not observed earlier in a one month pilot feeding study with unprocessed soybeans (parental line) fed to rats of comparable age at dietary levels up to 20 % w/w. Other postmortem findings observed at necropsy were randomly distributed among all the groups. Since the other pathologic findings observed at postmortem examination were not dose related and are commonly found in control animals at the testing facility, they were not considered related to feeding modified soybeans.

The pancreas was examined histologically from all animals on study since

feeding rats unprocessed soybeans has been reported to cause changes in the pancreas due to the presence of trypsin inhibitors. Minimal to mild microscopic changes were observed in the pancreas of test animals from all groups. The absence of marked histologic changes in the pancreas that could be attributable to feeding unprocessed soybeans is probably due to the fact that the diets also contained non-soy protein (corn) to make the diets isonitrogenous to the negative control diet. Subsequent to the completion of this study, it was learned that hypertrophy of the pancreas is observed only when the sole source of dietary protein is unprocessed soybeans ([redacted] personnel communication).

CONCLUSIONS

No adverse effects were observed in rats fed up to 10% w/w (100,000 ppm) unprocessed GTS meal in the diet compared to the meal from control soybeans. There were no statistically significant differences for the parameters measured between rats fed unprocessed soybean meal from the parental line and rats fed unprocessed soybean meal from glyphosate tolerant lines. Therefore, it was concluded that the wholesomeness of unprocessed soybean meal from the GTS lines is is not materially different than that of the parental line.

6. Quail (bobwhite) five day feeding study with raw soybean meal (117)

PURPOSE

Since birds such as quail may feed on soybean seeds in the field, a test of the wholesomeness of glyphosate tolerant varieties of soybean seed for quail was undertaken. Young quail are traditionally used in bird feeding studies. Therefore, it was necessary to feed quail raw soybean meal rather than intact seeds which are relatively large.

METHODS

Soybeans from the parental line (A5403) and glyphosate-tolerant lines (61-67-1, 40-3-2) were grown at the same time in the same field test plots. The raw soybeans were ground into raw meal at the Food Protein Research and Development Center, Texas A&M University System, College Station, Texas and sent to [redacted] for testing.

Northern bobwhite quail chicks were obtained from the [redacted] production flock. All test birds were from the same hatch. At study initiation, birds were 10 days of age. Thirty bobwhite chicks of mixed sex were assigned by random draw to each treatment and housed in groups of ten birds/pen. Each treatment group was fed nominal dietary concentrations of 20% w/w (200,000 ppm) raw soybean meal that was added directly to the game bird ration and mixed together in a Hobart mixer. This dietary level was selected based on the results of a pilot quail feeding study performed with raw control soybean meal. There were 2 lines of glyphosate tolerant soybean meal tested; line 40-3-2 and line 61-67-1. A control (parental line) was also tested; line A5403. An additional basal diet control group (no added soybean meal) of thirty birds fed only basal diet was also included in the study.

Similar to traditional toxicity studies with quail, each control and treatment group was fed the test diet for 5 days and then switched to basal (unsupplemented) diets for the last 3 days of the study. Food and water were provided *ad libitum*. Food consumption was recorded daily for each pen (days 1-5), and average food consumption/pen was recorded for days 6 to 8. Food consumption is an estimate due to the unavoidable spillage by the birds. Individual body weights were recorded at study initiation, on study day 5 and at study termination. The average temperature in the brooding compartment of the pens was 38° C ± 1°C. Average ambient temperature was 24.1°C ± 0.7 °C with an average relative humidity of 28% ± 9%. The photoperiod was 16 hrs. light/day during acclimation and throughout the study. Birds were observed twice daily during the study for mortality or signs of toxicity.

RESULTS

There was no mortality observed during the study in any of the groups tested. There were a few observations of birds that were nose picked or had feed caked

around their beaks in both the control and treated groups. One bird had its leg caught in a feeder. These findings were randomly distributed among all the groups and are common to similar studies with young quail. All other birds on study were normal in appearance. Body weight gain and food consumption were comparable for quail fed soybean meal from the glyphosate tolerant lines and those fed the control (parental line) and basal diet.

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

20% X 6 gm food/bird/day = 1.2 gm soybean meal/bird/day.

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

The average weight of a soybean seed of the A5403 variety is 0.135 gm (personal communication from [redacted] Project Coordinator for Soybeans, Monsanto Agricultural Group).

Approximate consumption of soybean seeds was therefore 356 seeds/kg body weight bird per day.

