

Evaluation of the safety and nutritional equivalence of a genetically modified cottonseed meal in a 90-day dietary toxicity study in rats

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Abstract

Meal prepared from Cry1F/Cry1Ac transgenic/genetically modified cottonseed (WIDESTRIKE™ Insect Protection, hereafter referred to as WIDESTRIKE™) was compared to cottonseed meal prepared from four conventionally bred lines of cotton (three commercial non-transgenic line controls (PHY72, PHY78 and 98M-2983), and a near isoline non-transgenic control (PSC355) in a 90-day dietary study to evaluate safety and nutritional equivalence. Diets were formulated with 10% WIDESTRIKE™ cottonseed meal equivalent to 7235 mg/kg/day for males and 7935 mg/kg/day for females. Animals were evaluated by cage-side and hand-held detailed clinical observations, body weight, and feed consumption. Functional tests, motor activity and ophthalmic examinations were conducted pre-exposure and prior to study termination. Standard hematology, clinical chemistry, prothrombin time and urinalysis parameters were evaluated. All rats had a complete necropsy and selected organs were weighed. Histopathologic examinations were performed on all rats fed the diets containing the near isoline non-transgenic control or WIDESTRIKE™.

Following 90 days of feeding, no adverse effects were observed during the conduct of clinical observations or in any of the parameters measured in this study. This study demonstrated that rodent diets prepared with 10% cottonseed meal from WIDESTRIKE™ cottonseeds do not produce any untoward effects and are nutritionally equivalent to cottonseed meals prepared from other, non-transgenic cottonseeds.

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1. Introduction

Meal prepared from Cry1F/Cry1Ac transgenic/genetically modified cottonseed (WIDESTRIKE™ Insect Protection, hereafter referred to as WIDESTRIKE™) was developed to offer farmers an alternative means to control Lepidopteran pests in cotton. Cry1F and Cry1Ac transformation events were developed by transforming a proprietary cotton *Acala* germplasm line known as GC510, released in 1984 in the US by Germain's Agribusiness,

Inc. Transformations were separately conducted via disarmed *Agrobacterium tumefaciens* methodology (Hoekema et al., 1983; Framond et al., 1983) using either the plasmid pAGM281 containing the Cry1F gene and the PAT (phosphinotricin acetyltransferase) gene (used as a selectable marker) isolated from *Streptomyces viridochromogenes* or the plasmid pMYC3006 containing the Cry1Ac gene isolated from *Bacillus thuringiensis* (B.t.) subspecies *aizawai* strain PS811, and the PAT isolated from *S. viridochromogenes*. Cotton lines derived from these two transformation events were interbred to produce individual plants expressing Cry1F, Cry1Ac and PAT proteins. In plants, the Cry1F and Cry1Ac proteins confer insect resistance to the most economically damaging pests in cotton production, the

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tobacco budworm and cotton bollworm. In 2001, these pests accounted for over \$100 million in lost yield value in the United States (Williams, 2002). The Cry1F and Cry1Ac proteins are also effective in the control of additional cotton pests including fall army worm, soybean loopers, and pink bollworm (Giannessi et al., 2002). The PAT protein confers tolerance to glufosinate-ammonium and has been the subject of several safety assessments throughout the world (Canadian Food Inspection Agency, 1998; EPA, 1995a, 1997; Health Canada, 1997; OECD, 1999). These safety assessments concluded that there was no concern for the safety and nutritional value of the PAT protein expressed in plants when present in foods.

WIDESTRIKE™ cotton has the same commercial uses as currently commercialized transgenic and non-transgenic cotton varieties. The raw agricultural product, cotton fiber, is used in clothing and other textile products. Human consumption of food products derived from cotton and cottonseed is limited. The refined cottonseed oil is used in food products and consumed by humans in a similar manner to vegetable oil. However, protein content in cottonseed oil is low and processing studies (EPA, 1995b, 1996) demonstrate that the refining process removes Cry1Ac and Cry1F to below the level of detection (LOD; <0.1 ng/mg). Cottonseed meal is used as a high protein supplement for livestock; hulls are used as exceptional roughage for cattle; and linters (short fibers that adhere to cottonseed after ginning) are a source of readily digestible cellulose for ruminants and humans. Lintners are also used in high fiber dietary products and as thickeners (Food Standards, 2005). Use of cottonseed meal is limited due to the presence of endogenous anti-nutrient gossypol. The recommended ration of cottonseed meal in swine feed is 5% in gestation, lactation, starter, grower and finisher rations (Walker, 1992).

The Cry1F and Cry1Ac proteins are readily inactivated by heat or mild acidic conditions and are not toxic to humans (EPA, 1995b, 1996). Cry1F and Cry1Ac proteins are contained in the plant matrix; therefore, there is little potential for human exposure to these proteins via dermal, eye, or inhalation exposure. The Cry1F and Cry1Ac proteins are classified as Toxicity Category III with a LD₅₀ value >700 mg/kg for Cry1Ac and a LD₅₀ value >600 mg/kg for Cry1F (EPA, 2005a). PAT proteins are acetyltransferase enzymes which are ubiquitous in nature and are found in microbes, plants, and animals (Dobson, 1995). These proteins rapidly degrade at elevated temperature and are destroyed under simulated gastric conditions (Herouet et al., 2005). In 1997, the EPA issued a final rule exempting PAT from the requirement of a tolerance in all raw agricultural commodities when used as a plant-incorporated-protectant (PIP) inert (EPA, 1997). In 2004, the EPA issued a permanent tolerance exception from the requirement of a tolerance for residues of Cry1F and Cry1Ac when used as a PIP (EPA, 2005b).

The purpose of this study was to evaluate the safety and nutritional equivalence of a diet formulated with 10%

cottonseed meal from WIDESTRIKE™ cotton (transgenic line) in rats following dietary administration for at least 90 days. For comparison, four non-transgenic cottonseed meal controls (three commercial and one near isoline non-transgenic control with similar genetic background as the genetically modified WIDESTRIKE™ but without the transgenes) were also evaluated in this study. As noted above, the normal processing of cottonseed to cottonseed meal, denatures Cry1F and Cry1Ac to levels below the LOD (0.025 ng/mg for both Cry1F and Cry1Ac). This denaturation was confirmed by ELISA assays which indicated no detectable levels of Cry1F, Cry1Ac or PAT proteins in the WIDESTRIKE™ cottonseed meal used in this study. Consequently, the dose was the percent of cottonseed meal in the prepared rat diet.

