Glyphosate-Tolerant Cotton: The Composition of the Cottonseed Is Equivalent to That of Conventional Cottonseed

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An important aspect of the safety assessment of genetically modified crops to be used for human food and animal feed is the product composition, including nutrients and antinutrients. Cotton lines have been developed that are tolerant to glyphosate, the active ingredient in the herbicide Roundup. The glyphosate-tolerant lines were generated by the stable insertion of a glyphosate tolerance gene in a common variety of cotton. The glyphosate tolerance gene encodes for 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium sp.* strain CP4 (CP4 EPSPS). The composition of the cottonseed and oil from two glyphosate-tolerant lines (GTCot), 1445 and 1698, was compared to that of the parental variety Coker 312 and to published values for other commercial cotton varieties. The nutrients measured in the cottonseed were protein, fat, fiber, carbohydrate, calories, moisture, ash, amino acids, and fatty acids. The antinutrients measured in the cottonseed included gossypol, cyclopropenoid fatty acids, and aflatoxin. In addition, the fatty acid profile and α-tocopherol levels were measured in the refined oil. These analyses demonstrated that the glyphosate-tolerant cotton lines are compositionally equivalent and as safe and nutritious as the parental and conventional cotton varieties commercially available.

Keywords: *Cotton; genetically modified; herbicide tolerant; Roundup*

INTRODUCTION

Genetic modification of crops offers the potential to improve crop varieties. New varieties which resist pests or diseases or possess improved quality characteristics are currently under development and nearing market introduction (Gasser, 1989; Fuchs et al., 1993a). Other crops in development are tolerant to nonselective herbicides, such as glyphosate-tolerant soybeans (Padgette et al., 1996b) and glyphosate-tolerant cotton (GTCot, denoted as Roundup Ready cotton, trademark of Monsanto Co.). Glyphosate is the active ingredient in the broad-spectrum, nonselective herbicide Roundup. Due to the sensitivity of cotton and other crops to glyphosate, the grower has been unable to use this herbicide over the top of growing crops to control weeds. The biochemical target site of action of glyphosate is the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) naturally present in plants, bacteria, and fungi (not in animals) as a component of the shikimate pathway of aromatic amino acid biosynthesis (Levin and Sprinson, 1964). Two cotton lines, 1445 and 1698, have been modified to become tolerant to glyphosate by expressing a gene that encodes a glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium sp.* CP4 (CP4 EPSPS) (Barry et al., 1992; Padgette et al., 1995b). The use of GTCot will provide the grower with an effective weed control option and enable the grower to take advantage of glyphosate's positive environmental and safety characteristics.

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Cottonseed provides an important source of oil for human consumption and meal for animal feed (*Cottonseed and Its Products*, 1989). The primary use of seed-cotton (non-delinted cotton seed) is for cattle feed (*Cottonseed and Its Products*, 1989). Cottonseed is processed into four major products as follows: oil, meal, hulls, and linters (Cherry and Leffler, 1984). Cottonseed oil is a premium quality oil that is used in a variety of foods including salad and cooking oils, mayonnaise, salad dressing, shortening, margarine, and packing oil. Cottonseed meal is principally sold as feed for cattle, swine, poultry, and fish (*Cottonseed and Its Products*, 1989). Therefore, the composition of the cottonseed has been extensively evaluated to confirm that these products derived from the glyphosate-tolerant cotton varieties are equivalent to the parental variety as well as to conventional cotton lines.

In addition to the CP4 EPSPS gene, the genes encoding neomycin phosphotransferase II (NPTII) as a plant selectable marker (DeBlock et al., 1984; Flavell et al., 1992) and aminoglycoside adenylyltransferase (AAD) as a bacterial selectable marker (Fling et al., 1985) are also present in both GTCot lines. The safety of the CP4 EPSPS and NPTII proteins has been demonstrated (Harrison et al., 1996; Fuchs et al., 1993b). NPTII has been approved by the FDA as a processing aid food additive for use in cotton, canola, and tomato (FDA, 1994) and exempted from the requirements of a tolerance by EPA (EPA, 1994).

There is no reported food use of protein products of cottonseed in the United States, due to the presence of the endogenous toxicants gossypol and cyclopropenoid fatty acids (Morgan, 1990; *Cottonseed Oil*, 1993). Refined, bleached, deodorized cottonseed oil and highly processed cottonseed linters (the fiber remaining after ginning seed-cotton) are the only cotton products used for human consumption. Since the linters are essentially comprised of cellulose (>99.9%), the composition of the fiber from GTCot lines has not been analyzed.

