

GRAS Notice (GRN) No. 471

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

ORIGINAL SUBMISSION



Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety-CFSAN
U.S. Food and Drug Administration
5100 Paint Branch Parkway (HFS-200)
College Park, MD 20740-3835

May 2, 2013

ATTN: Dr. Antonia Mattia, PhD

Our Reference: GRAS Notification and Exemption Claim for DeltaGold®, a tocotrienol rich extract

Dear Dr. Mattia,

AIBMR Life Sciences, Inc. has been retained as an agent by American River Nutrition, Inc. ('the Notifier') to submit a GRAS notification to the FDA for DeltaGold®, a tocotrienol-rich extract derived from the seeds of *Bixa orellana* L. intended for use as an ingredient in food. DeltaGold® is therefore exempt from the requirement of pre-market approval, consistent with section 201 (s) of the Federal Food, Drug and Cosmetic Act.

The GRAS determination is based on scientific procedures. The basis for the determination relies on acute, sub-chronic and chronic animal models of toxicology that support the safety of tocotrienols, as well as the long history of human exposure to tocotrienols and *Bixa orellana* extracts, and lastly, governmental organizations' positions on the safe use of tocotrienols in food.

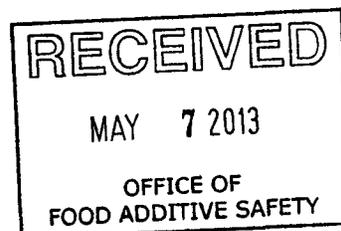
Please find enclosed three copies of the notification: *Notice to US Food and Drug Administration that the use of DeltaGold® is Generally Recognized as Safe*. Also enclosed are copies of all references cited in the notification. As stated in the exemption claim, the data and the information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of American River Nutrition, Inc, (31 Campus Plaza Road, Hadley, MA 01035) or will be sent to FDA upon request. Please do not hesitate to contact us with any questions.

Yours sincerely,

(b) (6)

John R. Endres
Chief Scientific Officer
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**Notice to US Food and Drug Administration
that the use of DeltaGold® is Generally
Recognized as Safe**

Submitted by the Notifier:

American River Nutrition, Inc.
31 Campus Plaza Road
Hadley, MA 01035 USA

Prepared by the Agent of the Notifier:

AIBMR Life Sciences, Inc
4117 S Meridian
Puyallup WA 98373

May 1, 2013

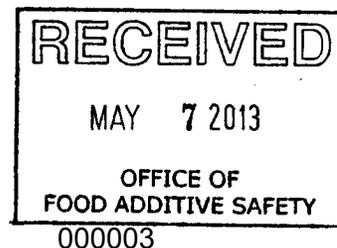


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GRAS Exemption Claim

American River Nutrition, Inc. (the notifier), has determined that DeltaGold®, a tocotrienol-rich Annatto seed extract, is Generally Recognized as Safe (GRAS) for its intended use, consistent with section 201 (s) of the Federal Food, Drug and Cosmetic Act. The determination has been made based on scientific procedures, and therefore the use of DeltaGold® for its intended purpose is exempt from the requirement of pre-market approval.

(b) (6)

May 1, 2013
Date

Barrie Tan
President
American River Nutrition, Inc.

Name and Address of the Notifier

Notifier

American River Nutrition Inc.
31 Campus Plaza Road
Hadley, MA 01035 USA

Agent of the Notifier

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Common or Usual Name

The subject of this notification is DeltaGold®, a tocotrienol-rich Annatto seed (*Bixa orellana* L.) extract.

Conditions of Use

The intended use of DeltaGold® is as an ingredient in food at an exposure level of up to 212 mg tocotrienols per day, consistent with current Good Manufacturing Practices (cGMPs).

Basis for GRAS Determination

Scientific procedures are the basis for the conclusion that the intended use of DeltaGold® as an ingredient in food is GRAS.

Data Availability Statement

The data that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of American River Nutrition Inc. located at: 31 Campus Plaza Road Hadley, MA 01035 USA; or will be sent to FDA upon request.

Characterization

The subject of this notification is DeltaGold®, a tocotrienol-rich extract derived from the seeds of the South American shrub annatto (*Bixa orellana* L.), manufactured by American River Nutrition, Inc. (31 Campus Plaza Rd. Hadley, MA 01035 USA).

Tocotrienol is one of the two groups of naturally occurring compounds that comprise vitamin E. Namely, tocopherols and tocotrienols. DeltaGold® is standardized to a minimum concentration of 70% tocotrienols, approximately 90% of which is δ -tocotrienol and 10% of which is γ -tocotrienol. DeltaGold® is virtually free of the tocopherol form of vitamin E.

The additional components of DeltaGold® include fatty acids, triglycerides, and annatto oleoresins that occur naturally in the seeds of *Bixa orellana*. Fatty acids make up approximately 10% of the ingredient, while annatto oleoresins make up the remaining 20%. The fatty acids and annatto oleoresins are derived from annatto seed; no additional ingredients are added during the manufacturing process. The annatto oleoresins are not different from oleoresins found in approved food additive annatto extracts.

Background information on *Bixa orellana*

Bixa orellana L. is a shrub native to tropical America, the seeds of which contain orange-red carotenoid pigments, which are widely used as food colorants throughout the world. The major carotene-derived pigments found in the seeds are bixin and norbixin.^{1,2} Mayans and Aztecs traditionally used the plant as a dye for foods and textiles, and the plant continues to play an important role as a colorant today.³ Annatto extracts of *Bixa orellana* are FDA-approved color additives for use in human food (in amounts consistent with good manufacturing practices (GMP) (21 CFR Section 73.30), drugs (21 CFR Section 73.1030), and cosmetics (21 CFR Section 73.2030).

According to the National Center for Biotechnology Information Taxonomy Database, 2009⁴, *Bixa orellana* (Annatto) is of the taxonomic lineage: *cellular organisms; Eukaryota; Viridiplantae; Streptophyta; Streptophytina; Embryophyta; Tracheophyta; Euphyllophyta; Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons; rosids; eurosids II; Malvales; Bixaceae; Bixa*.

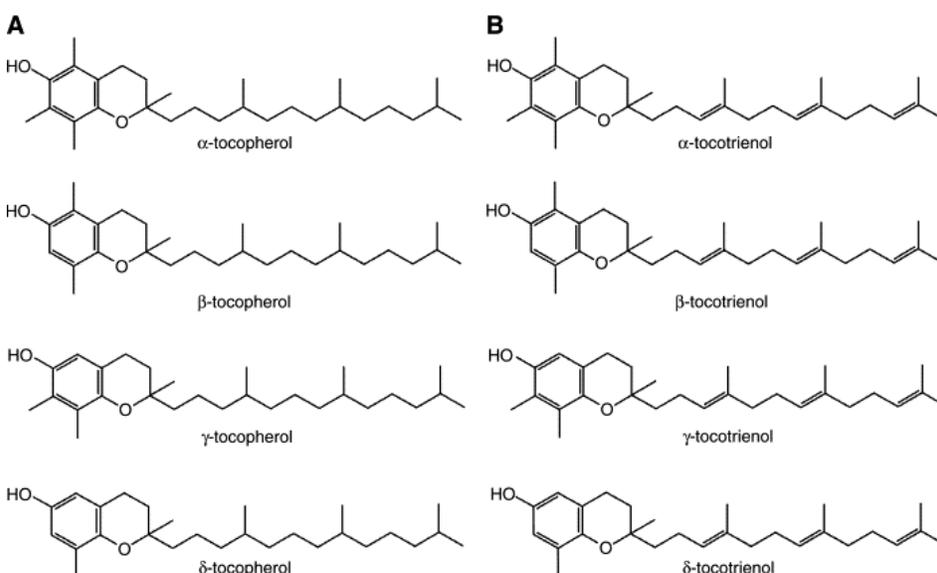
Background information on Tocotrienols

Vitamin E, as a complex of both tocopherols and tocotrienols, has been studied for about a century and is the subject of hundreds of thousands of publications in the public domain.⁵ The term “vitamin E” refers to a family of compounds possessing a chromanol head (comprised of two fused rings: one phenolic and one heterocyclic) with a 16-carbon side chain attached at the 2 position (see Figure 1). There are eight naturally occurring forms of vitamin E, including four tocopherols and four tocotrienols, all of which possess vitamin E biological activity.