2. Materials and methods

2.1. Animals

Male and female CrI:CD(SD) rats (Sprague-Dawley derived), were obtained from Charles River Laboratories Inc. (Portage, MI). Animals were stratified by body weight and randomly assigned to groups by a computer program. At the start of the study, animals were approximately 6 weeks old with males weighing between 163 and 220 g and females weighing between 132 and 177 g. The animals were housed singly in stainless steel wire-mesh cages in rooms with a temperature of 22 ± 1 °C (maximum permissible excursion of ±3 °C), 40–70% relative humidity, a 12-h light/dark photocycle and a room air exchange of 12–15 times/h.

2.2. Food and water

Food and water were provided *ad libitum* during acclimation and throughout the study. Prior to the start of the study, animals were provided LabDiet® Certified Rodent Diet #5002 (PMI Nutrition International, St. Louis, MO) in meal form. Analyses of the commercially available lot of LabDiet® Certified Rodent Diet #5002 were performed by PMI Nutrition International to confirm the diet provided adequate nutrition and to quantify the levels of contaminants. During the study, animals were provided diets uniquely prepared at Purina Test Diet (Richmond, IN). These diets were prepared utilizing the compositional analyses of the cottonseed meals from Covance Laboratories, Inc. (Madison, WI) and adjusting the standard recipe for LabDiet® Certified Rodent Diet #5002 to achieve an equivalent composition in meal form. Analyses of cottonseed meals and test diets uniquely prepared at Purina Test Diet (10% cottonseed meals incorporated into the standard recipe for LabDiet® Certified Rodent Diet #5002) for nutritional composition and contaminants were conducted by Covance Laboratories, Inc. (Madison, WI). Drinking water obtained from the municipal water source was periodically analyzed for chemical parameters, biological and chemical contaminants. There were no contaminants in the feed or water that were determined to affect the integrity of the study at the concentrations observed.

2.3. Cottonseed

Dow AgroSciences LLC (Indianapolis, IN) supplied the control substance (non-transgenic near isoline, PSC355), three reference substances [commercial non-transgenic line A (PHY72), non-transgenic line B (PHY78) and non-transgenic line C (98M-2983)], and WIDESTRIKE™ cotton (Cry1F/Cry1Ac) seeds. Characterization of transgene (near isoline control and WIDESTRIKE™ cotton) and transgene protein expression (near isoline control, three commercial controls and WIDESTRIKE™ cotton) were conducted on the cottonseed at Dow AgroSciences LLC,

Indianapolis, IN. The presence of Cry1F, Cry1Ac and PAT proteins was confirmed by ELISA analyses in the WIDESTRIKE™ cottonseed at 2.80 ng Cry1F/mg, 0.96 ng Cry1Ac/mg, and 0.46 ng PAT/mg. These proteins were not detected in any of the non-transgenic control cottonseed. Polymerase chain reaction (PCR) amplification, using the event specific primer sets, generated products of polymerase chain reactions (amplicons) in the WIDESTRIKE™ cottonseed but not in the near isoline control (PSC355). PCR products of the predicted size were obtained with both the 281-24-236 (Cry1F) and 3006-210-23 (Cry1Ac) event specific primer sets. As predicted, amplicons were obtained with the actin gene primers with both the test and control substance seed genomic DNA.

2.4. Cottonseed meals

All cottonseed was processed into meal at Food Protein Research and Development Center (Texas A&M University, College Station, TX). The test substance, WIDESTRIKE™ cotton (Cry1F/Cry1Ac) meal, was not characterized, assayed for purity or checked for structural confirmation because meal preparation degraded the transgenic proteins below the level of detection. Since Cry1F, Cry1Ac or PAT proteins were not detected in any of the cottonseed meals via ELISA analyses, no ELISA analyses were performed on any diets. The cottonseed meals (near isoline control, three commercial controls and WIDESTRIKE™ cotton) were also analyzed in accordance to Good Laboratory Practice (GLP) for nutrients, anti-nutrients, gossypol, mycotoxins (non-GLP), and pesticide residuals (organochlorinated and organophosphate screens) at Covance Laboratories, Inc. (Madison, WI). All meals contained comparable levels of crude protein, fat, ash, carbohydrates, calories, dry matter, crude fiber, detergent fiber, amino acids, minerals, vitamins, fatty acids, and heavy metals. Gossypol levels were 0.092–0.293% (free) and 1.41–2.17% (total). Pesticides were not detected in the cottonseed meals. All mycotoxins analyzed in cottonseed meals were below detection limits.

2.5. Diets

The diets were prepared at Purina Test Diet (Richmond, IN) by mixing the cottonseed meal into the standard recipe for LabDiet® Certified Rodent Diet 5002 (PMI® Nutrition International, St. Louis, MO) at a 10% concentration. Diet preparations were not documented according to GLP; however, the Quality Assurance Unit or designee at Purina Test Diet provided quality assurance oversight of the test diet preparations. All diets were stored throughout the study at 0 °F. Dose confirmation, homogeneity, and stability analyses were not conducted because the process for preparing the cottonseed meals resulted in degradation of Cry proteins. The diets were analyzed in accordance with GLP for nutrients, anti-nutrients, gossypol, and mycotoxins (non-GLP), and pesticide residues (organochlorinated and organophosphate screens) at Covance Laboratories, Inc. (Madison, WI). All diets contained comparable levels of crude protein, fat, ash, carbohydrates, calories, dry matter, crude fiber, detergent fiber, amino acids, minerals, vitamins, fatty acids, and heavy metals. Gossypol levels were 0.006–0.018% (free) and 0.132–0.216% (total). All mycotoxins analyzed in diets were below detection limits, except for deoxynivalenol which was detected in all diet samples at 0.1–0.2 ppm, and which was considerably below guidance/action levels established by the US FDA/USDA for animal diets (FDA, 2005). These data suggest that these diets were nutritionally comparable and contaminants were identified at levels that did not interfere with the study.