CONCLUSIONS

No treatment related mortality or differences in food consumption, body weight gain, or behavior occurred between birds fed 20% w/w raw GTS meal and birds fed raw soybean meal from the parental line or basal diet only. Based on the parameters measured, the wholesomeness of raw meal from the GTS lines is not materially different than that of the parental line.

Appendix C. Additional information on sequence searches for homology comparisons to known allergens and toxins

In order to gain insight on the potential allergenicity or toxicity of CP4 EPSPS, which has no known allergenic or toxic properties, 121 allergenic proteins and 1,935 toxin proteins were extracted from the Pir protein, Swissprot, and Genpept protein databases to compare amino acid sequence homology to the CP4 EPSPS protein (128). These sequences were compiled by searching the databases for proteins with keywords matching the text pattern "allergen" or "toxin". If the CP4 EPSPS protein were found to have meaningful homology with a known protein allergen or toxin, further investigation into the protein may be warranted.

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

Methods

All of the protein comparisons were assisted by the Genetics Computer Group (GCG) of Madison, WI., sequence analysis software package (version 7.1 March 1992) (21). The CP4 EPSPS peptide sequence used for comparisons between the database of allergen and toxin proteins was translated from the plasmid pMON17190 (nucleotides 1820-3186) containing the CP4 EPSPS coding region. Note that the amino acid sequence of the CP4 EPSPS mature protein encoded by pMON17190.pep is identical to the amino acid sequence of the CP4 EPSPS protein encoded by pMON21104, which was utilized to provide CP4 EPSPS for safety studies (17). Files containing lists of known allergen and toxin proteins were developed using the GCG command "STRINGSEARCH". "STRINGSEARCH" searches the user defined databases for matches of the user defined "text pattern". Each of the available protein databases were analyzed separately using the following logicals:

"Allergen"

LOGICAL	DATABASE	SEARCH THROUGH	VERSION/DATE	OUTPUT FILE
Swissprot:*	Swisprot	Complete Records	Release 25 (Apr '93)	Swissprotall.strings
Genpept:*	GenPept	Complete Records	Release 71 (Mar '92)	Genpeptall.strings
Pir1:*	Pir Protein	Complete Records	Release 36 (Apr 93')	Pir1all.strings
Pir2:*	Pir Protein	Complete Records	Release 36 (Apr 93')	Pir2all.strings
Pir3:*	Pir Protein	Complete Records	Release 36 (Apr 93')	Pir3all.strings

"Toxin"

LOGICAL	DATABASE	SEARCH THROUGH	VERSION/DATE	OUTPUT FILE
Swissprot:*	Swisprot	Complete Records	Release 25 (Apr '93)	Swissprotalltox.strings
Genpept:*	GenPept	Complete Records	Release 71 (Mar '92)	Genpeptalltox.strings
Pir1:*	Pir Protein	Complete Records	Release 36 (Apr 93')	Pir1alltox.strings
Pir2:*	Pir Protein	Complete Records	Release 36 (Apr 93')	Pir2alltox.strings
Pir3:*	Pir Protein	Complete Records	Release 36 (Apr 93')	Pir3alltox.strings

For example, at the prompt:

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

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

FASTa is the standard method (141-144) for rapid comparison of a query sequence, or defined sequence of interest, to a entire nucleotide or protein database. CP4 EPSPS (pMON17190.pep) was the query sequence used in all FASTa comparisons. Instead of comparing CP4 EPSPS to each entire protein database, the FASTa comparison was divided into two separate databases limited to proteins recognized from "STRINGSEARCH" using the text pattern "allergen" and text pattern "toxin". FASTa uses the algorithm developed by Pearson and Lipman (141) to search for similarities between the query, CP4 EPSPS peptide sequence, and any group of sequences. Basically, the FASTa algorithm uses four steps to calculate three scores (*init1, initn, and opt*) that characterize sequence similarity. The first step uses a rapid technique for identifying shared identities or similarities between the two sequences. This method is similar to the technique which has been described by