Tocotrienols differ in structure slightly from tocopherols by having an unsaturated farnesyl isoprenoid tail with three trans double bonds in their

structure, while tocopherols have a saturated phytyl tail.⁶⁻⁸ Both compounds have α , β , γ , and δ congeners that differ in the number and position of their methyl groups.⁹ Figure 1 shows the structures and chemical attributes of these vitamin E congeners.

Figure 1. Chemical Structures of Tocopherols and Tocotrienols



Tocopherols contain three chiral centers, making eight stereoisomers possible. Tocotrienols, on the other hand, have only one chiral center, to allow for two stereoisomers. However, the double bonds of the farnesyl tail allows for the existence of an additional four cis/trans isomers per tocotrienol, giving a total of eight isomers possible.⁸ Variations in biopotency are thought to occur between the eight alpha-tocopherol stereoisomers (in favor of the *RRR* form). The discrimination is related to a specific hepatic α -tocopherol transfer protein which preferentially recognizes the *RRR* configuration of the side chain methyl groups.⁸

The chromanol group of the vitamin E congeners is responsible for both the lipophilic properties and the antioxidant potential of both tocopherols and tocotrienols. The phytyl tail, on the other hand, is thought to be responsible for the lipophilic properties (and therefore, positioning in the cell membrane), but has no direct effect on the antioxidant potential.⁸ Therefore, the unsaturation of the phytyl tail, as well as the stereochemistry of the phytyl tail at the point where it meets the chromanol ring is thought to affect the biological activity of the compound.

Biological Activity

Vitamin E is considered a chain-breaking antioxidant, which can prevent the propagation of lipid peroxidation.¹⁰ The activity of vitamin E is often reported as α -tocopherol equivalents. The *RRR*- α -tocopherol form is the only α -tocopherol

form that occurs naturally in foods. When α -tocopherol is synthesized, 8 different “2-position” stereoisomers result (RRR-, RSR-, RSS-, RRS-, SRR-, SSR-, SRS-, and SSS-). This synthetic version is referred to as *all rac*- α -tocopherol.

The International Unit (IU) of measurement for vitamin E was assigned by the United States Pharmacopeia (USP) and was used before 1980. Although the IU is still currently used in the food industry, in 1980 the USP changed the IU to the USP unit; the definition however, remains the same.^{10,11}

One IU of vitamin E activity is defined as 1 mg of *all rac*- α -tocopheryl acetate (a mixture of RRR-, RSR-, RSS-, RRS-, SRR-, SSR-, SRS-, and SSS-). One mg of natural vitamin E (*RRR*- α -tocopherol or historically, and considered incorrectly, known as *d*- α -tocopherol¹⁰) is equivalent to 1.49 IU of vitamin E. Therefore, 1 IU of vitamin E is equivalent to 1 mg of *all rac*- α -tocopheryl acetate, 0.67 mg *RRR*- α -tocopherol, or 0.74 mg *RRR*- α -tocopheryl acetate.

Although the biological activities of tocotrienols have not been formerly accepted by a government agency, Table 1 compares the reported biological activities of the eight vitamin E congeners, measured by the rat resorption-gestation assay (in which female rats are given a vitamin E deficient diet and then mated, and levels of vitamin E that allow for normal childbearing is sought) compared to 1 mg of natural, *RRR*- α -tocopherol form. It is important to note that this is not a measurement of antioxidant biological activity.

Table 1. Biological Activity of Tocopherols and Tocotrienols Relative to Natural (*RRR*) α -Tocopherol (adapted from Kamal-Eldin and Appelqvist 1996⁸)

Compounds*	Relative Biological Activity
α -tocopherol	100%
β -tocopherol	50%
γ -tocopherol	10%
δ -tocopherol	3%
α -tocotrienol	30%
β -tocotrienol	5%
γ -tocotrienol	Not known (estimated to be ~1%)
δ -tocotrienol	Not known

**RRR* forms

Vitamin E activity in food is often referred to as α -tocopherol equivalents (α -TE). Previously, in the 1980 edition of the Recommended Allowances from the Food and Nutrition Board, total vitamin E activity in a food was given as mg of *d*- α -tocopherol plus the weights of other tocopherols or tocotrienols after correction to their equivalency as factors for the equivalence of *d*- α -tocopherol.¹² Alpha-TEs

equivalencies were defined as α -tocopherol 1 mg, β -tocopherol 0.5 mg, γ -tocopherol 0.1 mg, δ -tocopherol 0.03 mg; α -tocotrienol 0.3 mg; β -tocotrienol 0.05, γ -tocotrienol 0.01 mg and the biological activity/equivalency of δ -tocotrienols were unknown.^{10,13}

The Tolerable Upper Intake Levels (ULs) for vitamin E (α -tocopherol only), set by the Food and Nutrition Board (FNB) of the Institute of Medicine (IOM), are based on the hemorrhagic activity of tocopherols. The FNB declared doses up to 1,000 mg/day (1,500 IU/day of the natural form or 1,100 IU/day of the synthetic form) in adults appear to be safe for males and females over 19 years of age, including those pregnant and lactating. The tocopherols have a tendency to produce hemorrhaging in the order of α - > β - > γ - > δ -.¹⁴ Because tocotrienols have a similar range of biological activity (α - > β - > γ - > δ -) and the fact that δ -tocotrienol has the lowest known “biological activity” of the tocotrienols (as seen in the rat resorption-gestation assay) it is reasonable, that the UL of tocotrienols, particularly δ -tocotrienol, could exceed that of α -tocopherol, although there is some evidence that suggests that high doses of γ -tocotrienol are partly converted to α -tocopherol.¹⁵

Tocotrienols, as a whole, are similar in structure, function and chemical attributes (see Table 2). While there are some differences in the biological activity as previously mentioned, as well as the strength of antioxidant activity by the various tocotrienol congeners,^{7, 16, 17} there is no data that indicates the safety profile of one congener would be vastly different than another due to the great structural similarity between all vitamers of the tocotrienol complex.

Table 2. Tocotrienol Chemical Attributes

	CAS Number	Molecular Weight	Molecular Formula
α -tocotrienol	58864-81-6	424.65846	C ₂₉ H ₄₄ O ₂
β -tocotrienol	490-23-3	410.63188	C ₂₈ H ₄₂ O ₂
γ -tocotrienol	14101-61-2	410.63188	C ₂₈ H ₄₂ O ₂
δ -tocotrienol	25612-59-3	396.6053	C ₂₇ H ₄₀ O ₂

Absorption, Distribution, Metabolism and Excretion

The absorption and biokinetics of tocotrienols have not been fully elucidated. However, tocopherols and tocotrienols are thought to be absorbed in the intestines in a similar manner. There does not appear to be a competitive intestinal absorption between the two.¹⁸ Vitamin E absorption in the intestines is dependent upon multiple factors including biliary and pancreatic secretions, micelle formation, enterocyte uptake and chylomicron secretion.¹⁰ In the liver, however, α -tocopherol is preferentially recognized over the other tocopherol

isomers as well as the tocotrienols by the α -tocopherol transfer protein (α -TTP).¹⁹ In fact, the affinity of α -TTP for α -tocotrienol is reported to be 12% that of α -tocopherol.²⁰ Therefore, tocotrienol liver uptake and blood transfer is significantly lower than α -tocopherol, and subsequently, concentrations of tocotrienols in both plasma and lipoproteins are significantly lower than that of α -tocopherol.^{5, 18, 21, 22} It also appears that α -tocopherol, when taken in combination with tocotrienols, inhibit tocotrienol uptake.²⁰ Nevertheless, tocotrienol supplementation has been shown to increase plasma levels of tocotrienols significantly. It has been noted that plasma tocotrienol levels can reach 4.74 $\mu\text{mol/L}$ in humans and 21 $\mu\text{mol/L}$ in various animal species.^{21, 23-27} The absorption of tocotrienols has also been shown to be increased more than 2-fold when a human subject is fed rather than fasted.²⁸

A recent 2012 trial investigated the human metabolism of tocotrienols.¹⁸ After supplementation with either α -tocopherol (537 mg α -tocopherol exclusively) or a tocotrienol-rich palm fraction (526 mg vitamin E as 359.2 mg tocotrienols and 167 mg α -tocopherol), α -tocopherol was indeed the most abundant vitamin E isomer found in both plasma and lipoproteins in human subjects of both groups. Although α -tocopherol dominated in the plasma, supplementation with the tocotrienol-rich palm fraction did in fact result in a significant increase of α -, γ - and δ -tocotrienols in postprandial plasma, compounds that were not detected in plasma at baseline (β -tocotrienol was not detected throughout the study). All of detected isomers increased postprandially commencing at 2 hrs and peaking at 4 hours (δ -) and 5 hrs (α - and γ -) before declining. The absorption rates appeared in the order of α -tocotrienol > γ -tocotrienol > δ -tocotrienol, a ratio previously confirmed by others.²⁴⁻⁸ Tocotrienols were not detected in the plasma 24 hours after supplementation, indicating rapid metabolism, absorption, and/or excretion. In lipoprotein fractions, α -tocopherol was again the major vitamin E isomer found and followed similar absorption ratios as were found in plasma. Compared to α -tocopherol, the tocotrienol isomers were also more rapidly removed from circulating plasma and triacylglycerol-rich particles, LDL and HDL.