2.6. Study design

Groups of 12 male and 12 female Sprague-Dawley rats were fed diets containing 10% cottonseed meal for at least 90 days. Cage-side observations, detailed clinical observations, eye exams, functional tests, motor activity, body weights, feed consumption, hematology, clinical chemistry, prothrombin time, urinalysis, and organ weights were conducted. In addition, a gross necropsy was conducted and selected organs were weighed. Extensive histopathologic examination of tissues was performed

on all rats fed a diet containing the near isoline non-transgenic control or WIDESTRIKE™ (Cry1F/Cry1Ac, transgenic line). The eyes of all animals were examined pre-exposure and prior to the scheduled necropsy. Functional tests consisting of sensory evaluation, rectal temperature, grip performance and motor activity were conducted pre-exposure and during the 11th week of the study to evaluate functional performance in accordance to OPPTS 870.3100 guidelines (USEPA, 1988). Hindlimb grip performance was tested according to the procedure described by Mattsson et al. (1986). An automated system was used for motor activity data collection as described by Mattsson et al. (1996); except, instead of each test session being eight 6-min epochs or time segments for Fischer 344 rats, each test session consisted of 10 8-min epochs. A cage-side examination was conducted at least once a day. All animals were observed for morbidity, mortality, and the availability of feed and water at least twice daily. Detailed clinical observations (DCO) were conducted on all animals, pre-exposure and once/week throughout the study. The examination included cage-side, hand-held and open-field observations that were recorded categorically or using explicitly defined scales (ranked). All rats were weighed during the pre-exposure period and weekly thereafter. Body weight gains were calculated. Feed consumed was determined pre-exposure and weekly for all animals. Feed efficiency and test material intake were calculated.

The following guidelines were consulted during the design of the study: OPPTS 870.3100 (USEPA, 1988), OECD Guideline No. 408 (OECD, 1998), Part B.26, Directive 2001/59/EC (EEC, 2001) and Subchronic Oral Toxicity Study (JMAFF, 2000). The animal care and use activities required for conduct of this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with the US Department of Agriculture animal welfare regulations, 9 CFR, Subchapter A, Parts 1–4. The animal facility is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. This study was conducted under Good Laboratory Practice (GLP) Standards, except as previously noted.

2.7. Hematology and coagulation

Animals were fasted overnight prior to blood collection. Blood samples were obtained from the orbital sinus following anesthesia with CO₂ at the scheduled necropsy. Blood samples were mixed with ethylenediaminetetraacetic acid (EDTA) and assayed using an Advia 120 hematology analyzer (Bayer Corporation, Tarrytown, NY) for the following parameters: hematocrit, hemoglobin concentration, red blood cell (RBC) count, total white blood cell (WBC) count, differential white blood cell count, platelet count, reticulocyte count, mean corpuscular hemoglobin concentration, mean corpuscular volume, and mean corpuscular hemoglobin concentration.

Blood samples were collected in sodium citrate tubes, centrifuged and plasma collected and assayed for prothrombin time using an ACL9000 coagulation analyzer (Instrumentation Laboratory, Lexington, MA).

2.8. Clinical chemistry

Blood samples were collected and serum was separated from cells as soon as possible. The following serum parameters were measured using a Hitachi 912 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN): alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase activities; albumin, cholesterol, creatinine, electrolytes (sodium, potassium, phosphorus, chloride and calcium), globulin, glucose, total bilirubin, total protein, triglycerides, urea nitrogen, and albumin/globulin levels.

2.9. Urinalysis

Urine samples were obtained from all animals the week prior to the scheduled necropsy. Animals were housed in metabolism cages and urine samples were collected overnight (approximately 16 h). Feed and water were available *ad libitum* during this procedure. The following parameters were recorded: color, appearance, specific gravity (refractometer), and

urine volume. Semi-quantitative analyses (Multistix[®] Reagent Strips, Bayer Corporation, Elkhart, IN using a Clinitek 200+) were conducted for pH, bilirubin, glucose, protein, ketones, blood, and urobilinogen. Urine samples were also collected from each animal by manual compression of the urinary bladder. These urine samples were pooled from each group, and the micro sediments were characterized microscopically.

2.10. Gross necropsy and histopathology

Fasted rats submitted alive for necropsy were anesthetized by the inhalation of CO₂, weighed, and blood samples were obtained prior to euthanasia. A complete necropsy was conducted on all animals by a veterinary pathologist assisted by a team of trained individuals. The brain, liver, kidneys, heart, adrenals, testes, epididymides, uterus, ovaries, thymus, and spleen were trimmed and weighed immediately. The ratios of organ weight to terminal body weight were calculated. Representative samples of 54 tissues were collected and preserved in neutral, phosphate-buffered 10% formalin in accordance with the previously cited guidelines. Sections from all preserved tissues were processed by standard histologic procedures from control (non-transgenic near isolate (PSC355) and WIDESTRIKE[™] cotton (Cry1F/Cry1Ac) test animals. Paraffin embedded tissues were sectioned approximately 6 μm thick, stained with hematoxylin and eosin and examined by a veterinary pathologist using a light microscope.

2.11. Statistics

Dietary toxicity studies with cottonseed meal are unlike conventional dietary studies because the diet itself varied among the different treatment groups. In conventional dietary toxicity studies, the animals in all test groups are fed a standard diet such as Purina #5002 rat chow to which a test material is added. In contrast, in this cottonseed meal feeding study, 10% of the standard diet was replaced with test cottonseed meal. Since no two lots of cottonseed were identical, there was no specific control group for 'cottonseed'.

While the cottonseed meal from the "near isolate" cotton plants might be expected to be the closest control, there were differences between this and the test line that were unrelated to genetic transformation. For example, the level of gossypol was found to be higher in the feed derived from the near isolate than in the other feeds. Sources of variability in palatability and nutritional quality of different lots of cottonseed meal can arise from differences in the growing conditions (weather, soil, water, insects, fungi, etc.), the genetics of the plant, the post-harvest management of the grain, and differences in meal preparation.

In appropriately analyzing the data from experiments such as this, it is important to keep the objective of the study in mind. In the case of this study, the purpose was not to investigate the toxicity of the introduced proteins (not least because after processing the levels of the introduced Cry proteins were undetectable). Rather, the purpose was to investigate whether cottonseed meal derived from genetically-modified cotton was as toxicologically safe as cottonseed meal from conventional cotton. In this context, the most appropriate comparison was between the test material and the combined control (conventional) cottonseed meals.

