# *Memorandum*

# *Biotechnology Notification File No. 000163*
# CVM Note to the File

***Date:*** September 9, 2019

***From:*** Rial Christensen, Ph.D.

***To:*** Administrative Record, BNF No. 000163

***Subject:*** Cotton with reduced levels of gossypol in seed, event TAM66274

## *Keywords:*
Cotton, *Gossypium hirsutum*, cottonseed, low gossypol seed, RNA interference (RNAi), neomycin phosphotransferase II (NPTII), *Escherichia coli* Tn*5*, delta-cadinene synthase (dCS), OECD identifier TAM-66274-5, Texas A&M AgriLife Research

## *Purpose:*
This document summarizes the Food and Drug Administration (FDA) Center for Veterinary Medicine's (CVM, we) evaluation of biotechnology notification file (BNF) number 000163. Texas A&M AgriLife Research (Texas A&M) submitted a safety and nutritional assessment for a genetically engineered (GE) cotton, transformation event TAM66274 (TAM66274 cotton), which was received on September 25, 2017. The FDA received additional information from Texas A&M on February 16, 2018 and June 2, 2019. CVM evaluated the information in Texas A&M's submissions to ensure that regulatory and safety issues regarding animal food derived from TAM66274 cotton have been resolved prior to commercial distribution. FDA's Center for Food Safety and Applied Nutrition summarizes its evaluation of TAM66274 cotton in human food in a separate document.

In CVM's evaluation, we considered all of the information provided by Texas A&M as well as publicly available information and information in the agency's files. Here we discuss the outcome of the consultation for animal food use, but do not intend to restate the information provided in the final consultation in its entirety.

## *Intended Effect:*
The intended effect of the modification in TAM66274 cotton is to significantly reduce the concentration of total gossypol in the seed,[1] while maintaining normal gossypol levels in the rest of the plant.[2] To confer this trait, Texas A&M introduced DNA

[1] Gossypol is the predominant terpenoid in the seed.

[2] There are conventional cotton varieties that have reduced levels of gossypol throughout the plant. These varieties are known as "glandless" cotton because they lack the glands where gossypol and other related

sequences containing inverted repeat nucleotide sequences of the delta-cadinene synthase (*dcs*) gene under the control of a seed specific promoter, which produces double-stranded ribonucleic acid (dsRNA) transcripts that trigger RNA-mediated silencing mechanism. The *dcs* gene encodes the delta-cadinene synthase (dCS) protein, the enzyme that catalyzes the first committed step (cyclization of farnesyl diphosphate to (+)-delta-cadinene) in the synthesis of gossypol and related terpenoids.

## *Regulatory Considerations:*

The purposes of this evaluation are (1) to assess whether Texas A&M has introduced into animal food a substance requiring premarket approval as a food additive and (2) to determine whether use of the new plant variety in animal food raises other regulatory issues with respect to the Federal Food, Drug, and Cosmetic Act (FD&C Act).

## *Genetic Modification and Characterization:*

Texas A&M transformed segments of Coker 312 cotton seedlings using disarmed *Agrobacterium tumefaciens*-mediated transformation. The transfer DNA (T-DNA) contains two expression cassettes between left and right border sequences. The first cassette contains nucleotide sequences from the coding region of the cotton (*Gossypium hirsutum*) *dcs* gene, an intron from the *Flaveria trinervia* pyruvate orthophosphate dikinase gene, and nucleotide sequences complementary to the *dcs* coding region sequence in reverse orientation, under the control of the seed specific *alpha-globulin B* gene from *G. hirsutum*. The second cassette includes a *neomycin phosphotransferase II*[3] (*nptII)* gene derived from *Escherichia coli* Tn*5* under the control of the constitutive nopaline synthase promoter from *A. tumefaciens*.[4]

Following transformation, somatic embryos were cultivated on selection media[5], plants were regenerated, and plants were then grown to maturity. Genomic DNA was isolated from the leaves of GE plants to confirm the presence and determine the number of copies of the T-DNA. Additional breeding steps (up to 7 generations) were conducted to generate plants used in the characterization of the genetic insertion, inheritance studies, and gene expression studies.