Husain et al. (2009) studied the pharmacokinetics of δ -tocotrienol specifically in female athymic nude mice. Twenty-two mice were administered 100 mg/kg δ -tocotrienol by gavage. The δ -tocotrienol plasma peak occurred at 2 hours, with a half-life of 3.5 hours, and total clearance by 24 hours.²⁹ Yap et al. (2001) reported the elimination half-lives of α -, γ -, and δ -tocotrienol in humans to be 4.4, 4.3 and 2.3 hours, respectively.²⁸ The half-lives of tocotrienols are known to be much shorter than that of α -tocopherol.²²

Tocopherols and tocotrienols alike are absorbed and transported within chylomicrons and are involved in the lipoprotein cascade. It is also well established that tocotrienol concentrations within these particles are overall significantly lower than tocopherols.¹⁸ Alpha-tocopherol is primarily incorporated into LDL and HDL (compared to VLDL and IDL). Tocotrienols, on

the other hand, are thought to be transported nonspecifically, most likely incorporated with triglycerides in the core of the chylomicron.³⁰

The differences in tissue uptake of tocotrienols and tocopherols have been noted in both animal and human trials.^{30, 31} After supplementation in humans, tocotrienols can be found at low concentrations in most vital organs and tissues; with the greatest reservoir being adipose tissue.²³ In animals, only adipose tissue accumulates more tocotrienols than tocopherols — primarily γ -tocotrienol.³⁰

Tocotrienols, like tocopherols, are metabolized in humans by ω -oxidation followed by five cycles of β -oxidation of the side chain. The final products of both vitamin E compounds are carboxyethyl hydroxychromans (CEHC), found in the urine.^{19, 32} However, tocotrienols appear to have a more complex degradation, most likely due to the unsaturated side chain and the need for additional enzymes for their metabolism, similar to the β -oxidation of unsaturated fatty acids. The biliary/fecal route is the main route of excretion for unabsorbed and unutilized vitamin E.^{19, 22}

History of Consumption

Use of *Bixa orellana* L. seed and seed extracts as food

Annatto seeds and extracts have been consumed as food for over two centuries², and the seed is sometimes referred to as the “poor man’s saffron”. The Aztecs used annatto extract to color the cocoa and vanilla-based drink cacahuatl.³

Annatto extracts are FDA-approved color additives for use in foods, drugs, and cosmetics (21 CFR 73.30, 73.1030, and 73.2030) in amounts consistent with good manufacturing practices. Commercially produced extracts are manufactured by extracting the colored pigment from the seeds using water, vegetable oil, or solvents. Extracts typically contain 1–10% of the carotenoids bixin and norbixin (the pigments used for coloring), although solvent extractions can raise these levels up to 97%.² Annatto functions as a colorant for foods such as cheese, edible oils, ice cream, smoked fish, custards, peanut butter, margarine, and butter.³³ It is also used to color tablets, pills, and granules in drugs and dietary supplements.

Additionally, the World Health Organization reported that based on data from 1997, approximately 28% of Brazil’s population (44 million people) consume whole annatto seeds as a condiment and have done so for many years.³⁴ A search for information available in the public domain on the Internet reveals numerous recipes for annatto oils and pastes.

As of 2003, the average annual production of annatto seeds was approximately 10,000 metric tons. Two-thirds were traded as seeds, the other one-third as seed extracts. Sixty percent was produced by Latin America; the rest by Africa and Asia.³

Presence of tocotrienols in food

Sources of tocotrienols in nature are numerous, and include but aren't limited to annatto seeds, palm oil, rice bran oil, barley, wheat, corn, oats, coconut, and rosemary seeds. Annatto seeds are unique in that they are high in tocotrienols, while virtually free of tocopherols.⁷ The amount of tocotrienols in annatto seeds has been investigated using liquid and gas chromatography, and has been reported as 140–147 mg per 100 grams (3.5 ounces) of dry seeds, or 5.2–5.5% weight of the lipid extract.³⁵ The majority of tocotrienol in annatto is delta-tocotrienol, making it the richest source of δ -tocotrienol amongst plants. In comparison, palm oil and rice bran oil contain 6.0 mg and up to 10.0 mg of δ -tocotrienol, respectively, per 100 grams.³⁵

Palm oil extracted from the palm plant *Elaeis guineensis* contains up to 800 mg of tocotrienols per kg, mainly consisting of gamma- (γ -) and alpha- (α -) tocotrienol, but also some δ -tocotrienol.⁷ Cereal grains also have a relatively high tocotrienol content; barley contains up to 52 mg per kg, and rice up to 201 mg per kg. Commercial eggs contain approximately 0.11 mg of tocotrienol per egg, but have been engineered to contain up to 0.62 mg by feeding hens rice bran oil.³⁶

Franke and colleagues analyzed 79 food items commonly consumed in Hawaii for their levels of vitamin E. The average total level of tocotrienol in the foods ranged from non-detectable to 432 mg/kg. The sum of all E vitamins (tocopherols plus tocotrienols) in the foods ranged from 0.6 to 827.7 mg/kg. Mean levels of δ - and γ -tocotrienol in the foods were 37.0 mg/kg and 36.4 mg/kg respectively.³⁷

Several researchers have published calculations of tocotrienol levels typically found in diets. In 2010, the tocotrienol consumption of a Japanese population was estimated by analyzing 242 food items and 64 meal items. The daily intake of tocotrienols ranged from 1.9–2.1 mg /day/person.³⁸ A typical Finnish diet contains between 2.96 and 4.21 mg of tocotrienols per day.³⁹ Tocotrienols are also regularly sold and consumed at much higher levels in dietary supplements.