Means and standard deviations were calculated for all continuous data. Body weights, feed consumption, organ weights, urine volume and specific gravity, clinical chemistry, prothrombin time, and appropriate hematologic were evaluated by Bartlett's test ($\alpha = 0.01$; Winer, 1971) for equality of variances. One planned *a priori* contrast of each continuous variable was calculated in a one-way analysis of variance (ANOVA), with seed type as a factor (Steel and Torrie, 1960). The contrast compared the group of four controls with the WIDESTRIKE[™] cottonseed meal.

Descriptive statistics only (means and standard deviations) were reported for body weight gains, feed efficiency, RBC indices, and differential WBC counts. Statistical outliers were identified by a sequential test ($\alpha = 0.02$; Grubbs, 1969), but routinely excluded only from feed consumption statistics.

DCO incidence data (ranked observations only) and sensory evaluation (post-treatment only) were statistically analyzed by a *z*-test of pro-

portions comparing the WIDESTRIKE[™] cottonseed meal with the averaged proportion for the four control cottonseed meals (Bruning and Kintz, 1987). DCO data collected at different time points were analyzed separately by sex.

For rectal temperature, grip performance and motor activity, an ANOVA was selected that included gender and rat feed group as factors; contrasts were conducted to compare the group fed WIDESTRIKE[™] cottonseed meal to the combination of the four groups fed control cottonseed meals. Including gender as a factor nearly doubled the degrees of freedom, and considerably increased the statistical power of the ANOVA. To retain gender as a factor, the sex-by-group interactions had to be non-significant at $\alpha = 0.05$. If significant at $\alpha = 0.05$, analyses were done separately for each sex. By using a statistical contrast, the one *a priori* analysis retained the information on differences among the control grains (between-group variance for control cottonseed meals) and the information on data variability within each control group. Thus, this ANOVA addressed the question: Do rats (male and female) fed a rodent chow supplemented with 10% cottonseed meal derived from WIDESTRIKE[™] cottonseed differ significantly from rats fed chow supplemented with cottonseed meal derived from four other cottonseed sources?

Post-treatment data for rectal temperature and grip performance were analyzed. The group-by-sex interactions were examined, and if significant at $\alpha = 0.05$, analyses were done separately for each sex. A linear contrast compared the four control cottonseed meals with the WIDESTRIKE[™] cottonseed meal.

Motor activity counts were reported as their square roots to minimize problems of heterogeneity of variance and departure from normality that commonly occur from treatment (Pryor et al., 1983). Post-treatment motor activity data were analyzed by a factorial repeated-measure design (using the multivariate approach), with factors of sex and group and the repeated factor of epoch. The group-by-sex interactions were examined, and if significant at $\alpha = 0.05$, analyses were run separately for each sex. A linear contrast compared the four control cottonseed diets with the WIDESTRIKE[™] cottonseed diet. The Pillai's Trace *p*-values were used for the repeated factor.

As previously described, there was one planned *a priori* contrast of each discrete variable. The statistical significance of these contrasts was judged using the Benjamini–Hochberg method, which adjusts for the number of contrasts made to control the false discovery rate and keep it below 5% (Ellis et al., 2000). This method was applied to each of the following groups of data: body weights, feed consumption, hematology, prothrombin time, clinical chemistry, electrolytes, urine specific gravity with urine volume, organ weights, relative organ weights, motor activity, rectal temperature, and grip performance. Similarly, probability values for the *z*-test of proportions were also adjusted.

3. Results

3.1. Mortality and in-life observations

All rodents survived the 90-day test period. The detailed clinical observations of rats from all groups were within normal limits at all times. Cage-side examinations had no treatment-related findings.

3.2. Ophthalmology

Pre-exposure examinations of all rats placed on study indicated that all rats were within normal limits, except for one control female (non-transgenic line A, PHY 72) that had a cloudy cornea and one control female (non-transgenic line C, 98M-2983) with a pale fundus. Prior to study termination (day 86), ophthalmologic observations of pale fundus were noted in a limited number (0–2 per

treatment group) of rats. These observations were interpreted to be spontaneous lesions and not an effect of ingestion of any particular cottonseed meal due to their low incidence and common occurrence.

3.3. Functional tests

Sensory evaluations performed on rats (week 11) represented normal responses for rats of this age and strain and there were no group-related differences between cottonseed meal groups. There were no group-related sex differences in rectal temperature ($p = 0.296$), hindlimb ($p = 0.179$) or forelimb ($p = 0.341$) grip performance across the five different groups of rats given cottonseed meals. The contrasts of Groups 1–4 (near isoline and three commercial non-transgenic controls) versus Group 5 (WIDESTRIKE™) were also not significant for rectal temperature ($p = 0.811$), hindlimb ($p = 0.179$), or forelimb ($p = 0.341$) grip performances, which indicated that these measures in rats given WIDESTRIKE™ cottonseed meal were not different from those in rats given the non-transgenic cottonseed meals.

There was a statistically significant group by sex interaction in motor activity ($p = 0.036$) across the five different groups of rats given cottonseed meals (Fig. 1). The statistical significance was thought to be due to the different patterns in motor activity between the groups of males and females, particularly in Groups 2 (PHY72) and 4 (98M-2983). The analysis was subsequently conducted separately for each sex. The contrasts of Groups 1–4 (near isoline and three commercial non-transgenic controls) versus Group 5 (WIDESTRIKE™) were not significant in males ($p = 0.433$) or females ($p = 0.266$), which indicated that the total motor activity of rats given WIDESTRIKE™ cottonseed meal was not different from rats given the non-transgenic cottonseed meals. The contrasts of epoch by Groups 1–4 versus Group 5 were also not significant in males ($p = 0.851$) or females ($p = 0.990$), indicating that the distribution of motor activity counts of rats given WIDESTRIKE™ cottonseed meal was not different from rats given the non-transgenic cottonseed meals.

3.4. Body weights/body weight gains/feed consumption

There were no statistically identified group-related differences in the body weights/body weight gains (Fig. 2), or feed consumption (Table 1) of male or female rats given WIDESTRIKE™ cottonseed meal when compared to the controls. There were no group-related differences in the feed efficiency of male or female rats given WIDESTRIKE™ cottonseed meal when compared to the controls.