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The composition of the seed and oil from the two GTCot lines across six locations for 2 years was compared to the Coker 312 control seed composition and literature ranges previously reported for cotton varieties. Data presented in this paper demonstrate that the levels of nutrients (protein, fat, fiber, ash, carbohydrates, calories, amino acids, and fatty acids) and antinutrients (gossypol, cyclopropenoid fatty acids, and aflatoxin) in the glyphosate-tolerant cottonseed are comparable to those in the parental variety and other commercial cotton varieties. In addition, the fatty acid profile, α-tocopherol levels, and gossypol levels in refined oil were also measured and shown to be similar to the parental control values and within established ranges for commercial varieties. The results confirm that the cottonseed and the products produced from these glyphosate-tolerant cotton lines are compositionally equivalent to the parental variety and other commercial varieties.

MATERIALS AND METHODS

Glyphosate-Tolerant Cotton Lines. Two independent glyphosate-tolerant cotton lines, 1445 and 1698, were generated and characterized as described in Nida et. al. (1996). Seed from R_4 and R_5 generations were planted for the 1993 and 1994 field trials, respectively, to generate seed material of lines 1445 and 1698 for analysis. Parental control line Coker 312 was obtained from SeedCo., Inc. (Lubbock, TX). These lines were grown at the following field sites in 1993: Starkville, MS; Bossier City, LA; West Sinton, TX; Tifton, GA; Maricopa, AZ; and Loxley, AL. The field sites utilized for the 1994 study were located in Proctor, AR; Waukena, CA; Washington, LA; Choctaw, MS; Raymondville, TX; and Levelland, TX. All field studies were conducted under good laboratory practices (GLP) guidelines. Samples of seed-cotton from individual field sites were harvested, ginned, acid-delinted, and analyzed for composition.

Processing Experiments. A portion of the 1993 seed samples was composited by line to produce a bulk sample for processing into selected fractions for subsequent analyses. Cottonseed was processed into refined oil and toasted meal at the Food Protein Research and Development Center (formerly the Engineering and Biosciences Research Center) at Texas A&M University (College Station, TX) under GLP using a solvent extraction method (Cherry and Leffler, 1984). The processing procedure was performed to mimic commercial procedures, although the scale was much smaller. The bulk samples were mechanically delinted, dehulled, and solvent extracted to produce oil and meal. The processed fractions generated were full fat flour, toasted meal, and refined oil.

Compositional Analysis. All compositional analyses were performed under GLP at Hazleton-Wisconsin (Madison, WI) with the exception of the 1993 analyses for gossypol levels and fatty acid profiles, which were performed at USDA-ARS Southern Crop Research Laboratory (College Station, TX) and Texas A&M University (College Station, TX), respectively. All analyses on seed samples were performed from both the 1993 and 1994 studies, with the exception of the aflatoxin measurements, which were performed only for the 1993 studies. Analyses on the processed fractions (gossypol in full fat flour, toasted meal, and refined oil and α-tocopherol in refined oil) were not repeated in 1994 because no differences were observed in the processed fractions from 1993, and the processing was not repeated.

Proximate Analysis. Proximate analysis (protein, fat, ash, moisture, carbohydrates, and calories) was performed on ginned, acid-delinted cottonseed. Protein levels were estimated by determining the total nitrogen content using the Kjeldahl method, as previously described (Bradstreet, 1965; Kalthoff and Sandell, 1948; AOAC, 1990a). Protein was calculated from total nitrogen using N × 6.25.

Fat content was estimated by the Soxhlet extraction method (AOAC, 1990b). The sample of seed tissue was dried to remove excess moisture followed by extraction with pentane. The extract was evaporated, dried, and quantitated gravimetrically to calculate percent fat. Ash content was measured according to AOAC methods (AOAC, 1984). The sample was ignited to ash at 550 °C in a muffle or electric furnace to remove volatile organic matter. The residue was quantitated gravimetrically, and calculations were made to determine percent ash. Moisture content was determined by loss of weight upon drying in a vacuum oven at 100 °C to constant weight, as previously described (AOAC, 1990c). Carbohydrate levels were estimated by using the fresh weight-derived data and the following equation (USDA, 1975a):

$$\%\ \text{carbohydrate} = 100\% - (\%\ \text{protein} + \%\ \text{fat} + \%\ \text{ash} + \%\ \text{moisture})$$

Calories were calculated using the Atwater factors with the fresh weight-derived data and the following equation (USDA, 1975b):

$$\text{calories (kcal/100 g)} = (4 \times \%\ \text{protein}) + (9 \times \%\ \text{fat}) + (4 \times \%\ \text{carbohydrates})$$

Amino Acid Composition. Acid-delinted, ground cottonseed samples were hydrolyzed with hydrochloric acid, adjusted to pH 2.2. Individual amino acids were quantitated using an automated amino acid analyzer. This assay was based on previously published references (AOAC, 1990d). The analytical standards used for these analyses were K18 (Beckman, Fullerton, CA; lot no. A304008), L-tryptophan (Sigma Chemical Co., St. Louis, MO; lot no. 52H0717), cysteic acid monohydrate (Sigma, lot no. 50H2616), and methionine sulfone (Sigma, lot no. 49F0113).