Manufacturing

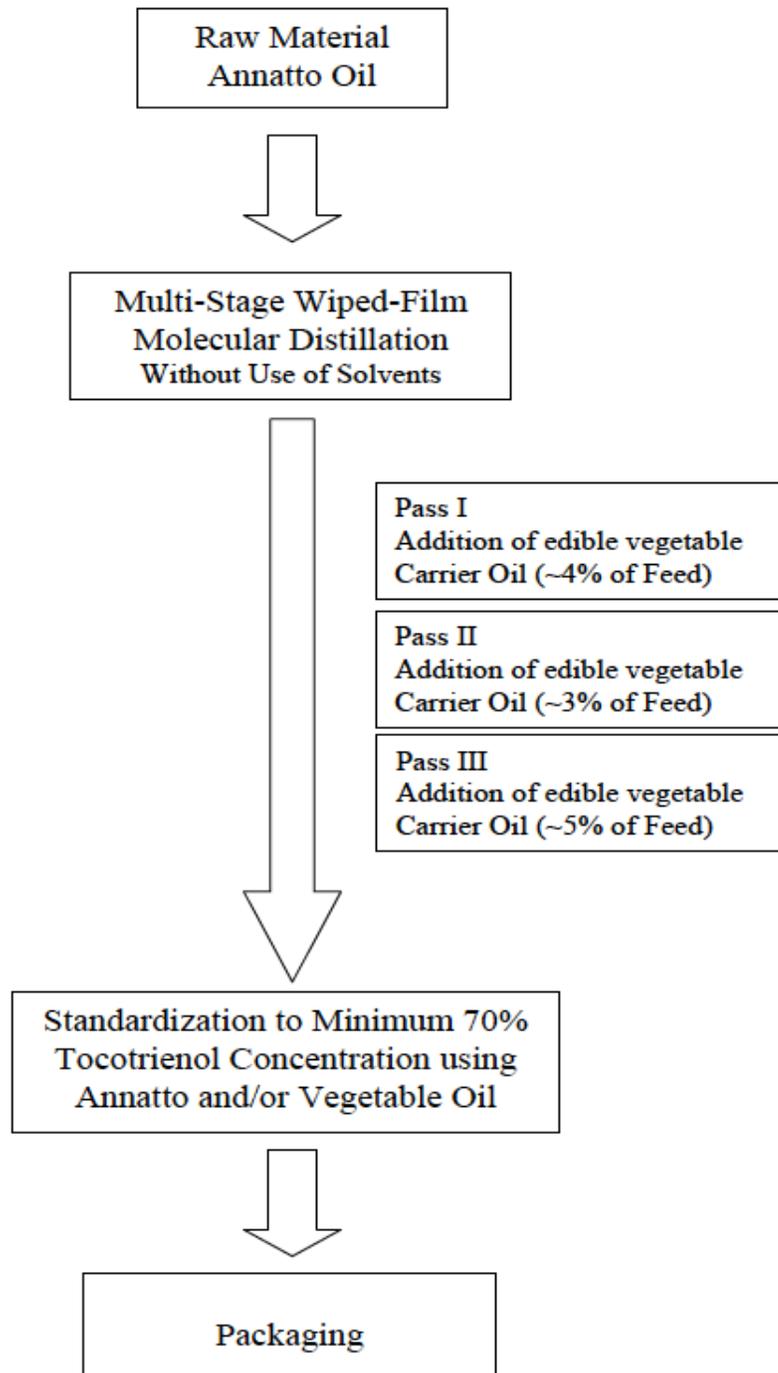
Manufacturing Overview

DeltaGold® is manufactured using annatto (*Bixa orellana* L.) seed oil, by American River Nutrition, Inc. (31 Campus Plaza Road, Hadley, MA 01035 USA).

Described herein is a simplified version of the manufacturing process, which employs evaporative techniques without the use of chemical or hydrocarbon solvents. Annatto oil is either lipophilically or hydrophilically extracted from annatto seeds (*Bixa orellana*). A schematic overview of the manufacturing process for the oil product is presented in Figures 2 and 3. Details of the manufacturing process are described in US Patent No: 6,350,453.

Figure 2. Schematic Overview of the Manufacturing Process for DeltaGold®. Not mentioned in the drawing is the addition of an edible and common vegetable oil (a food grade carrier oil) as a lubricant.

MANUFACTURING PROCESS OF DELTAGOLD® 70% TOCOTRIENOL
AMERICAN RIVER NUTRITION, INC. US PATENT # 6,350,453



Good Manufacturing Practice

DeltaGold® is manufactured according to procedures which follow quality control and assurance standards, as well as food GMPs.

Raw Materials

The starting raw material is annatto oil, obtained through qualified suppliers from South America and other countries. Suppliers are qualified through occasional site visits, as well as vendor questionnaires that are updated every two years. Upon receipt, raw material is quarantined, sampled, and released for processing only after satisfactory analysis has been completed to ensure compliance to specifications. An edible vegetable oil is used as a food-grade carrier oil during the molecular distillation process.

DeltaGold® Specifications

Current product specifications are listed below in Table 3.

Table 3. Specifications of DeltaGold®

	Method	DeltaGold® Specifications
Tocotrienols (T3)*		
Total Tocotrienols	HPLC	NLT 70% (700 mg/g)
δ-Tocotrienol	HPLC	84-92% of total T3, typical
γ-Tocotrienol	HPLC	8-16% of total T3, typical
Physical/Chemical		
Appearance	Visual	Orange-red oil
Moisture	USP/NF <731>	NMT 1% max
Peroxide Value, initial	AOAC 965.33	NMT 5 meq/kg
Microbiology		
Total Aerobic Count	USP <2021>	NMT 1,000 cfu/g
Yeast and Mold	USP <2021>	NMT 100 cfu/g
<i>Salmonella</i>	USP <2022>	Absent
<i>E. coli</i>	USP <2022>	Absent
<i>Staphylococcus aureus</i>	USP <2022>	Absent
Heavy Metals		
Total Heavy Metals	USP/NF <231>	NMT 10 ppm
Arsenic (As)	ICP/MS	NMT 2 ppm
Lead	ICP/MS	NMT 0.5 ppm
Mercury	ICP/MS	NMT 1 ppm
Cadmium	ICP/MS	NMT 2 ppm

Pesticides		
Pesticide Residues	USP 33<561>	Complies

* The product is “tocopherol-free”, defined to be below the measurable limit of α -tocopherol by HPLC, which is less than 0.1%

Shelf-Life Stability

An accelerated shelf-life stability assessment was conducted on DeltaGold®. The ingredient was tested over a four-month period at room temperature, 40°C and 20% relative humidity.

The δ -tocotrienol content changed from 669.97 mg/g (88.12%) to 593.99 mg/g (88.16%) in the DeltaGold® oil, from the initiation of the study until the study end. Similarly γ -tocotrienol content decreased from 90.35 mg/g (11.88%) to 79.74 mg/g (11.84%). Total tocotrienols decreased from 760.32 mg/g (76.32%) to 673.73 mg/g (67.37%).

Thus, the DeltaGold® levels of δ -tocotrienol and γ -tocotrienol remained within current specifications for the entire accelerated shelflife stability study; however, while the total tocotrienol content remained within current specifications until the 6 month time point, it dropped just below the specification of 700 mg/g (698.32 mg/g at 6 months and to 673.73 at 9 months). Shelf-life stability studies at normal conditions are currently in progress for all products.

Self-Limiting Levels of Use

While DeltaGold® does not add texture or taste to foods, it may confer a color change if used at higher concentrations. Additionally, it would be cost-prohibitive to add more than 100 mg of tocotrienols per serving to one particular food item.

Safety Assessment - Toxicology Studies

In Vitro Genotoxicity Studies

The mutagenic activity of an edible-grade rice bran oil (specific content of tocotrienols not described) was evaluated in an Ames study using *Salmonella typhimurium* strains TA98 and TA100 with or without metabolic activation. This study reported no evidence of mutagenic activity.⁴⁰

Tasaki et al. (2008) briefly mentions the results of unpublished studies on a mixed tocotrienol mixture obtained from palm oil. The tocotrienol mixture was reportedly negative for mutagenic activity in the Ames test, negative for chromosomal aberration and negative for induction of micronuclei.⁴¹

Chromosomal Aberration Study

The absence of genotoxicity of red palm oil was investigated on bone marrow cells from 8–10 week old Balb/C female mice.⁴² Experimental groups (n=10) were

administered, by gavage, 4.5 g/kg daily of supernatant and sediment of red palm oil and a mixture of both for five consecutive days. The negative control group received corn oil and the positive control group received cyclophosphamide 20 mg/kg intraperitoneally. There were no significant differences in the frequency of chromosomal aberrations and mitotic index between mice that were treated with red palm oil and those that received corn oil. While red palm oil is known to contain significant concentrations of tocotrienols, no information about its content was provided in the publication of this study.

Single Dose Toxicity Studies

Geh and Chan (1992) conducted a study in cats using a single intravenous dose (ranging between 2-20 mg/kg) of a vitamin E extract of palm oil (Palm vitee; 80% tocotrienols and 20% tocopherols) diluted in olein solution in anaesthetized cats (number of animals was not reported). Minor and inconsistent fluctuations in mean arterial blood pressure and heart rate seen were not significantly different from cats given injections of olein alone.⁴³

Repeated Oral Toxicity Studies

A repeated oral toxicity study was conducted on a vitamin E extract of palm oil (80% tocotrienols and 20% α -tocopherol) in young albino mice (weighing 15–20 g) and Sprague-Dawley rats (weighing 150–180 g). Four groups of each species (n=10 per group, authors state “acute toxicity studies were carried out in two species of animals of either sex”, however, sex per group not clearly specified) were administered 250, 500, 1,000 or 2,500 mg/kg body weight daily of palm oil extract (Palmvitee) diluted in refined, bleached and deodorized olein for 30 days. Daily observations were made including behavior, motor activity and body weight. No appreciable adverse effects were observed in the animals with respect to physical manifestations or behavioral changes. However, some sluggish motor activity was observed in both species and in all dose groups. At the end of the treatment period mice, but not rats, had increased body weight ($p < 0.05$). The authors concluded that these extremely high doses of palm oil extract did not appear to produce appreciable adverse effects in mice and rats. No information on percentages of each vitamer of tocotrienol was provided and furthermore no NOAEL was stated by the authors.⁴³