3.5. Test material intake

Controls (near isoline PSC355, PHY72, PHY78 and 98M-2983) given 10% of their respective cottonseed meal in rodent diet, resulted in time weighed average (TWA) intakes of approximately 7074–7352 mg/kg/day for males

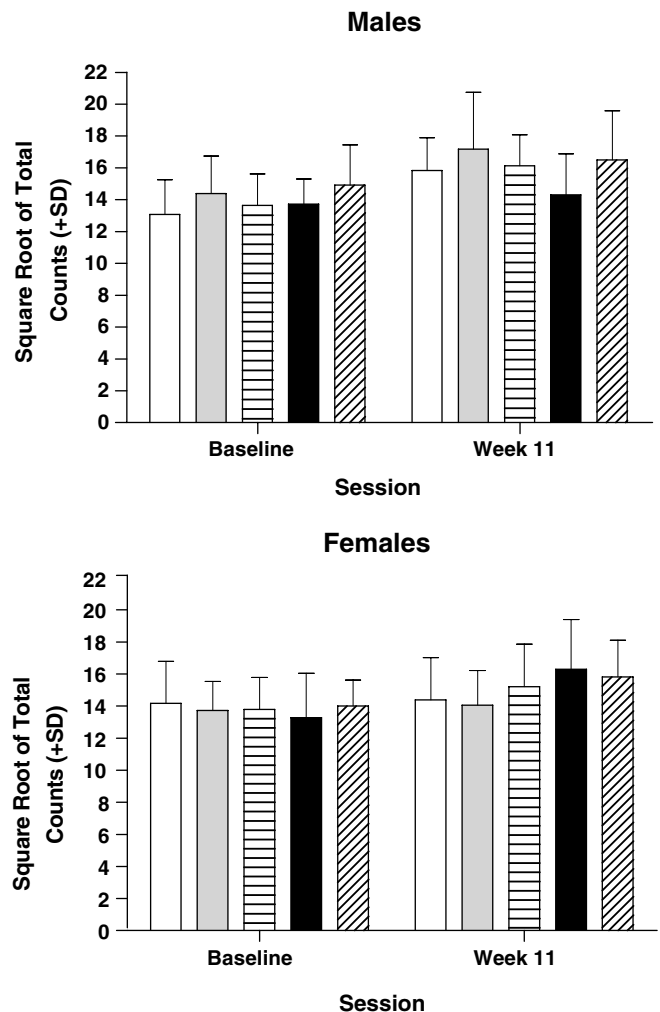


Fig. 1. Motor activity. Group 1 (near isoline control); Group 2 commercial non-transgenic line A (PHY72); Group 3 commercial non-transgenic line B (PHY78); Group 4 commercial non-transgenic line C (98M-2983); Group 5 (WIDESTRIKE™).

and 7905–8239 mg/kg/day for females. Rats given 10% WIDESTRIKE™ cottonseed meal in rodent diet had TWA intakes of approximately 7235 and 7935 mg/kg/day for males and females, respectively. The TWA intakes for rats given WIDESTRIKE™ cottonseed meal were within the range of controls.

3.6. Hematology and coagulation

There were no group-related changes or statistically identified differences in any of the hematologic parameters or group-related alterations in prothrombin times for male and female rats given the WIDESTRIKE™ cottonseed meal versus the non-transgenic groups.

3.7. Clinical chemistry

There were no group-related alterations or statistically-identified differences in any of the clinical chemistry param-

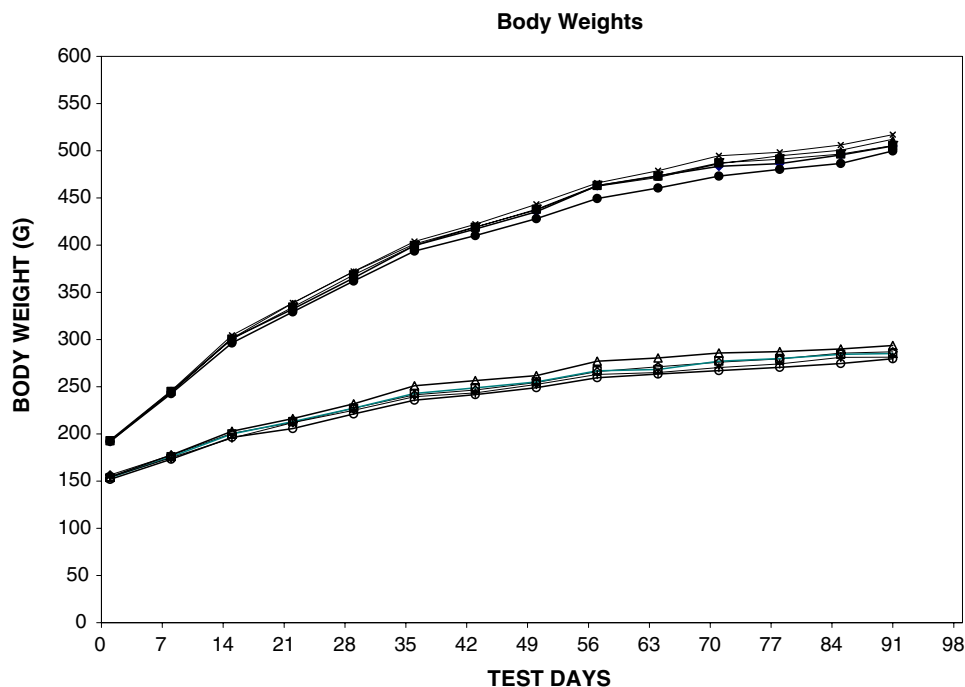


Fig. 2. Body weights. (—◆—) Group 1 males (near isoline control); (—■—) Group 2 males (PHY72); (—▲—) Group 3 males (PHY78); (—●—) Group 4 males (98M-2983); (—×—) Group 5 males (WIDESTRIKE™); (—◇—) Group 6 females (near isoline control); (—□—) Group 7 females (PHY72); (—△—) Group 8 females (PHY78); (—○—) Group 9 females (98M-2983); (—+—) Group 10 females (WIDESTRIKE™).

eters for male and female rats given WIDESTRIKE™ cottonseed meal versus the non-transgenic groups (Table 2). Slightly higher mean alanine aminotransferase and aspartate aminotransferase activities (not statistically significant) occurred in males assigned to Group 5 (WIDESTRIKE™); however, these differences were due to outlying values for individual rats and in the absence of a consistent histologic correlate was not attributed to ingestion of WIDESTRIKE™ cottonseed meal. The majority of the animals identified as statistical outliers for AST values (13 animals) also had elevated levels of ALP activities (11 of 13 animals) and 9 of the 11 elevated ALP activities were also identified as statistical outliers. Review of the statistically identified outliers included in the mean value revealed 2/13 of the outliers with histopathologic observations of the liver consisting of aggregates of macrophages-histocytes; adjacent to necrotic or degenerative hepatocytes; multifocal; very slight. Degenerative hepatocytes were not seen in the other animals; however, if the liver samples were serially sectioned, additional foci of necrotic or degenerative hepatocytes would likely be detected.