α-Tocopherol. Refined oil samples (from the 1993 trial) were saponified to release the α-tocopherol, which was then extracted with organic solvent followed by quantitation on an HPLC silica gel column using fluorescence detection (Cort et al., 1983; Speek et al., 1985; McMurray et al., 1980). The analytical standard for this method was USP (Rockville, MD) α-tocopherol, lot K.

Aflatoxin. The levels of aflatoxins B_1, B_2, G_1, and G_2 were determined on ground, acid-delinted cottonseed samples from the 1993 study. The seed samples were washed with a dilute hydrochloric acid solution and chloroform extracted. A portion of the extract was applied to a silica gel column. Aflatoxins were eluted with methylene chloride/acetone and concentrated with a rotary evaporator. The extracts were separated by high-performance liquid chromatography and compared to a known standard (Third International Congress of Food Science and Technology, 1994; JAOAC, 1988a−c). The analytical standard for this method was aflatoxin mix-M (Supelco, Bellefonte, PA; lot no. LA39657).

Lipid Determination and Fatty Acid Analyses. Lipids were extracted using a double-Bligh and Dyer procedure (Bligh and Dyer, 1959), as described by Wood (1991). Lipid was extracted from samples using a chloroform/methanol solvent. The dry weight of the sample and the weight of the extracted lipid were used to calculate the total percentage lipid in the sample.

An aliquot of the lipid was saponified with mild alkaline hydrolysis procedure to obtain free fatty acids (Wood, 1986a). The saponified lipid was extracted using ethyl ether:hexane (1:1), and the sample was dried by evaporation (Wood and Lee, 1983). The phenacyl derivatives were analyzed by high-performance liquid chromatography according to Wood (1986a,b). Peak elution order and peak shape were monitored by a strip chart recorder. The absorption data for each peak were collected directly from the ultraviolet monitor and integrated for percent of total peak area. Peak area for each fatty acid was directly proportional to the percent of each fatty acid contained in total lipid. The analytical standard for the fatty acid analyses was commercial cottonseed oil.

Measurement of Free and Total Gossypol Levels. For the 1993 study, free and total gossypol levels were measured in cottonseed, full fat flour, toasted meal, and refined cottonseed oil under GLP at the USDA-ARS Southern Crop Research Laboratory (College Station, TX). Prior to gossypol analysis,

Table 1. Summary of Proximate Analysis of Cottonseed from Glyphosate-Tolerant and Coker 312 Cotton Lines

	1993 mean[d] (range)[b]			1994 mean[d] (range)[b]			
characteristic[a]	C312	1445	1698	C312	1445	1698	lit. range
protein, %	27.8 (24.6−28.9)	29.6[c] (25.6−31.3)	29.5[c] (25.7−30.7)	28.8 (27.0−30.6)	30.6[c] (28.2−31.9)	29.4 (27.9−30.9)	12−32[e]
fat, %	23.3 (20.5−24.8)	23.8 (19.5−26.1)	23.8 (20.8−25.6)	24.4 (23.8−25.5)	25.3[c] (24.6−26.7)	24.9 (24.1−26.3)	16.1−26.7[f]
ash, %	4.5 (4.1−4.9)	4.7 (4.2−5.2)	4.6 (4.1−5.1)	4.4 (3.7−4.9)	4.5[c] (3.8−5.0)	4.3 (3.5−4.7)	4.1−4.9[g]
carbohydrate, %	44.4 (41.9−46.2)	41.9[c] (39.2−44.0)	42.1[c] (39.2−44.1)	42.4 (41.0−44.4)	39.6[c] (38.0−42.0)	41.4[c] (40.4−42.2)	
calories/100 g	499 (483−505)	500 (477−512)	501 (484−510)	505 (501−513)	508 (504−514)	508 (503−514)	
moisture, %	11.6 (9.1−14.1)	11.1 (9.0−13.0)	11.1 (9.0−13.8)	6.7 (5.5−7.4)	7.5 (5.8−13.5)	6.9 (5.8−8.6)	5.4−10.1[h]

[a] Protein, fat, ash, carbohydrate, and calories reported as percent dry weight of sample. [b] Range denotes the lowest and highest individual values across sites for each line. [c] Statistically significant from Coker 312 control line at the 5% level (paired *t*-test). [d] Value reported is least-squares mean of six samples. [e] Turner et al., 1976; Cherry et al., 1978a; Kohel et al., 1985. [f] Cherry and Leffler, 1984; Cherry et al., 1978a; Cherry et al., 1978b. [g] Cherry et al., 1978b; Belyea et al., 1989. [h] Cherry et al., 1978a.

the toasted meal samples were placed in a vacuum desiccator to reduce moisture percentage so that it would be similar to the ground cottonseed samples. In the 1994 study, free and total gossypol were measured at Hazleton-Wisconsin (Madison, WI) in cottonseed. Evaluation of free gossypol levels was completed via high-performance liquid chromatography according to the procedure described by Stipanovic et al. (1988) and AOCS (1989a). Total gossypol levels (corrected for moisture) were measured spectrophotometrically using aniline as a complexing agent (Pons *et al*., 1958; AOCS, 1989b). The analytical standard for these analyses was gossypol acetic acid obtained from Sigma, lot no. 102H4038.