Yu et al. (2006) examined the effects of dietary supplementation with large amounts of α -tocopherol, α -tocotrienol, γ -tocotrienol, δ -tocotrienol and a purified tocotrienol-rich fraction (TRF) of palm oil (16.8% α -tocopherol, 26.5% α -tocotrienol, 42.5% γ -tocotrienol, and 14.1% δ -tocotrienol), on weight gain, organ weights, and serum lipid profiles in chickens. Two hundred and four White Leghorn 1-week-old female chickens, weighing 40–50 g, were equally divided into 34 groups. Experimental groups were supplemented for 4 weeks with 0, 50, 100, 250, 500, 1,000, or 2,000 ppm in the diet of α -tocopherol, α -tocotrienol, γ -tocotrienol, δ -tocotrienol, or TRF. Supplementation (50–500 ppm diet) with α -tocotrienol, γ -tocotrienol, δ -tocotrienol, and TRF decreased serum cholesterol levels ($p < 0.05$) in 17%, 20%, 27%, and 17%, respectively. Supplementation (50–

2,000 ppm/kg diet) with α -tocotrienol, γ -tocotrienol, δ -tocotrienol, and TRF reduced serum LDL cholesterol levels ($p < 0.05$) by 33%, 66%, 66% and 52%, respectively. HDL cholesterol and triglyceride levels were minimally impacted by tocotrienols. Alpha-tocopherol did not affect total cholesterol and had a modest lowering of LDL-cholesterol levels at the 2000 ppm level. Although specific data was not reported, the authors stated that food consumption, body weight of chickens at sacrifice, and weights of liver, lungs, pancreas, kidneys, and heart, did not differ between treatments or within treatments. Although the total daily intake of food was not reported in the publication and therefore, no specific daily tocotrienol dose could be calculated, based on the results of this study the authors concluded that the safe dose of α -, γ -, or δ -tocotrienols for human consumption is in the range of 200–1,000 mg/day.¹⁶

In another feeding study, Male golden Syrian hamsters, approximately 6 weeks old, were administered either no treatment, simvastatin, purified γ -tocotrienol (0.6% α -tocotrienol, 7.0% β -tocotrienol, 86.1% γ -tocotrienol, 0.1% δ -tocotrienol, and 6.2% tocopherols) or a tocotrienol mixture (29.5% α -tocotrienol, 3.3% β -tocotrienol, 41.4% γ -tocotrienol 0.1% δ -tocotrienol, and 25.1% tocopherols). Food consumption was monitored daily and the animals were weighed twice per week throughout a 4-week treatment period. The daily doses of tocotrienols were calculated from the daily food intake and body weight of the animals and equated to 39 and 263 mg/kg/day of mixed tocotrienol, and 23, 58 and 263 mg/kg/day γ -tocotrienol. Weight gain and mean food intake were not significantly different in the tocotrienol treatment groups compared to controls. Furthermore, tocotrienols had no significant effect on liver weights in any dose group.

Husain et al. (2009) studied the toxicity of an orally-administered 97% δ -tocotrienol supplement in mice. In repeated dose experiments, 18 athymic mice were randomly assigned to be fed vehicle control (saline/ethanol extracted olive oil) or δ -tocotrienol dissolved in saline/ethanol extracted olive oil at a dose of 50 or 100 mg/kg twice per day 5 times per week and once per day on weekends for a maximum of 6 weeks. The daily dose of δ -tocotrienol was 194 mg. Animals were monitored daily and weight was recorded. The authors reported no mortality and no significant change in body weight after oral administration of δ -tocotrienol for 6 weeks. Histopathological examination revealed normal histology of the liver, heart, kidney, and pancreas with no evidence of any pathology.²⁹

Nakamura et al. (2001) evaluated the oral toxicity of a tocotrienol concentrate (21.4% α -tocotrienol, 3.5% β -tocotrienol, 36.5 % γ -tocotrienol, and 8.6% δ -tocotrienol) from palm oil in 5-week-old Fisher 344 rats of both sexes for 13 weeks. The concentrate also contained 20.5% α -tocopherol, 0.7% β -tocopherol, 1.0% γ -tocopherol, and 0.5% δ -tocopherol. Four groups (10 rats/sex/group) were administered 0, 0.19, 0.75, and 3% of the powdered tocotrienol preparation in an *ad libitum* CRF-1 powder basal diet, which corresponded to 0, 119, 474, and 2130 mg/kg bw/day for male rats and 0, 130, 491, and 2047 mg/kg bw/day for female rats. Acute clinical observations were performed daily and measurements

of body weight and food intake were conducted weekly. Other examinations were as follows:

Exam	Time of examinations	Parameters
<i>Hematological and serum biochemical examinations, necropsy</i>	End of experiment	<p>Hematological parameters included: red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), white blood cell count (WBC), and white blood cell differential count</p> <p>Biochemical parameters included: total protein (TP), albumin /globulin ratio (A/G), albumin (AIB), total bilirubin (T. Bil), triglyceride (TG) and total cholesterol (T.Cho), blood urea nitrogen (BUN), creatinine (CRN), asparagine transaminase (AST), alanine transaminase (ALT), γ-glutamyl transaminase (γ-GT), alkaline phosphatase (ALP), calcium (Ca), inorganic phosphate (P), sodium (Na), potassium (K), chloride (Cl),</p>
<i>Organ weights</i>	At necropsy	Brain, heart, lungs, thymus, liver, spleen, adrenals, kidneys, testes, ovaries, uterus
<i>Histopathology</i>	At necropsy	Brain, heart, lungs, thymus, liver, spleen, adrenals, kidneys, testes, ovaries, uterus, nasal cavity, pituitary, eyeballs, Harderian glands, spinal cord, salivary glands, stomach, small and large intestine, pancreas, urinary bladder, skin, mammary gland, mesenteric lymph nodes, trachea, esophagus, thyroid gland, tongue, skeletal muscle, ischiatic nerve, epididymis, seminal vesicles, prostate gland, vagina and bone marrow (femur and sternum)
<i>Immunohistochemical procedures</i>	At necropsy	Hepatocellular nodules (spongiotic lesions) examined

No deaths occurred and no remarkable changes in general appearances were observed throughout the study. While food intake was comparable between rats of both sexes throughout the study, there was a decrease in body weight gain in the male rats fed the highest concentration (3% group). Statistical significance was not reported and no food intake changes were recorded, therefore, the authors stated that the cause for this decrease was unclear.

In male rats, small but significant decreases in MCV were observed in all dose groups and a decrease in MCH was observed in males in the highest dose group (3%). However, no changes were seen in RBC, Hb, or HCT, suggesting that these findings are of no toxicological significance. An increase in WBC and a reduction of monocytes were also observed in male rats in the highest dose group (3%) tested, however no histopathological changes related to these findings were found. Platelet count was significantly reduced in males rats (not observed in female rats) of the two highest dose groups, in a dose-dependent manner. While the authors suggest that this may suggest a hemorrhagic response, no hemorrhagic lesions were found at necropsy. In females, Hb, MCV, MCH, and MCHC were all significantly decreased in females in the two highest dose groups tested and HCT was significantly reduced in females of the highest dose group. These findings were associated with an increase in spleen weights, however, similar to the males, histopathological examination of RBCs did not reveal any pathological changes. In female rats in the two highest dose groups tested, the WBC differential revealed significant decreases in segmented leukocytes and monocytes, and an increase in lymphocytes. In the highest dose group, there was also a significant decrease in eosinophils. These changes were minimal and histopathological examination of the bone marrow from the femur and sternum did not reveal any toxicological concern.

Clinical chemistry studies revealed a significant increase in the A/G ratio and ALP in all treated males. Significant increases in ALB, BUN, Ca, and ALT (text reports significant increases in ALT in males of highest group but tables in the publication do not) and a significant decrease in P were observed in males in the highest dose group (3%). A significant decrease in Na was observed in the male 0.75% group without any dose relationship. Total cholesterol was significantly decreased in male rats in the two highest dose groups. Female rats in high dose group had significant increases in A/G, TG, BUN, P, ALT, γ -GT, and ALP (tables indicate these as significant increases although text mistakenly states "decreased") AST levels were also significantly increased in the high dose group females. Ca levels were significantly increased in female rats in the 0.19% group and AST was significantly decreased in female rats that received 0.19% tocotrienols in the diet.