3.8. Urinalysis

The mean urine volume of females given WIDESTRIKE™ cottonseed meal (Group 5) was slightly higher than the control groups (Table 2). The contrast of mean urine volume of Groups 1–4 (near isoline and three commercial non-transgenic controls) versus Group 5 females

was significant ($p = 0.027$) indicating that the urine volume of females fed WIDESTRIKE™ cottonseed meal was different from rats treated with the non-transgenic cottonseed meals. This difference was interpreted to reflect normal variation and was not related to the consumption of diets containing WIDESTRIKE™ cottonseed meal. This opinion was supported by a lack of statistical differences in mean urine specific gravity of Group 5 females, absence of group-related microscopic effects in tissues of these females, and absence of similar effects in Group 5 males. Other urinalysis parameters for male and female rats given WIDESTRIKE™ cottonseed meal were similarly unaffected versus the non-transgenic groups.

3.9. Gross necropsy, organ weights and histopathology

There were no group-related alterations or statistically identified differences in organ weights of male and female rats given WIDESTRIKE™ cottonseed meal versus the non-transgenic groups (Tables 3 and 4). There were also no group-related gross or histopathologic observations. All observations were considered to be the result of spontaneous alterations, unassociated with exposure to WIDESTRIKE™ cottonseed meal.

4. Discussion and conclusions

The assessment of the safety of foods derived from genetically modified (GM) crops typically requires

Table 1
Feed consumption (g/day) for male and female rats given near isoline, commercial non-transgenic lines, or WIDESTRIKE™ cotton in the diet for 90 days

Group		Days on test													
		–6–1	1–8	8–15	15–22	22–29	29–36	36–43	43–50	50–57	57–64	64–71	71–78	78–85	85–91
<i>Males</i>															
1	Mean	22.5	24.1	27.8	28.6	28.1	29.0	29.4	29.5	28.3	28.9	29.4	27.1	27.6	27.4
	SD	1.4	3.3	2.6	2.8	2.2	2.5	2.4	2.8	3.0	2.6	2.5	2.7	2.3	2.7
	N =	12	10	9	11	11	11	10	9	10	10	8	10	11	10
2	Mean	22.4	24.4	27.2	27.7	27.6	27.9	28.1	27.8	27.4	27.3	27.3	25.7	26.2	26.1
	SD	2.1	2.7	3.1	1.1	1.3	1.3	1.3	1.5	1.2	1.4	1.7	1.7	1.6	1.3
	N =	12	10	11	10	10	11	11	9	9	10	11	10	11	10
3	Mean	22.7	24.0	27.1	28.4	28.6	28.5	28.9	28.3	27.4	27.4	27.1	26.4	26.7	26.9
	SD	1.9	2.9	2.1	1.8	2.1	2.0	2.1	2.1	2.7	2.0	2.0	1.8	1.9	1.9
	N =	12	8	10	9	11	11	10	10	11	10	11	12	12	11
4	Mean	22.0	23.0	26.4	26.6	26.8	27.7	28.1	27.4	27.2	27.0	27.4	25.5	26.9	26.3
	SD	1.5	1.5	1.3	0.4	0.8	1.4	2.3	2.0	1.9	1.9	2.4	2.4	2.0	2.5
	N =	12	11	11	9	10	10	11	10	9	10	10	10	11	11
5	Mean	22.5	23.8	27.5	28.6	29.0	29.2	28.8	28.8	28.9	28.6	28.9	27.3	27.6	27.7
	SD	1.8	1.2	1.6	2.4	2.5	2.1	2.3	2.1	2.6	3.1	2.4	1.8	1.5	2.0
	N =	12	10	12	12	11	10	10	11	11	11	12	12	12	11
<i>Females</i>															
1	Mean	18.0	17.5	19.1	19.0	18.3	19.2	18.0	19.7	18.6	18.8	18.9	18.3	20.1	17.3
	SD	2.0	3.0	3.5	3.2	2.9	2.5	2.0	2.9	2.8	2.5	2.9	2.3	3.3	2.4
	N =	12	10	9	10	9	9	7	11	8	9	8	9	11	9
2	Mean	17.4	17.8	19.3	19.8	19.7	20.1	19.8	19.4	19.5	19.8	20.6	19.1	19.9	18.8
	SD	1.1	1.0	1.9	1.4	1.6	1.0	1.1	0.2	1.4	1.7	1.8	1.8	1.6	1.4
	N =	12	10	12	12	10	9	10	8	9	11	12	11	12	11
3	Mean	17.7	17.8	19.0	19.9	20.0	20.3	20.0	20.0	19.4	18.9	19.3	17.8	18.8	17.9
	SD	1.5	1.4	1.6	1.9	2.0	1.5	1.7	1.6	1.3	1.2	1.6	1.5	1.5	1.6
	N =	12	12	11	11	10	9	9	9	7	9	10	11	11	11
4	Mean	17.3	17.7	18.3	18.7	19.2	19.5	19.0	19.3	19.0	18.6	17.9	17.1	17.5	17.2
	SD	1.0	1.4	2.0	2.0	2.0	2.3	1.6	1.4	1.5	1.2	1.0	1.0	1.4	1.2
	N =	12	11	11	11	11	10	10	11	10	10	8	10	8	11
5	Mean	17.2	17.5	18.7	18.1	19.0	18.9	18.7	19.5	18.8	18.1	19.1	17.8	18.5	17.9
	SD	1.4	1.8	2.0	1.4	1.3	1.7	1.5	1.9	2.0	1.6	1.8	1.2	1.2	2.1
	N =	12	10	12	9	9	10	8	11	11	10	12	11	10	11

1 = near isoline (PSC355); 2 = commercial non-transgenic line A (PHY72); 3 = commercial non-transgenic line B (PHY78); 4 = commercial non-transgenic line C(98M-2983); 5 = WIDESTRIKE™ cotton.

demonstration of substantial nutritional equivalence to foods derived from conventional crops. Compositional analyses and comparison of key nutrients and anti-nutrients along with feed utilization studies in livestock are typically conducted to demonstrate substantial equivalence to most regulatory agencies. Although these studies are purposely designed to evaluate the nutritional performance of a food derived from a GM crop, the studies are not designed to characterize potential toxicity associated with either the transgenic proteins or some other change in the nutritional composition not captured in the compositional analyses. Thus, the findings from these studies are not designed for the derivation of reference doses for human risk assessments. However, the 90-day rodent dietary toxicity study, reported here, does provide an experimental design for the determination of the dietary safety of foods

derived from GM crops and also provides additional analyses of the nutritional performance.