Wilbur and Lipman (142). This rapid technique is based on a lookup table to locate identities between the query, CP4 EPSPS, and the database of allergens and toxins. The *ktup,* or word size, indicate how many consecutive identities are required for a match. By searching protein databases, the amino acid's chemical similarity or mutational similarity is also considered when a match is assigned. A *ktup* value of 2 is the standard or default value for protein database searches (21). A *ktup* value of 1, may be applied for a more sensitive database search, but this value also increases false positive matching between the query and database sequences. The first step in the calculation of the scoring matrix in FASTa is to identify the highest pairs (*ktup*=2) of identities shared between the two sequences. Next, the ten highest regions, without gaps in the sequence, that contribute to the highest scores, are rescanned using the PAM250 matrix (PAM = percent accepted mutation). The PAM250 (MDM_{78}) matrix (196,197) was derived from the amino acid replacement analysis among related proteins. It specifies a range of positive scores for common or likely mutations and a range of negative scores for unlikely substitutions or mutations. Specifically, the values are the log of the probability that the amino acid residue arose from the mutation of a common ancestor, divided by the probability that the sequences are related by chance. Positive values indicate that the amino acid residues are more likely than chance to have come from a common ancestor and negative numbers indicate that an evolutionary relationship is less likely than chance. The PAM250 scores of the initial alignment are summed and reported as the *init1*. In general, the higher the PAM250 score, the longer the stretches of homology between the two sequences. Next the *initn* score is calculated by joining the initial alignments (*init1*) minus a gap penalty (usually 20 for each gap). The *initn* scores are used to rank the homologies generated between the query and the database, from the highest homology to the lowest homology. Finally an *opt* (optimized) score is calculated considering only those residues that lie in a band 32 amino acid residues wide centered on the highest scoring region (*init1*). The results of the FASTa comparison with CP4 EPSPS to the allergen and toxin protein database are described in the next section.

Results

CP4 EPSPS was compared to the generated database of known allergens and toxins using the FASTa algorithm for rapid database comparison. The results of the FASTa comparison used *ktup*=2 for each of the protein database. The PAM250 scores used for ranking the sequences (*initn*) ranged from 38 to 9 for the allergen database. A range was not generated for the toxin database since only the top 50 scores for each of the protein databases are reported.

A low PAM250 score or *initn* score indicates that the length of homology or similarity between the two sequences is short. A low *initn* score does not necessarily indicate that significant homology does not exist. For example, the length of the allergenic proteins or allergenic protein fragments ranged between 10 amino acids and 398 amino acids. Since the generated database of allergens consisted of several short allergenic peptides, 100% identity between the

pMON17190.pep and the short allergenic peptide may generate a low *initn* score. For example, a peptide of ten amino acid identical to the first ten N-terminal amino acids of CP4 EPSPS was compared using the FASTa algorithm. An *initn* score of only 43 was calculated, even though there was 100% identity between the two sequences. Also another test of the FASTa algorithm was performed by comparing CP4 EPSPS to the entire database of protein sequences. *Initn* scores of up to 805 were generated from the protein database to the CP4 EPSPS, and the highest ranking *initn* scores were from *Aro* genes (encoding EPSPS proteins), in which considerable homology is expected. Therefore, it is the task of the biologist to independently examine each of the alignments for potential biological significance. If the proteins in the database are of comparable length to the query, then a low *initn* score indicates little or no biological homology, unless the homology can be assigned to an active site in the protein or a site known to be important for its function or activity.

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

The significance of the alignments was tested using the GCG command "SHUFFLE" to randomize pMON17190.pep, but keeping the amino acid composition identical. This type of analysis using a randomized protein to determine the statistical significance of the alignments is known as Monte Carlo analysis (198). The resulting randomized CP4 EPSPS was named pMON17190.shufl. The amino acid alignment between pMON17190.pep and pMON17190.shufl was constructed using the GCG program- "GAP". The gap comparison using the default conditions of the GAP program determined that the two proteins shared only 19.570% identity and 44.869% similarity. The shuffled protein (pMON17190.shufl) was compared to the identical database of allergenic proteins and toxin proteins using the same word size (*ktup*=2) as pMON17190.pep. The range of *initn* scores for the randomized peptide was compared to the database of allergens was between 41 and 12. A range was not generated for the toxin sequences since only the top 50 scores are reported. However, the *initn* scores from the allergen database and the toxin database, generated with the randomized CP4 EPSPS, resembled the *initn* scores generated with the unrandomized CP4 EPSPS. This indicates that the generated alignments between the database of allergen proteins and toxin proteins was determined by random distribution of amino acids in a peptide sequence. There also was no alignment which generated 100% homology to the pMON17190.shufl or CP4 EPSPS (pMON17190.pep).

The 10 highest scores from each database were compared between the CP4 EPSPS and the scrambled CP4 EPSPS. The results from one randomization of the CP4 EPSPS peptide sequence clearly indicates that CP4 has no relationship, other than a random distribution of its amino acids, to the database of allergens and toxins.

Conclusion

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