The decrease in total cholesterol in males rats in the two highest dose groups ($p < 0.01$), increases in ALT in both sexes of the highest dose group ($p < 0.01$), and an increase in AST in males rats of the highest dose group ($p < 0.01$) were considered consistent with the reported inhibition of 3-hydroxy-3-methylglutaryl (HMG) CoA reductase activity by tocotrienols in rats.^{44, 45} These effects were only seen in the highest dose groups. No significant changes in these parameters were seen in the lowest dose group (0.19%). The increases in ALP in both sexes may suggest cholestasis or bone remodeling, however, no increase in total bilirubin,

or histopathological evidence of these changes were found. Changes noted in both Ca and P may also be reflective of bone remodeling, however, the changes were not dose-dependent, and therefore, not considered toxicologically significant. Increases in BUN, and increases in K and decreased Na in females were not associated with any histopathological findings such as kidney abnormalities. Increases in A/G in both sexes and an increase in albumin in males were also not considered of toxicological significance due to the small degrees of changes.

Relative organ weights of brain, heart, liver, kidneys, and testes were significantly increased in male rats in the highest dose group and were thought to be observed because of the decreased body weight gain. Lung weights showed a tendency for reduction in all dose groups, but no corresponding histopathological changes were observed. Relative liver weights for both sexes in the highest dose groups, relative adrenal weights in male rats in all dose groups tested were increased and finally relative ovary and uterus weights in females in the highest dose groups were decreased. Histopathological examination revealed no abnormalities in these reproductive organs. Slight hepatocellular hypertrophy in male rats of the two highest dose groups and reduction of cytoplasmic vacuolation in the adrenal critical region in male rats in the highest dose group, suggested a possible relationship to the hypocholesterolemic activity of tocotrienols.

The authors concluded that because of the histopathological changes in the liver in male rats, and the hematological changes in female rats, the No Observed Adverse Effect Level (NOAEL) was concluded as 0.19% (1.9 g/kg) in the diet amounting to exposure to 120 mg/kg bw/day for male rats and 130 mg/kg bw/day for female rats.⁴⁶

Shibata et al. (2012) examined the effects of a 98.7% tocotrienol rice bran extract, administered to male Fischer 344/slc rats for 13 weeks.⁴⁷ The extract was composed of 2.5% α -tocotrienol, 92.0% γ -tocotrienol and 4.2% δ -tocotrienol. The rats were assigned to one of 4 groups (8 rats/group) and given either a basal diet, or the basal diet containing 0.02%, 0.06%, or 0.20% of the tocotrienol extract. Acute clinical observations and food intakes were evaluated daily, while body weights were evaluated once per week. Other examinations are outlined below:

Exam	Time of examinations	Parameters
<i>Hematological and serum biochemical examinations, necropsy</i>	End of experiment	Hematological parameters included: red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) white blood cell count (WBC), and platelets (PLT) Biochemical parameters

		included: total protein (TP), albumin/globulin ratio (A/G), blood urea nitrogen (BUN), creatinine (CRN), asparagine transaminase (AST), alanine transaminase (ALT), triglyceride (TG), total cholesterol (T-Cho), high density lipoprotein cholesterol (HDL-Cho), low-density lipoprotein cholesterol (LDL-cho), and total bilirubin (T-Bil)
<i>Relative organ weights/macrosopic evaluation</i>	At necropsy	Adrenal gland, brain, epididymal fat, heart, kidneys, lungs, liver, perirenal fat, gastrointestinal, spleen, testes and visceral fat
<i>Histopathology</i>	At necropsy	Liver, small intestine, large intestine
<i>Tocotrienol and Tocopherol levels (measured by fluorescence-high performance liquid chromatography)</i>	At necropsy	Adrenal gland, aorta, brain, ear, epididymal fat, gastrointestinal tract, heart, kidneys, lungs, liver, muscles, skin, perirenal fat, spleen, testes, visceral fat, plasma and RBC

No deaths or adverse clinical signs were observed during the study. No significant changes were noted with respect to weight gain or food consumption. The mean exposure to tocotrienols was calculated at 4.7, 14.6, and 42.2 mg/rat/day for the 0.02%, 0.06% and 0.20% groups respectively.

In this study, exposure to tocotrienols did not significantly affect RBC, WBC, Hb, Ht, MCV, MDH, MCHC, or PLT. Furthermore, no significant differences were found in TP, A/G, CRN, BUN, LDL, T-Bil, AST, or ALT for any of the groups. Total cholesterol tended to be higher in the 0.02% group (although not statistically significantly different from controls), and thought to be the result of a non-significant increase in HDL cholesterol in the same group. A trend towards tocotrienol exposure having lowered triglycerides was observed, although these reductions were also not statistically significant.

Relative liver weights were significantly higher in the 0.02% group compared to those in the 0.06% and 0.20% groups, for unknown reasons. Tocotrienols also appeared to decrease the weight of perirenal fat tissues, although not significantly. Macroscopic examinations found no treatment related changes in any organs examined. No histopathological changes were found in the liver, small intestine or large intestines.

Tocotrienols accumulated predominantly in white adipose tissue (epididymal fat, perirenal fat, and visceral fat) and skin. Comparatively high levels of tocotrienols were also found in the ear, adrenal gland, gastrointestinal tract,

heart, lungs and muscle, while relatively low levels were detected in the brain, kidney, testes, liver, plasma, RBC, spleen, and aorta.

While a NOAEL was not determined, the authors suggest that tocotrienols are safe if used at a dose less than 0.20% of the diet, comparable to 170 mg/kg/day. This dose is somewhat higher than what was seen in the NOAEL concluded in the Nakamura study mentioned above (120–130 mg/kg bw/day). The authors suggest that because this study was conducted on a pure tocotrienol product (98.7% tocotrienols, with negligible tocopherols), that the data presented is more relevant to the safety of tocotrienols themselves as compared to other toxicological studies on lower percentage tocotrienol products, such as Nakamura⁴⁶ (61.4% tocotrienols) and Tasaki⁴¹ (70% tocotrienols),

Chronic Oral Toxicity Study

Tasaki et al. (2008) performed a one-year chronic feeding study on a tocotrienol mixture.⁴¹ Wistar Hannover male and female rats, 5 weeks old, were acclimated for one week and then assigned to one of four groups. The rats were fed (*ad libitum*) a diet containing 0%, 0.08%, 0.4% and 2% of a tocotrienol mixture for 52 weeks. Each group consisted of 10 males and 10 females, with the exception of the highest dose group, which consisted of 20 male and 20 female rats. The tocotrienol mixture consisted of 21.4% α -tocotrienol, 3.5% β -tocotrienol, 36.5% γ -tocotrienol, 8.6% δ -tocotrienol, 20.5% α -tocopherol, 0.7% β -tocopherol, 1.0% γ -tocopherol and 0.5% δ -tocopherol. Total exposure to tocotrienol in this feeding study in the 0.08%, 0.4% and 2% diets were 8.76, 35.25, and 183.68 g/kg/day respectively for males, and 5.03, 38.51, and 140.02 g/kg/day for females. Clinical observations were conducted daily, and body weights and average food intake was measured weekly. Additional examinations performed are outlined below:

Exam	Time of examinations	Parameters
Hematological and serum biochemical examinations, necropsy	End of experiment	<p>Hematological parameters included: red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), white blood cell count (WBC), white blood cell differential count, activated partial thromboplastin time (APTT), prothrombin time (PT), and fibrinogen (Fib)</p> <p>Biochemical parameters included: total protein (TP), albumin/globulin ratio (A/G), albumin (ALB), blood urea nitrogen (BUN), creatinine (CRN), asparagine transaminase (AST), alanine transaminase (ALT), γ-glutamyl transaminase (γ-GT), alkaline phosphatase (ALP), lactate dehydrogenase</p>

		(LDH), choline esterase (ChE), calcium (Ca), inorganic phosphate (P), sodium (Na), potassium (K), chloride (Cl), total bilirubin (T. Bil), direct bilirubin (D. Bil), glucose (Glu), triglyceride (TG) and total cholesterol (T.Cho)
<i>Organ weights</i>	At necropsy	Brain, heart, lungs, thymus, liver, spleen, adrenals, kidneys, testes, ovaries, uterus
<i>Histopathology</i>	At necropsy	Brain, heart, lungs, thymus, liver, spleen, adrenals, kidneys, testes, ovaries, uterus, Harderian glands, spinal cord, salivary glands, stomach, small and large intestine, pancreas, urinary bladder, skin, mammary gland, mesenteric lymph nodes, trachea, esophagus, thyroid gland, tongue, skeletal muscle, ischiatic nerve, epididymis, seminal vesicles, prostate gland, vagina and bone marrow (femur and sternum)
<i>Immunohistochemical procedures</i>	At necropsy	Hepatocellular nodules (spongiotic lesions) examined

At week 50, 6 males in the 2% tocotrienol group had died from hemorrhaging at several organ sites; therefore, the highest dose was reduced to 1% in both sexes thereafter. At necropsy, hemorrhage was observed at the cerebral base and in the thoracic cavity, testis, prostate and/or bladder.