In this 90-day rodent toxicity study, there was no evidence of differences in nutritional quality in rodent diets prepared with 10% WIDESTRIKE™ cottonseed meal compared to diets from non-transgenic cottonseed. All rats survived the 90-day test period. There were no group-related effects in clinical signs, ophthalmic examinations, functional tests, motor activity, body weights, feed consumption, hematology, prothrombin time, clinical chemistry, or urinalysis parameters. In addition, there were no group-related effects in organ weights, gross or histopathology. Therefore it can be concluded that the no-observed-effect level (NOEL) for Sprague-Dawley rats of either sex was the targeted concentration of 10% cottonseed meal prepared from WIDESTRIKE™ cottonseed, which was

Table 2

Selected clinical chemistry and urinalysis parameters for male and female rats given near isoline, commercial non-transgenic lines, or WIDESTRIKE™ cotton in the diet for 90 days

Group		UN MG/DL	ALT U/L	ALP U/L	AST U/L	TP G/DL	ALB G/DL	GLOB G/DL	A/G Ratio	CHOL MG/DL	TRIG MG/DL	TBIL MG/DL	CREA MG/DL	GGT U/L	GLUC MG/DL	Urine volume (ML)	Specific gravity
<i>Males</i>																	
1	Mean	16	57	77	116	6.8	3.7	3.1&	1.2&	52	43	0.2	0.4	<3	151	12.5	1.048
	SD	4	31	11	56	0.3	0.2	0.2	0.1	11	15	0.1	0.1	- ^A	23	9.5	0.018
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2	Mean	14	47	84	96	6.6	3.7	2.9&	1.3&	46	44	0.1	0.3	<3	133	13.4	1.042
	SD	2	25	18	21	0.4	0.1	0.3	0.1	10	20	0.1	0.0	- ^A	26	6.3	0.013
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
3	Mean	14	87	82	146	6.8	3.9	3.0&	1.3&	51	49	0.2	0.4	<3	156	11.0	1.046
	SD	1	111	17	132	0.3	0.2	0.2	0.1	12	17	0.0	0.1	- ^A	28	5.0	0.011
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
4	Mean	14	53	83	107	6.8	3.8	3.0&	1.3&	52	44	0.2	0.3	<3	153	11.0	1.046
	SD	1	25	28	31	0.2	0.2	0.1	0.1	15	14	0.1	0.1	- ^A	23	5.0	0.013
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
5	Mean	15	100	79	155	7.0	3.8	3.1&	1.3&	51	61	0.1	0.4	<3	157	12.2	1.048
	SD	2	145	15	150	0.5	0.2	0.3	0.1	15	22	0.1	0.1	- ^A	49	8.6	0.016
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
<i>Females</i>																	
1	Mean	16	42	42	104	7.7	4.5	3.2&	1.4&	67	40	0.3	0.4	<3	115	6.3	1.055
	SD	2	12	7	26	0.5	0.3	0.2	0.1	17	10	0.1	0.1	- ^A	21	3.4	0.029
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2	Mean	14	49	57	115	7.3	4.2	3.1&	1.4&	72	38	0.3	0.3	<3	116	7.0	1.051
	SD	2	39	21	66	0.6	0.4	0.2	0.1	27	13	0.1	0.1	- ^A	16	3.8	0.019
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
3	Mean	13	31	49	83	7.3	4.2	3.1&	1.4&	67	35	0.2	0.3	<3	118	6.4	1.047
	SD	3	5	15	13	0.4	0.2	0.3	0.1	12	9	0.0	0.1	- ^A	23	3.6	0.014
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
4	Mean	13	35	46	86	7.4	4.3	3.1&	1.4&	66	38	0.2	0.4	<3	116	6.1	1.053
	SD	2	9	13	14	0.4	0.2	0.2	0.1	12	7	0.1	0.1	- ^A	22	2.3	0.025
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
5	Mean	14	35	47	94	7.6	4.3	3.3&	1.3&	59	39	0.2	0.4	<3	118	9.7	1.042
	SD	2	9	10	38	0.3	0.3	0.2	0.1	12	13	0.0	0.0	- ^A	21	5.8	0.018
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12

UN = urea nitrogen, ALT = alanine aminotransferase, ALP = alkaline phosphatase, AST = aspartate aminotransferase, TP = total protein, ALB = albumin, CHOL = cholesterol, TRIG = triglycerides, TBIL = total bilirubin, CREA = creatinine, GGT = gamma glutamyl transpeptidase, GLUC = glucose, albumin/globulin (A/G) ratio.

& Indicates no statistical comparison of means.

<3 Below analyzer detection limit.

A = Unable to calculate SD.

1 = near isoline (PSC355); 2 = commercial non-transgenic line A (PHY72); 3 = commercial non-transgenic line B (PHY78); 4 = commercial non-transgenic line C (98M-2983); 5 = WIDESTRIKE™ cotton.