Texas A&M characterized the introduced DNA in TAM66274 cotton using restriction enzyme digestion of genomic DNA followed by Southern blot analyses. The parental cultivar, Coker 312, (control) was used as the comparator in these analyses. Texas A&M reported that a single, intact copy of the T-DNA was inserted into the genome. Southern

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terpenoids are primarily stored. These glands are located in the stem, leaves, seeds and flower buds of the cotton plant. Gossypol protects cotton plants from some insects and pathogens; consequently, glandless cotton is more susceptible to these insects and pathogens.

[3] Neomycin phosphotransferase II, also known as aminoglycoside 3´-phosphotransferase II (*aph(3´)II*) and kanamycin resistance (*kan*$^r$), is a gene that encodes the NPTII (APH(3´)II and KAN$^R$) protein.

[4] An, G., B.D. Watson, S. Stachel, M.P. Gordon, and E.W. Nester. 1985. New cloning vehicles for transformation of higher plants. EMBO J. 4:277-284. The first 24 nucleotides of the *nptII* gene from *E. coli* transposon Tn*5* were replaced with 51 nucleotides from the *nos* gene and the remaining 768 nucleotides are identical to the *nptII* gene that was isolated from *E. coli* Tn*5*.

[5] The selection media contained kanamycin for selection of transformants and carbenicillin for inhibition of *A. tumefaciens* growth.

blot analysis was also used to demonstrate the absence of vector backbone sequences, the region outside of the T-DNA borders, in TAM66274 cotton. Texas A&M also used overlapping PCR amplification followed by DNA sequencing of the amplified products to confirm the nucleotide sequence and genomic organization of the inserted DNA. Texas A&M identified a 44 base pair deletion of genomic DNA at the insertion site.[6]

Texas A&M evaluated the stability and integrity of the insert across three self-pollinated generations (T1, T2, and T3) using Southern blot analysis. Texas A&M observed similar band migration patterns in restriction digested genomic DNA, when compared to genomic DNA from the control and concluded that the T-DNA insert is stably transmitted from generation to generation. Texas A&M also assessed inheritance of the T-DNA insert in a segregating population using phenotypic (visible color difference in the glands of TAM66274 cottonseed kernels) data and event-specific PCR analyses on individual cottonseeds. Texas A&M analyzed the results of these analyses by Chi-square goodness-of-fit analysis and showed that the desired genotype segregated according to the expected Mendelian principles. These data also support the conclusion that TAM66274 cotton contains a single insert integrated into a single chromosomal locus.

Texas A&M also performed bioinformatics analyses using the nucleotide sequence for the insert and 50 nucleotides of genomic sequences flanking on either side of the T-DNA in TAM66274 cotton to determine whether insertion of the introduced DNA has the potential to create open reading frames (ORFs) that would encode for putative polypeptides that share homology with known toxins. Based on the results of bioinformatics analyses, Texas A&M concludes that even in the unlikely occurrence of translation of any ORFs, the resulting putative polypeptides would not share homology with known toxins and would not constitute a safety concern.

### *Intended Effect: Reduced Expression of dcs Messenger RNA*

To determine the effectiveness and tissue specificity of the *dcs* RNA interference (RNAi) cassette in suppressing transcript levels of the *dcs* gene(s), Texas A&M performed quantitative real-time PCR (qRT-PCR). Messenger RNA was obtained from root, leaf, bract, floral bud, axillary bud and seed embryo tissues of TAM22674 cotton and control. Texas A&M reported that relative values for *dcs* gene transcript levels in developing embryos (described as unopened bolls) to be 0.14 and 1.00 for TAM66274 cotton and control, respectively. Further, Texas A&M reports that there were no significant differences between TAM66274 cotton and control in *dcs* gene transcript levels in non-seed tissues. Texas A&M concludes that these data demonstrate that the *dcs* RNAi cassette selectively reduces *dcs* transcript levels in embryos, with no effect on transcript levels in other plant parts. This conclusion was also supported by the results of phenotypic analysis.