Statistical Analysis of the Data. Statistical analyses were performed using the SAS statistical program (SAS Institute, 1990) on compositional parameters of the cottonseed. Each characteristic analyzed consisted of six values (from the six locations) for each of the two GTCot lines and the Coker 312 control line. The individual values were converted from fresh weight to a dry weight (DW) basis using the percent moisture values, as follows: DW value = fresh weight value/(1 − moisture/100). The exceptions to this conversion were the fatty acid values (reported as percent lipid) and moisture (reported on fresh weight basis). The amino acid values were analyzed by dry weight on per unit of protein basis. The data collected in the 1993 and 1994 studies were analyzed independently. The experiments had a randomized complete block design, with line as a treatment effect and location as a blocking effect. The means and standard errors were calculated; the means of Coker 312 and the test lines were compared using a *t*-statistic and pooled error. No statistical analysis was performed on components measured on the bulk processed samples because there was only one sample per line.

RESULTS

The strategy employed for assessing the safety of the GTCot lines was 3-fold: (1) each genetic insertion was characterized to identify the portion of the plasmid DNA that inserted into the plant genome and to show that the inserted DNA behaved in a typical Mendelian fashion (Nida et al., 1996); (2) the safety of the two expressed proteins (CP4 EPSPS and NPTII) was demonstrated by producing the plant equivalent protein in *Escherichia coli*, confirming the rapid digestion of each protein in a simulated mammalian model system, and establishing the lack of adverse effects for each protein in acute toxicity test (Harrison et al., 1996; Fuchs et al., 1993b); and (3) the composition of the cottonseed and refined oil from GTCot lines 1445 and 1698 was evaluated and demonstrated to be substantially equivalent to that of the control line and similar to that of other commercial varieties. The focus of the compositional studies was cottonseed, since cottonseed is the starting material for all other processed cottonseed products. Processed cottonseed oil and cottonseed meal were generated in the 1993 study for analyses.

Proximate Analysis of Cottonseed. The levels of the major components of cottonseed (protein, fat, ash, moisture, carbohydrate, and calories) were determined for cottonseed from each of the field test sites in 1993 and 1994. The results are presented in Table 1. In the 1993 study, there were several minor differences between the GTCot lines and the Coker 312 control line that were determined to be statistically significant ($p < 0.05$). These differences were noted for protein (29.59% for line 1445 and 29.53% for line 1698 versus 27.76% in the control) and carbohydrate (41.91% in line 1445 and 42.06% in line 1698 versus 44.35% in the control). Although the differences are statistically significant, they are not considered to be meaningful differences in nutritional value of the seed for the following reasons. The levels of protein for all of the lines evaluated fall well within reported ranges for cottonseed from commercial cultivars. In addition, the ranges for percent protein and carbohydrate in GTCot lines overlap with the levels for cottonseed from Coker 312 (Table 1). The percent moisture content for all three lines in 1993 was higher than that previously published for cottonseed. No published data were available for carbohydrate and calorie content of cottonseed, so comparisons to literature values could not be made. These parameters are not standard for assessing the composition of cottonseed.

In the 1994 study, statistical differences were apparent with line 1445 for protein (30.55% versus 28.80% for Coker 312 control), fat (25.27% versus 24.43%), ash (4.53% versus 4.35%), and carbohydrate (39.62% versus 42.40%) and with line 1698 for carbohydrate (41.37% versus 42.40%). With the large number of components included in the SAS comparison, a number of statistical differences would be expected by chance. Adjusting the *p*-value by the number of comparisons to obtain a conservative critical *p*-value to identify clear significance indicated that the decrease in the carbohydrates observed with line 1445 in the 1994 study was clearly significant, whereas the other differences were not. All of the differences noted were small and within the previously reported ranges for cottonseed; therefore they were regarded as biologically insignificant.