Table 3
Organ weights for male and female rats given near isoline, commercial non-transgenic lines, or WIDESTRIKE™ cotton in the diet for 90 days

Group		Final body	Adrenal glands		Heart		Kidneys		Liver		Brain		Spleen		Thymus	
		Wt. (G)	(G)	(G/100)	(G)	(G/100)	(G)	(G/100)	(G)	(G/100)	(G)	(G/100)	(G)	(G/100)	(G)	(G/100)
<i>Males</i>																
1	Mean	467.4	0.067	0.014	1.489	0.318	3.504	0.750	12.977	2.776	2.124	0.458	0.784	0.168	0.278	0.059
	SD	46.0	0.009	0.002	0.213	0.026	0.513	0.089	1.748	0.249	0.101	0.046	0.167	0.031	0.085	0.017
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2	Mean	466.9	0.065	0.014	1.502	0.322	3.344	0.717	13.211	2.818	2.132	0.461	0.754	0.163	0.288	0.062
	SD	46.3	0.010	0.002	0.197	0.027	0.357	0.050	2.313	0.274	0.097	0.054	0.092	0.023	0.071	0.015
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
3	Mean	475.6	0.067	0.014	1.512	0.319	3.498	0.738	13.693	2.874	2.119	0.448	0.778	0.164	0.259	0.055
	SD	37.5	0.011	0.002	0.090	0.021	0.319	0.066	1.705	0.226	0.122	0.044	0.105	0.019	0.052	0.013
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
4	Mean	461.4	0.068	0.015	1.520	0.330	3.271	0.709	12.472	2.705	2.084	0.454	0.729	0.158	0.564	0.127
	SD	36.6	0.009	0.002	0.169	0.036	0.357	0.061	1.121	0.156	0.099	0.043	0.113	0.019	1.123	0.261
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
5	Mean	480.8	0.064	0.013	1.572	0.327	3.500	0.732	13.682	2.856	2.090	0.438	0.754	0.158	0.306	0.064
	SD	44.9	0.008	0.002	0.164	0.026	0.286	0.072	1.929	0.419	0.139	0.044	0.098	0.022	0.044	0.009
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
<i>Females</i>																
1	Mean	262.0	0.076	0.029	0.962	0.367	1.999	0.764	7.626	2.904	1.911	0.740	0.510	0.195	0.231	0.087
	SD	32.0	0.020	0.006	0.145	0.029	0.322	0.082	1.254	0.242	0.077	0.103	0.090	0.025	0.062	0.016
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
2	Mean	261.1	0.070	0.027	1.023	0.393	2.009	0.773	7.685	2.941	1.969	0.757	0.549	0.211	0.321	0.121
	SD	23.2	0.017	0.006	0.103	0.040	0.102	0.061	1.012	0.245	0.118	0.045	0.067	0.024	0.178	0.056
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
3	Mean	268.8	0.080	0.030	1.013	0.377	2.004	0.745	7.648	2.841	1.918	0.716	0.531	0.197	0.268	0.100
	SD	22.5	0.023	0.007	0.108	0.032	0.220	0.044	0.817	0.120	0.151	0.055	0.062	0.013	0.056	0.019
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
4	Mean	255.1	0.070	0.028	0.977	0.384	1.957	0.768	7.312	2.861	1.964	0.778	0.508	0.199	0.287	0.112
	SD	27.2	0.016	0.007	0.089	0.021	0.232	0.057	0.986	0.151	0.079	0.091	0.082	0.024	0.071	0.024
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
5	Mean	258.7	0.076	0.029	0.985	0.381	1.976	0.764	7.421	2.860	2.008	0.778	0.514	0.199	0.250	0.096
	SD	17.6	0.010	0.004	0.118	0.037	0.153	0.040	0.991	0.237	0.093	0.033	0.052	0.020	0.080	0.028
	N=	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12

1 = near isoline (PSC355); 2 = commercial non-transgenic line A (PHY72); 3 = commercial non-transgenic line B (PHY78); 4 = commercial non-transgenic line C (98M-2983); 5 = WIDESTRIKE™ cotton.

Table 4

Reproductive organ weights for male and female rats given near isolate, commercial non-transgenic lines, or WIDESTRIKE™ cotton in the diet for 90 days

Group		Final body			Epididymides	
		Wt. (G)	Testes (G)	(G/100)	(G)	(G/100)
1	Mean	467.4	3.612	0.781	1.377	0.297
	SD	46.0	0.240	0.103	0.070	0.025
	N =	12	12	12	12	12
2	Mean	466.9	3.593	0.779	1.356	0.293
	SD	46.3	0.335	0.121	0.145	0.044
	N =	12	12	12	12	12
3	Mean	475.6	3.617	0.764	1.355	0.287
	SD	37.5	0.252	0.068	0.096	0.035
	N =	12	12	12	12	12
4	Mean	461.4	3.534	0.770	1.391	0.303
	SD	36.6	0.359	0.099	0.107	0.026
	N =	12	12	12	12	12
5	Mean	480.8	3.420	0.716	1.352	0.283
	SD	44.9	0.508	0.122	0.113	0.037
	N =	12	12	12	12	12
			Ovaries		Uterus	
			(G)	(G/100)	(G)	(G/100)
1	Mean	262.0	0.117	0.045	0.671	0.261
	SD	32.0	0.019	0.006	0.160	0.078
	N =	12	12	12	12	12
2	Mean	261.1	0.117	0.045	0.745	0.290
	SD	23.2	0.028	0.012	0.224	0.102
	N =	12	12	12	12	12
3	Mean	268.8	0.117	0.044	0.793	0.293
	SD	22.5	0.018	0.006	0.297	0.103
	N =	12	12	12	12	12
4	Mean	255.1	0.119	0.046	0.817	0.325
	SD	27.2	0.021	0.005	0.163	0.081
	N =	12	12	12	12	12
5	Mean	258.7	0.120	0.046	0.800	0.313
	SD	17.6	0.021	0.008	0.294	0.127
	N =	12	12	12	12	12

1 = near isolate (PSC355); 2 = commercial non-transgenic line A (PHY72); 3 = commercial non-transgenic line B (PHY78); 4 = commercial non-transgenic line C (98M-2983); 5 = WIDESTRIKE™ cotton (PHY440W).

equivalent to dietary intake of 7235 mg/kg/day for males and 7935 mg/kg/day for females.

The results of this study showing the lack of any toxicity of genetically modified WIDESTRIKE™ cottonseed are consistent with Bren (2003), which states the FDA is confident that genetically engineered food products are as safe as their conventionally bred counterparts and do not pose any risks for consumers. Similarly, Betz et al. (2000), states that plants modified to express insecticidal proteins from *Bacillus thuringiensis* (BT-protected plants) and their expressed insecticidal proteins (Cry) have undergone extensive testing over the last 40 years to establish their safety to humans, animals, and the environment.

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