[6] The deletion appears to be within the last intron of a putative alpha-hydrolase gene. Texas A&M measured alpha-hydrolase gene expression using qRT-PCR and Texas A&M concluded that there was minimal impact on messenger RNA (mRNA) expression from this gene. Based on bioinformatics analysis and results of qRT-PCR, Texas A&M does not expect the insertion to interrupt or alter the expression of the putative alpha-hydrolase gene.

## *Safety of Expression Products:*

The expression of the *dcs* RNAi cassette in TAM66274 cotton results in the synthesis of double stranded RNA that, after processing, specifically reduces the levels of dCS in the seed. Double stranded RNA is not translated into protein. Previously, the FDA has stated that "nucleic acids are present in the cells of every living organism, including every plant and animal used for food by humans and animals and do not raise a safety concern as a component of food."[7]

TAM66274 cotton was also genetically engineered to express NPTII derived from *E. coli* Tn*5*. NPTII confers tolerance to neomycin, kanamycin, G418 and other related antibiotics. NPTII has been used as a selectable marker in the production of some GE plants.

Texas A&M notes that the amino acid sequence for NPTII protein in TAM66274 cotton is not identical to the amino acid sequence of the NPTII protein expressed by *E. coli* Tn*5*. Texas A&M shows that, for the NPTII protein in TAM66274 cotton, the first 17 amino acids of the NOS protein replaced the first eight amino acids of the *E. coli* expressed NPTII protein, resulting in an in-frame fusion protein. Texas A&M provided information to demonstrate that modification of the n-terminus of the NPTII protein does not modify the amino acid sequences for the conserved domains within the NPTII protein. It also does not alter the enzyme's ability to impart resistance to kanamycin, the selection antibiotic used in the production of TAM66274 cotton.

Texas A&M summarizes scientific literature[8] about NPTII from *E. coli* Tn*5* to support the safety of the NPTII protein in TAM66274 cotton. These studies show that NPTII protein in *E. coli* Tn*5* is rapidly digested in simulated mammalian gastric and intestinal fluids and that consumption of exaggerated levels of this protein did not affect the health of mice. Texas A&M highlights that FDA conducted a comprehensive safety review and approved use of the NPTII protein from *E. coli* Tn*5* as a food additive in GE cotton, canola and tomatoes for use in food for humans and animals.[9]

Texas A&M states that the levels of NPTII protein in plant tissues of TAM66274 cotton, as measured by enzyme-linked immunosorbent assay, did not exceed the amount reasonably required for selection of plant cells carrying the *nptII* gene and the protein was expressed at no more than 41.1 nanograms/gram of dry weight in the seed of TAM66274 cotton. Based on this data and information, Texas A&M concludes that the NPTII protein expressed in TAM66274 cotton has the same animal food safety characteristics as the NPTII expressed in other commercial GE new plant varieties.

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[7] Statement of Policy – Foods derived from new plant varieties. Federal Register Vol. 57 No. 104 Friday, May 29, 1992 22984 - 22990.

[8] Fuchs, R.L., J.E. Ream, B.G. Hammond, M.W. Naylor, R.M. Leimgruber, and S.A. Berberich. 1993. Safety assessment of neomycin phosphotransferase II (NPTII) protein. Nature Biotechnol. 11:1543-1547.

[9] Title 21 of the Code of Federal Regulations Part 173.170 (21 CFR 173.170) and 573.130 (21 CFR 573.130), respectively.