Amino Acid Composition. Of the 18 amino acids measured, there were no statistically significant differences in the levels of any of the amino acids compared

Table 2. Amino Acid Composition of Cottonseed from Glyphosate-Tolerant and Coker 312 Cotton Lines[a]

	literature[c]		1993[d]			1994[d]		
amino acid	max	min	C312	1445	1698	C312	1445	1698
aspartic acid	9.5	8.8	9.6	9.6	9.7	9.4	9.4	9.3
threonine	3.2	2.8	3.5	3.4	3.4	3.4	3.4	3.4
serine	4.4	3.9	4.8	4.7	4.7	4.7	4.7	4.7
glutamic acid	22.4	20.5	18.9	19.6	19.6	19.8	19.3	19.5
proline	4.0	3.1	4.1	4.0	4.0	3.7	3.6	3.7
glycine	4.5	3.8	4.3	4.3	4.3	4.4	4.3	4.3
alanine	4.2	3.6	4.1	4.1	4.0	3.8	3.7	3.7
cysteine	3.4	2.3	1.7	1.6	1.6	1.6	1.6	1.6
valine	4.7	4.3	4.2	4.3	4.3	4.0	4.0	4.0
methionine	1.8	1.3	2.0	1.7	1.7	1.5	1.5	1.5
isoleucine	3.4	3.0	3.1	3.1	3.1	2.9	2.8	2.9
leucine	6.1	5.5	6.0	6.0	6.0	5.9	5.8	5.9
tyrosine	3.3	2.8	2.9	2.9	2.9	2.7	2.7	2.7
phenylalanine	5.6	5.0	5.2	5.3	5.3	5.2	5.1	5.1
lysine	4.1	3.9	4.7	4.7	4.6	4.6	4.5	4.5
histidine	2.8	2.6	3.0	2.9	2.9	3.0	3.0	3.0
arginine	12.3	10.9	11.6	11.6	11.6	11.0	11.1	11.1
tryptophan	1.4	1.0	1.0	1.0	1.0	1.1	1.1	1.1

[a] Amino acids reported as mg/kg dry weight of protein in the cottonseed. [b] Significantly different from the Coker 312 control line at the 5% level (paired *t*-test). [c] Lawhon, 1977. [d] Value reported is least-squares mean of six samples.

to the control line, across both 1993 and 1994 (Table 2). The amino acid values were evaluated on a per unit of protein basis to compare the resulting profiles. The results were also within the ranges previously reported for cottonseed (Lawhon et al., 1977).

Since EPSPS catalyzes a step in the aromatic amino acid biosynthetic pathway, it was important to determine if expression of CP4 EPSPS influenced the levels of the aromatic amino acids in GTCot lines. EPSPS is not the rate-limiting step in aromatic amino acid biosynthesis (Herrmann, 1983; Weiss and Edwards, 1980). Therefore increased EPSPS activity would not be expected to increase the levels of aromatic compounds in plants. Data from GTCot lines 1445 and 1698 grown in six sites for 2 years establish that no statistically significant increase in the aromatic amino acids tyrosine, phenylalanine, or tryptophan accompanies the presence of the CP4 EPSPS gene or enzyme. These data are consistent with that of glyphosate-tolerant soybean, in which no impact of the CP4 EPSPS was detected on the levels of aromatic compounds (Padgette et al., 1996a).

Total Lipid Content and Fatty Acid Profile. Percent lipid and fatty acid profiles were evaluated in cottonseed for both 1993 and 1994 studies and in refined oil for 1993. No differences in total lipids in the GTCot lines compared to the control line were detected. The fatty acid profiles for cottonseed from the GTCot lines were similar to cottonseed from the Coker 312 control (Table 3). No significant differences were detected in 1993. Minor differences were detected in 1994, but these values were within the literature ranges available for refined cottonseed oil.

The fatty acid composition of refined oil was assessed from processing of the composited seed sample for each line. Therefore, the data were not subjected to statistical analyses. A summary of the fatty acid profiles (including cyclopropenoid fatty acids) for the refined oil samples showed that the values for lines and the Coker 312 control were consistent with each other and with literature ranges for fatty acid levels of cottonseed oil from commercial varieties (Table 4).

Included in the fatty acid profile are the cyclopropenoid fatty acids which are considered as antinutrients. The levels of cyclopropene acids must be minimized due to undesirable effects in food and feed products (Cherry and Leffler, 1984; Phelps et al., 1965). Malvalic acid and sterculic acid are unique fatty acids common in cotton. Malvalic and sterculic acids are 17 and 18

Table 3. Lipid and Fatty Acid Composition of Cottonseed from Glyphosate-Tolerant and Coker 312 Cotton Lines