Body weight gain was significantly decreased in both male and female rats at the 2% dose. Significant decreases in food consumption were also observed in the 0.4% treatment group and above in male rats and in the 0.08% group in females. Final body weights (at week 52) of both male and female rats in the 2% group were significantly decreased.

Relative organ weights for brain, lung, heart, adrenal, and kidney weights were significantly increased in males in the 2% group; however, no absolute weight changes in any individual organs were seen. The female rats had absolute organ weights that were significantly increased for heart, liver and kidney in the 0.4% group, and decreased for lung in the 2% group, but were not associated with relative organ weight changes. Female rats in the highest treatment group had relative organ weights that were significantly increased for brain, heart, liver, adrenals and kidneys.

In male rats in the 0.08% group, Ht and WBC were significantly decreased and APTT was significantly shortened. A significant decrease of MCV was noted in

the 0.4% and 2% groups in male rats. PT was significantly prolonged in male rats in the high dose (2%) group. Significant increases in A/G, P, ALT, ALP and D.Bil and decreases in LDH and TG were observed in male rats in the 2% dose group. Significant decreases in Glu and increases in Cl were also observed in males in the dose groups of 0.4% and greater, but the values were within historical control ranges. In female rats, decreases in Hb, Ht, MCV and MCH were observed in the highest dose group. Also in females, ALP was significantly increased and T.Bil was significantly decreased in the 2% group and significant increase in PL was observed in females in the 0.08% group.

A high incidence of multiple cystic nodules was found at necropsy in the livers of both male and female rats in the 2% group. Significant hepatocellular nodular hyperplasia was noted that resulted in distortion of hepatic cords, compressing the surrounding tissue, almost all associated with spongiosis hepatis. However, lobular architecture was found intact and the hepatocytes showed little atypia. Hepatocellular altered foci of eosinophilic type were found in females in the 2% group; other types of altered foci were sporadically observed. In both males and females, foamy cell infiltration in the lung was observed at elevated incidence (only reaching statistical significance in females), but the toxicological significance was uncertain. Immunohistochemical analyses for desmin, vimentin and α -smooth muscle actin were performed in spongiotic lesions. Most nodules were GST-P negative (glutathione S-transferase placental form; a predictor of carcinogenic potential), however some nodules included GST-P positive foci in part. Constituent hepatocytes demonstrated elevated PCNA (proliferation cell nuclear antigen) labeling rates. On GST-P quantitative analysis of all areas, the numbers of areas per square centimeter of GST-P positive foci were significantly increased in female rats in the 2% group.

In conclusion, 52 weeks of a 2% tocotrienol-enhanced diet induced hepatocellular nodules in the liver accompanied by spongiosis in male and female rats. Despite distortion of the hepatic cords, the basic lobular architecture was not affected by the hepatic lesions, suggesting they were of a non-neoplastic nature. Additionally, almost all of the nodules were negative for GST-P, although some were positive (quantitative analysis determined that the GST-P positive lesions were only significantly increased in female rats in the 2% group). However, the characteristics of these nodules did not appear to be neoplastic in nature. The authors of this study concluded that based on the morphological features of the lesions, it could be considered "nodular hepatocellular hyperplasia" (NHH). The six deaths of male rats due to hemorrhagic reasons were considered to be from the possible interaction of vitamin E and vitamin K, considering the prolonged PT in male rats of the 2% group. No changes considered to be of toxicological significance were found in rats of both sexes in the 0.4% group. Therefore, the authors concluded a NOAEL of 303 mg/kg bw/day for male rats and 472 mg/kg bw/day for female rats in this feeding study.

In an extended study, Tasaki et al. conducted a two-year combined chronic toxicity/carcinogenicity study to further investigate and clarify the pathology of the hepatic lesions associated with the aforementioned chronic toxicity study

(rats were fed tocotrienol for a total of 104 weeks, or two years).⁴⁸ This study was conducted under the same experimental conditions as in the chronic study discussed above. Male and female Wistar Hannover rats were administered 0, 0.4 and 2% tocotrienols (50 rats/sex/group) for 50 weeks, and subsequently the high dose group (2%) was reduced to 1% from week 51 on due to the six hemorrhagic deaths that occurred in the 2% high-dose male group. At necropsy, similarly to the chronic toxicity study, multiple cyst-like hepatic nodules were observed in both sexes in the high dose (1%) group. These nodules were “strikingly enlarged” in size compared to the previous one-year study. In contrast to the chronic toxicity study, some of the hepatic nodules observed were not accompanied by spongiosis, and instead angiectasis was prominent. In this extended study, it was found that the affected hepatocytes had minimal atypia, almost no GST-P reactivity and heterogenous proliferation, indicating a non-neoplastic nature of proliferation. The nodules were again, suggested to be “nodular hepatocellular hyperplasia”, and although non-neoplastic, were successively enlarged by the further treatment of tocotrienols. In contrast, in the high-dose females, tocotrienols appeared to induce low levels of hepatocellular adenomas. The authors concluded that the hepatic nodules found in this study are consistent with nodular hepatocellular hyperplasia (NHH) diagnoses in the previous one-year study and concomitantly, tocotrienols induced non-neoplastic hepatocellular adenomas are based on non-genotoxic mechanisms. This is further confirmed with an absence of genotoxicity demonstrated in Ames, chromosomal aberration and micronucleus tests (unpublished data) conducted by these authors.

Reproductive and Developmental Toxicity Study

Rukmini (1988) investigated the toxicological effects of consumption of rice bran oil by rats over three generations. Thirty (15 females, 15 males) weanling albino rats of NIN/Wistar strain were fed a diet containing 20% protein and 10% rice bran oil or 10% groundnut oil (control). The rice bran oil did not affect the balance of nitrogen, phosphorus and calcium in rats (calculated based on dietary and fecal levels of these nutrients) or percentages of conception, birth weight, litter size, weaning weight and pre-weaning mortality. In addition, no differences in mutagenicity and teratogenicity were observed. No specific information about tocotrienol content was provided.⁴⁹ However, in a later publication, Rukmini lists the content of tocotrienols in rice bran oil as follows: 49 ppm α -tocotrienol, 292 ppm γ -tocotrienols, and 28 ppm δ -tocotrienols (total tocotrienol content equal to 369 ppm).⁵⁰

Additional Scientific Studies

Human studies

Some of the major human clinical studies spanning over two decades conducted on tocotrienols are summarized in Table 4. These studies have been included in this dossier solely to demonstrate how various forms and doses of tocotrienols have been well tolerated in humans. These published clinical trials have demonstrated the lack of adverse events of a daily dose of 200 mg of a

tocotrienol-rich palm fraction for up to 5 years and 3200 mg for 14 days. These studies have been void of reported adverse events, with the exception of one study on tocotrienol acetates reporting mild gastrointestinal effects.