## *Animal Food Use:*

Following cleaning and ginning, whole cottonseed (after removal of the cotton fibers) is processed into four major human and animal food products: oil, linters, meal, and hulls. The first two ingredients are almost exclusively used in human food. Whole cottonseed, acid delinted cottonseed, cottonseed meal, hulls, and cotton gin trash are used as ingredients in food for ruminant animals. The amount of cottonseed meal that can be used in monogastric animal diets is normally limited by the presence of gossypol. Cottonseed meal which contains not more than 0.04% (400 parts per million (ppm)) free gossypol, can be used as a source of protein in food for animals.[10] For example, cottonseed meal from glandless varieties of cotton have been used in animal food for monogastric species, such as swine and poultry, and aquaculture.[11]

## *Composition:*

### *Scope of Analysis:*

Texas A&M analyzed the nutrient composition of TAM66274 cotton and the control that were grown and harvested under similar conditions. Texas A&M selected components for analysis from those recommended in the Organization for Economic Cooperation and Development (OECD) cotton composition consensus document.[12]

### *Study Design:*

Texas A&M conducted field trials during the 2014 (two sites in North Carolina and one site in Mississippi) and 2015 (two sites in North Carolina, two sites in Mississippi, and one site in Texas) growing seasons. The field sites were selected to be representative of cotton growing regions in the United States. The cotton varieties were planted using a randomized block design with four replicate plots at each field site. Ginned and acid delinted TAM66274 cotton and control cottonseed were collected (by combining samples from the four replicates) at each site and were analyzed according to published analytical methods. As part of a separate analysis, 25-boll samples were collected from each replicate plot at each location and were processed to remove cotton fibers, linters, and seed coat (hulls) and kernels were used for total gossypol analysis.

Texas A&M statistically compared each component for TAM66274 cotton with the control across-locations by year using a mixed-design model with a residual maximum likelihood approach. Texas A&M identified statistically significant differences at the level of $P \leq 0.05$. Any observed differences between TAM66274 cotton and control were

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[10] Low gossypol cottonseed meal, which contains not more than 0.04% free gossypol, that was obtained by mechanical or solvent extraction are defined (24.50 and 24.51, respectively) in the Official Publication of the Association of American Feed Control Officials as ingredients for use in food for animals.

[11] Ryan, J.R., F.H. Kratzer, C.R. Grau, and P. Vohra. 1986. Glandless cottonseed meal for laying and breeding hens and broiler chicks. Poultry Sci. 65:949-955; LaRue, D.C., D.A. Knabe, and T.D. Tanskley, Jr. 1985. Commercially processed glandless cottonseed meal for starter, grower, and finisher swine. J. Anim. Sci. 60:495-502; Robinson, E.H., S.D. Rawles, and R.R. Stickney. 1984. Evaluation of glanded and glandless cottonseed products in catfish diets. Aquaculture 38:145-154.

[12] Organization for Economic Cooperation and Development. 2009. Consensus document on compositional considerations for new varieties of cotton (*Gossypium hirsutum* and *Gossypium barbadense*): Key food and feed nutrients and anti-nutrients. Series on the Safety of Novel Foods and Feeds No. 11. ENVJM/MONO(2004)16.

compared with ranges published for conventional cotton varieties in the International Life Sciences Institute (ILSI) Crop Composition Database and in the scientific literature.

*Results of analyses:*

Texas A&M reported results for proximates (moisture, crude protein, total fat, crude fiber, carbohydrates by calculation, and ash), calories by calculation, fiber components (total dietary fiber, acid detergent fiber, and neutral detergent fiber), 18 amino acids, 11 fatty acids[13], nine minerals, α-tocopherol, phytic acid, secondary metabolites (malvalic, sterculic, and dihydrosterculic acids), and total and free gossypols for TAM66274 cotton and control.

For compositional components other than gossypol, Texas A&M reported statistically significant differences between TAM66274 cotton and control in the levels of 16 components for samples collected in 2014, and 20 components for samples collected in 2015. However, Texas A&M notes that, when statistical differences were observed, the mean values for these components in TAM66274 cotton fell within the range of values in the ILSI database and scientific literature. Texas A&M concluded that mean values for proximates, calories, fiber components, amino acids, fatty acids, minerals, α-tocopherol, phytic acid, and secondary metabolites fell within the range of values for these components in conventional cotton varieties with a history of safe use in animal food.