	1993 mean[d] (range)[a]			1984 mean[d] (range)[a]		
component[c]	C312	1445	1698	C312	1445	1698
lipid	32.7 (31.2−33.9)	32.2 (30.2−34.5)	33.4 (31.2−35.9)	26.2 (23.5−28.1)	26.7 (26.3−27.2)	26.8 (25.6−28.8)
myristic (14:0)	0.97 (0.89−1.17)	0.95 (0.84−1.03)	1.06 (0.96−1.15)	0.78 (0.66−1.00)	0.69 (0.52−0.90)	0.76 (0.58−1.00)
pentadecanoic (15:0)	1.0 (0.5−2.3)	0.56 (0.3−0.8)	1.3 (0.4−2.4)	0.16 (0.1−0.2)	0.15 (0.1−0.2)	0.16 (0.1−0.2)
palmitic (16:0)	27.7 (25.8−28.6)	26.8 (26.0−27.8)	26.0 (24.2−27.6)	24.6 (22.7−27.1)	24.8 (22.6−28.2)	26.3[b] (22.6−28.2)
palmitoleic (16:1)	0.64 (0.56−0.77)	0.65 (0.61−0.69)	0.69 (0.61−0.79)	0.39 (0.30−0.45)	0.35 (0.29−0.40)	0.42 (0.31−0.51)
stearic (18:0)	2.7 (2.4−3.4)	2.7 (2.3−3.0)	2.5 (2.2−2.8)	2.0 (1.7−2.8)	2.3 (1.9−2.8)	1.8 (1.7−2.1)
oleic (18:1)	15.3 (13.9−15.8)	15.5 (14.4−16.8)	14.8 (13.8−15.7)	15.3 (14.7−16.0)	15.0 (13.7−16.4)	14.2[b] (13.1−15.0)
linoleic (18:2)	43.2 (36.3−47.3)	45.9 (43.9−47.0)	43.8 (36.1−51.5)	55.3 (52.3−57.3)	55.4 (52.7−58.5)	55.2 (53.4−58.9)
linolenic (18:3)	0.16 (0.08−0.31)	0.21 (0.13−0.38)	0.20 (0.14−0.30)	0.14 (0.11−0.17)	0.13 (0.10−0.19)	0.16 (0.11−0.23)
arachidic (20:0)	0.24 (0.21−0.29)	0.29 (0.24−0.34)	0.28 (0.23−0.37)	0.18 (0.15−0.22)	0.21[b] (0.17−0.24)	0.18 (0.15−0.19)
behenic (22:0)	0.15 (0.10−0.27)	0.17 (0.11−0.38)	0.11 (0.02−0.16)	0.10 (0.10−0.12)	0.11 (0.10−0.12)	0.11 (0.10−0.12)
malvalic (C-17)	0.43 (0.25−0.58)	0.41 (0.21−0.58)	0.41 (0.29−0.60)	0.33 (0.31−0.36)	0.34 (0.29−0.40)	0.28[b] (0.19−0.33)
sterculic (C-18)	0.71 (0.52−0.92)	0.70 (0.56−0.98)	0.74 (0.48−0.94)	0.24 (0.12−0.77)	0.18 (0.11−0.37)	0.14 (0.10−0.20)
dihydrosterculic	1.12 (0.34−3.39)	0.58 (0.27−1.07)	1.69 (0.34−3.46)	0.13 (0.11−0.15)	0.13 (0.10−0.15)	0.10[b] (0.10−0.13)

[a] Range denotes the lowest and highest individual value across sites for each line. [b] Significantly different from the Coker 312 control at the 5% level (paired *t*-test). [c] Value of lipid is % of dry sample weight. Value of fatty acid is % of total lipid. [d] Values presented are least-squares mean and ranges of six samples per line.

Table 4. Oil Composition from Glyphosate-Tolerant Cotton Lines and Coker 312 Control

component	lit. range	1993 refined oil C312	1445	1698
	Fatty Acids[a]			
myristic (14:0)	0.5−2.5[d] 0.68−1.16[e]	0.95	0.84	0.93
palmitic (16:0)	17−29[d] 21.63−26.18[e]	25.54	25.14	25.42
palmitoleic (16:1)	0.5−1.5[d] 0.56−0.82[e]	0.64	0.61	0.63
stearic (18:0)	1.0−4.0[d] 2.27−2.88[e]	2.46	2.41	2.53
oleic (18:1)	13−44[d] 15.17−19.94[e]	15.03	14.53	14.51
linoleic (18:2)	33−58[d] 49.07−57.64[d]	50.10	51.27	50.44
linolenic (18:3)	0.1−2.1[d] 0.23[f]	0.14	0.16	0.14
arachidic (20:0)	<0.5,[d] 0.41[f]	0.26	0.27	0.24
behenic (22:0)	<0.5[d]	0.12	0.08	0.11
sterculic (C-18)	0.08−0.56[g]	0.44	0.50	0.53
malvalic (C-17)	0.22−1.44[g]	0.35	0.56	0.46
dihydrosterculic (C-19)		0.23	0.23	0.36
	Antinutrients/Vitamins[c]			
total gossypol	≤0.01% (1 ppm)[e]	ND[b]	ND	ND
free gossypol	≤0.01% (1 ppm)[e]	ND	ND	ND
α-tocopherol[h]	136−660[i]	670	588	624