*Intended Compositional Changes:*

Texas A&M analyzed gossypol levels in dehulled kernels obtained from TAM66274 cotton and control using two analytical methods.[14] In samples obtained in 2014 and 2015, Texas A&M reported statistically lower levels of total gossypol in kernels obtained from TAM66274 cotton when compared to control with the two analytical methods producing similar results. Mean values for total gossypol by the aniline method were 440 and 420 milligrams per kilogram (equal to ppm) on a dry weight basis in TAM66274 cotton and 9,630 and 9,410 ppm in control for samples obtained in 2014 and 2015, respectively. Mean values for free gossypol were 300 and 260 ppm in TAM66274 cotton and 7,770 and 8,300 ppm in control for samples obtained in 2014 and 2015, respectively. Texas A&M also reported (-)-gossypol isomer levels by HPLC were 109 and 123 ppm in TAM66274 cotton and 2,820 and 2,728 ppm in control for samples obtained in 2014 and 2015, respectively. The corresponding concentrations of (+)-gossypol isomer were 148 and 160 ppm in TAM66274 cotton and 3,893 and 4,204 ppm in control for samples obtained in 2014 and 2015, respectively. Texas A&M concludes that total gossypol levels in TAM66274 cotton kernels were reduced to approximately 3% of levels in control kernels and that free gossypol levels in these kernels are below 400 ppm.

[13] Values for 16 other fatty acids were below the limits of quantitation.

[14] The aniline method is a relatively fast colorimetric method that determines the concentration of free gossypol and total gossypol in a cottonseed sample, but it tends to overestimate the levels of gossypol because the method also detects impurities and other terpenoids in cottonseed. The HPLC method measures each terpenoid separately and total gossypol is calculated as the sum of the terpenoids.

*Summary of Compositional Analyses:*

Texas A&M states that the genetic modification does not meaningfully affect nutrient composition and nutritional value of cottonseed derived from TAM66274 cotton except for the intended change in total and free gossypol. Texas A&M concludes that animal food from TAM66274 cotton is as safe as and, with the exception of reduced levels of gossypol in seed, does not differ in composition from cotton-derived animal food currently on the market.

## *Labeling Considerations:*

It is a producer's or distributor's responsibility to ensure that labeling of the foods it markets meet applicable legal requirements, including disclosure of any material differences in the food. In evaluating the common or usual name appropriate for animal food ingredients from TAM22674 cotton, CVM considered that this new cotton variety was genetically engineered to reduce gossypol in seed alone, and its dehulled seed contains no more than 0.04% free gossypol. CVM recognizes that when used in animal food, the appropriate name for dehulled cottonseed derived from TAM66274 cotton is "low gossypol dehulled cottonseed", and the appropriate name for dehulled cottonseed meal derived from TAM66274 cotton is "low gossypol dehulled cottonseed meal". CVM recognizes that for all other uses of TAM66274 cotton and derived products in animal food, "cotton" is the appropriate name (for example, "cottonseed hulls", "cottonseed screenings", and "cotton plant byproduct").

## *Conclusion:*

CVM evaluated Texas A&M's submissions to determine whether TAM66274 cotton raises any safety or regulatory issues with respect to its uses in animal food. Based on the information provided by Texas A&M and other information available to the agency, CVM did not identify any safety or regulatory issues under the FD&C Act that would require further evaluation at this time.

Texas A&M has concluded that TAM66274 cotton and the animal foods derived from it are as safe as and, with the exception of lower free gossypol levels in dehulled seed, are not materially different in composition or any other relevant parameter from other cotton varieties now grown, marketed, and consumed. At this time, based on Texas A&M's data and information, CVM considers Texas A&M's consultation on TAM66274 cotton for use in animal food to be complete.

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