[a] Reported as % of total lipids. One sample per line from a composite seed sample. [b] ND = not detected (limit of detection was 0.04% and 0.002% for measurement of total and free gossypol in oil, respectively). [c] Free and total gossypol reported as % weight; tocopherol reported as mg/kg. [d] FAO/WHO Codex Alimentarius committee on fats and oils (*Cottonseed Oil*, 1993). [e] Cherry and Leffler, 1984. [f] Cherry, 1983. [g] Phelps et al., 1965. Values reported for crude cottonseed oil. [h] α-Tocopherol reported as mg/kg of oil. [i] Rossel, 1991; Dicks, 1965.

carbons long, respectively, and contain a double bond at the propene ring. The cyclopropenoid fatty acids affect membrane permeability and increase the melting point of oils by inhibiting the desaturation of stearic to oleic acid. The levels of cyclopropenoid fatty acids are decreased during processing, with the greatest point of deactivation during the deodorization of the refined oil (*Cottonseed Oil*, 1993). The levels of the cyclopropenoid fatty acids, as well as of dihydrosterculic acid, were similar in cottonseed and refined oil from Coker 312 and the GTCot lines (Table 3). The levels of the cyclopropenoid fatty acids were also within the range reported in the literature for refined oil from commercial varieties (Table 4).

Gossypol Analyses. Gossypol is a terpenoid substance produced in discrete glands present in various cotton tissues, including the seed (Abou-Donia, 1976). It is associated with discoloration and toxicity problems in food and feed products of cottonseed (Berardi and Goldblatt, 1980). Gossypol is typically measured in two forms, free and total. Free gossypol is the physiologically active form. The levels of free gossypol are decreased during heat processing due to the binding of gossypol with proteins, thus making it unavailable and essentially biologically inactive (Cherry and Leffler, 1984; Berardi and Goldblatt, 1980).

For the 1993 study, total and free gossypol levels were evaluated in full fat flour, toasted meal, and refined oil. In addition, levels of total gossypol were measured in cottonseed collected across field test locations. As expected the levels of free gossypol in the full fat flour were similar to that in the seed and decreased to nondetectable levels after processing to generate toasted meal. Similarly, no gossypol was detected in the refined oil (Table 4). Because only one bulk sample of full fat flour, toasted meal, and refined oil was available for the analyses, no statistics were performed on those values. The FDA guidelines allow a maximum of 0.06% free and 1.2% total gossypol for cottonseed meal used as chicken feed and no greater than 0.01% free gossypol when cottonseed meal is used as a protein supplement for swine (Berardi and Goldblatt, 1980).

Table 5. Gossypol Levels Determined in Seed, Full Fat Flour, and Toasted Meal from Glyphosate-Tolerant and Coker 312 Cotton Lines

	total gossypol, %, mean[b] (range) 1993	1994	free gossypol, %, mean[b] (range) 1993	1994
		Cottonseed		
C312	1.19 (0.99−1.46)	0.90 (0.67−1.02)	NA[d]	0.77 (0.55−0.86)
1445	1.32[a] (1.13−1.63)	1.02[a] (0.84−1.17)	NA	0.90[a] (0.75−1.01)
1698	1.01[a] (0.81−1.22)	0.88 (0.72−1.07)	NA	0.75 (0.61−0.84)
		Full Fat Flour[c]		
C312	1.05	NA	0.70	NA
1445	1.35	NA	0.83	NA
1698	0.97	NA	0.66	NA
		Toasted Meal[c]		
C312	0.99	NA	ND[d]	NA
1445	1.30	NA	ND	NA
1698	0.86	NA	ND	NA

[a] Values are statistically significant compared to the Coker 312 at $p = 0.05$ using a pairwise *t*-test. [b] Values, expressed as % dry weight, reported for seed samples are the least-squares mean (from statistical analyses) of six samples; ranges represent the lowest and highest values. [c] Values reported from full fat flour and toasted meal samples are one value obtained from processing fractions generated from the composite of seed across field sites. [d] NA = not analyzed, ND = not detectable (limit of detection for measurement of free gossypol in toasted meal = 0.007%).

The total gossypol levels in the cottonseed were measured in the cottonseed, and the values were statistically analyzed (Table 5). The GTCot line 1698 value was statistically lower than the Coker 312 control value (1.01% versus 1.19%), and the line 1445 value was statistically higher than the Coker 312 control value (1.32% versus 1.19%). The ranges of values overlapped for the three lines, and all values obtained were within the range of previously reported levels for cottonseed grown under various field conditions, 0.39−1.70% (Berardi and Goldblatt, 1980; Abou-Donia, 1976).

For the 1994 study, the total and free gossypol levels in the seed were measured and statistically analyzed. No significant differences were apparent with line 1698; however, line 1445 was again statistically higher than the Coker 312 control for both total and free gossypol levels (1.023% versus 0.902% total gossypol and 0.903% versus 0.774% free gossypol). As before, the results were well within the previously published ranges for gossypol in cottonseed. However, the results were clearly significant using the conservative critical *p*-value. Therefore, to determine if the gossypol increase was associated with the glyphosate tolerance locus, three commercial varieties which had been crossed with line 1445 to transfer the glyphosate-tolerant traits into commercial material were evaluated for gossypol levels. Three genotypes containing the glyphosate-tolerant trait and the negative control isoline (a sister line generated during the cross that did not receive the glyphosate-tolerant trait) were analyzed for free and total gossypol at Hazleton-Wisconsin (Madison, WI). There were no trends for higher gossypol (total or free) with the

Table 6. Gossypol Levels in Cottonseed from Commercial Varieties Crossed with Line 1445l

genotype	free gossypol	total gossypol
variety A negative[a]	0.87	0.95
variety B negative	0.80	0.84
variety C negative	0.70	0.78
variety A positive[b]	0.85	0.96
variety B positive	0.77	0.95
variety C positive	0.72	0.76

[a] Negative refers to the control isoline that did not contain the glyphosate tolerance trait. [b] Positive refers to the isoline that contained the glyphosate-tolerance trait.

positive lines containing the glyphosate tolerance trait versus the negative control lines which did not contain the trait (Table 6). Therefore the isoline experiment provides supporting evidence that the increase in gossypol is not associated with the glyphosate tolerance trait. We conclude that the increased gossypol levels are not associated with the glyphosate tolerance locus.

Aflatoxin Analyses. Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* that may contaminate food and feed products (Jorgensen and Price, 1981). Cottonseed is one of the commodities most commonly contaminated by aflatoxins (Bagley, 1979). Contamination can be very difficult to prevent or control because it may occur either in the field before harvest or during storage after harvest (Goldblatt and Dollear, 1977). The detection and detoxification of aflatoxin in food and feed products are critical due to the human and animal health risks (Scott, 1991). The maximum action level allowed by the FDA is 20 μg/kg (20 ppb) (Jorgensen and Price, 1981). The levels of the four primary aflatoxins (B1, B2, G1, and G2) in the cottonseed for the glyphosate-tolerant lines and the control line at all field 1993 sites were shown to be undetectable at a sensitivity of 1 ppb.

α-Tocopherol Analysis. Tocopherols are compounds naturally present in cottonseed oil. They serve as antioxidants which enhance storage properties and have vitamin E potency. The levels of tocopherol vary in nature and are affected by processing. They are reduced primarily during the steps of refining and deodorizing (*Cottonseed Oil*, 1993). α-Tocopherol levels, measured in refined oil prepared from GTCot lines 1445 and 1698 and the Coker 312 line, were 588, 624, and 670 mg/kg of oil, respectively (Table 4). These levels were similar to levels of 136−660 mg/kg of oil previously reported in the literature (Rossel, 1991; Dicks, 1965).

DISCUSSION

The FDA Food Policy document (FDA, 1992) provides the basis for establishing that a new plant variety produced through genetic modification is as safe and nutritious as the currently available varieties. The compositional data generated over the period of 2 years demonstrate that the seed from both GTCot lines and the resulting products are substantially equivalent to the parental Coker 312 line as well as to other commercial varieties of cotton. Numerous compositional analyses have been completed with only a few instances of statistically significant differences between the GTCot lines and control. In these instances where a difference was noted and in the instances where available data did not permit statistical analyses, the values are well within the established ranges reported in the scientific literature for cotton.

The levels of the nutrients (protein, fat, ash, moisture, carbohydrate, calories, amino acids, fatty acids, and α-tocopherol) for all three lines were comparable to the parental Coker 312 control values, as well as to the values reported for other commercial cotton varieties. Most importantly, from a safety perspective, the levels of the endogenous antinutrients (gossypol and cyclopropenoid fatty acids) in the glyphosate-tolerant cotton lines were also similar to the reported values for other cotton varieties. In the case of higher gossypol levels in line 1445 compared to the Coker 312 control, additional analyses supported that this effect is not associated with the glyphosate tolerance trait. The levels of aflatoxin in the cottonseed samples from the 1993 field trials showed undetectable levels for both the glyphosate-tolerant lines and the Coker 312 control.

On the basis of the data collected and the heterogeneity reported for different cotton varieties, it is concluded that except for tolerance to glyphosate, the GTCot lines are substantially equivalent to and are as safe and nutritious as the cotton varieties currently available in the marketplace.

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