Table 4. Summary of Notable Clinical Studies on Tocotrienol (T3)

Study	Number of study Subjects	Duration	Total T3 Dose (mg/day)	Details of T3 Composition	Estimated dose δ -T3 (mg/day)	Source	Adverse Events
Qureshi et al., 1991 ⁵¹	25	4 weeks	200	Mixed (25–30% δ -)	60	Palm	No adverse events were reported.
Qureshi et al., 1995 ⁵²	36	4–8 weeks	220 and 200	Mixed in 220 group (27% δ -), and γ in 200 group.	60	Palm	No adverse events were reported.
Qureshi et al., 1997 ⁵³	41	4 weeks	200	Mixed (10% δ -)	20	Rice bran	No adverse events were reported.
Qureshi et al., 2001 ¹⁵	14 14	35 days 70 days	50	Mixed (5.2% δ -)	3	Rice bran	No side effects were reported.
Qureshi et al., 2002 ⁵⁴	90	35 days	25, 50, 100, and 200	Mixed (5.2% δ -)	10	Rice Bran	No adverse events were reported.
Qureshi et al., 2012 ⁵⁵	96	4 weeks	50	98% δ - purified from DeltaGold®50	50	Annatto	No adverse events were reported
Tomeo et al., 1995 ⁵⁶	50	18 months	224–336	Mixed α - and γ - forms	0	Palm	No adverse events were reported.
O’Byrne et al., 2000 ²⁶	51	8 weeks	250	Individual doses of α -, γ - and δ - (81%) tocotrienyl acetate forms	203	Palm	Subjects tolerated the T3 acetates, except four receiving γ -T3 acetate who had transient abdominal distention, gastric upset, and pain during the first week. Two reported persistent flatulence. One subject, who had a hiatal hernia and gastric reflux,

							reported nausea and vomiting 1 hour after taking α -T3 acetate with evening meal— symptoms stopped when taken with the afternoon meal.
Baliarsingh et al. 2005 ⁵⁷	19	8 weeks	390	Mixed (30% δ -)	117	Rice bran	No adverse events were reported.
Rasool et al., 2006 ⁵⁸	36	8 weeks	80–320	Mixed (15% δ -)	48	Palm	Treatment was well-tolerated with no serious adverse events recorded.
Rasool et al., 2008 ⁵⁹	36	8 weeks	50–200	Mixed 9.83% δ -	5–20	Palm	Treatment was well-tolerated; no adverse events requiring withdrawal from supplement.
Ajuluchukwu et al., 2007 ⁶⁰	28	4 weeks	73	Mixed (8.8% δ -)	6	Palm	No adverse events were reported.
Kooyenga et al., 2001 ⁶⁰	50	3 years 1 year	52	Not described	Not described	Palm Rice bran	Study states: “no reports of side effects such as headache, intestinal upset, muscle weakness, or persistent liver function abnormalities”.
Tan, 2005 ⁶¹	10 (5x2)	2 months	75	Mixed (90% δ -)	68	Annatto	No adverse events were reported.
Mahalingam et al. 2010 ⁶²	108	2 months	400	Tocotrienol-rich fraction (13% δ -)	51.36	Palm	No adverse events were reported.
Chin et al. 2011 ⁶³	62	6 months	160	Tocotrienol-rich fraction (74% tocotrienols)	67.2	Palm	No adverse events were reported.
Patel et al. 2012 ²³	80	Mean duration 20 weeks up to 96 weeks	400	Mixed (13% δ -)	51.36	Palm	No adverse events were reported.

Nesaret et al. 2010 ⁶⁴	240	Median duration 5 years (60 months)	200	Tocotrienol-rich fraction; % of tocotrienols not described	Not described	Palm	Combination of tamoxifen and tocotrienol-rich fraction was well tolerated. Liver function was not altered. No adverse effects were reported.
Houston 2010 ⁶⁵	30	2 months	75	δ-tocotrienol	75	Annatto	No adverse events reported.
Springett et al. 2011 ⁶⁶	12	13-15 days	Escalating doses (200, 400, 600, 800) day)	δ-tocotrienol	Up to 800 mg/day	Not known	Well tolerated, no related toxicities
Springett et al. 2012 ⁶⁷	17	14 days	Escalating doses (200, 400, 600, 800, 1,600 and 3,200mg)	δ-tocotrienol	Up to 3200 mg/day	Not known	Well tolerated, no related toxicities
Qureshi et al. 2012 ⁵⁵	96	4 weeks	50	δ-tocotrienol	50	Annatto	No adverse events reported.
Qureshi et al. 2013 ⁶⁸	98	6 weeks	100	δ-tocotrienol	100	Annatto	No adverse events reported.

Allergenicity

No reports of allergenicity were found in the public domain associated with vitamin E or tocotrienol consumption. There are rare reports of allergic reactions to *Bixa orellana* (annatto) extracts in sensitive persons.⁶⁹

Commercially Available Tocotrienol Products

Previous Sales of DeltaGold® and Reported Adverse Events

American River Nutrition, Inc. has verified that, to date, approximately 36.7 million doses of DeltaGold® have been sold since 2002, along with similar levels of diluted versions of the ingredient (totaling 83.6 million doses of DeltaGold®). These estimates are based on dosages consisting of 100 mg active tocotrienol. To

the best of their knowledge, no adverse events have been reported to them or to the FDA via Medwatch since sales began in 2002.

Similar Products in the Marketplace

Table 5. Examples of dietary supplements containing tocotrienols

Company	Brand Name	Total Tocotrienols Per serving
Nutraceutical Sciences Institute	Tocomin Supra Bio	60 mg
A.C. Grace	Unique-E Tocotrienol (Annatto)	125–250 mg
Solgar	Tocotrienol Complex	46–92 mg
TwinLab	Maxilife Rice Tocotrienols	50–100 mg
Allergy Research	CoQ10 with Tocotrienols	25–75 mg
Jarrow Formulas	Toco-Life	150 mg
Complementary Prescriptions	Annatto Tocotrienols	100 mg
Cardiovascular Research	Palm Tocotrienols	50–100 mg
Vitamin Research Products	Annatto Tocotrienols	100 mg
Yasoo Health Inc	Vitamin E Factor Tocotrienols	68 mg or more
Designs for Health	Tocotrienols (Annatto)	100 mg
Montiff	Super E Plus	50–200 mg

Current Regulatory Status and Governmental Opinions

Tocotrienols are found in annatto seeds, as well as in numerous other plants and are presumed to be present in annatto extracts approved by FDA for color additive use in foods (21 CFR Section 73.30), drugs (21 CFR Section 73.1030), and cosmetics (21 CFR Section 73.2030)—especially those that utilize oil for their extraction method.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) originally determined an acceptable daily intake (ADI) level for annatto extract, based on the available research at the time, as 0–0.065 mg/kg bw/day of annatto extract expressed as bixin. In a more recent 2004 committee meeting, however, new data was evaluated. Six different preparations of annatto extract were reviewed, and new toxicological data (in the form of 28-day and 90-day animal studies) was available for four of the extracts. Because a generic ADI for the various types of annatto extract preparations that were reviewed could not be established, temporary ADIs for the different preparations were determined.^{2,70}

- Annatto B (*solvent-extracted extract containing 92% pigment; 97% was bixin and 1.7% norbixin*)

- ADI = 0–7.0 mg/kg bw (based on no observed effect levels (NOELs) of 1311 mg/kg bw per day and 1446 mg/kg bw per day in male and female mice respectively).
- Annatto C (*solvent-extracted extract containing 91.6% norbixin*)
 - ADI = 0–0.4 mg/kg bw (based on NOELs of 69 mg and 76 mg/kg bw per day in male and female rats respectively).
- Annatto E (*aqueous-processed extract containing 26% pigment, of which 90% was bixin and 4.2% norbixin*)
 - ADI = 0–4.0 mg/kg bw (based on NOELs of 734 mg and 801 mg/kg bw per day in male and female rats respectively)
- Annatto F (*alkali-processed norbixin*)
 - ADI = 0–0.4 mg/kg bw (based on NOELs of 79 mg and 86 mg/kg bw per day in male and female rats respectively).

Additionally, it may be interpreted that FDA considers tocotrienols GRAS, as is stated in a proposed 1978 FDA rule (Federal Register, Vol 43, No. 209, Friday October 27, 1978, pp. 50193–50198, Docket No. 78N-.213). In this proposed rule, GRAS vitamin E is defined as “eight d-form tocopherols” that occur naturally. The eight forms of vitamin E include the four tocopherols and the four tocotrienols, the latter of which were discovered in 1964.⁷ Tocotrienols were referred to as tocopherols in some literature of that time, as in the manuscript entitled “Tocopherols in Foods and Fats” by Hal Slover published in 1971.⁷¹ The 1976 Merck manual also lists 8 “tocopherols”; α -tocopherol acid succinate, β -tocopherol, γ -tocopherol, δ -tocopherol, ϵ -tocopherol, ζ_1 -tocopherol, ζ_2 -tocopherol, and η -tocopherol. This nomenclature extended into the 1983 and 1996 versions of the Merck manual. However, in the 2006 version, there is a clear distinction between tocopherols and tocotrienols, where α -, β -, δ , and γ -tocopherols are listed, and ϵ -tocopherol, ζ_1 -tocopherol, ζ_2 -tocopherol, and η -tocopherol are seemingly replaced with α - and β -tocotrienol.⁷²⁻⁷⁵ The 1978 proposed rule has been cited as a clear indication that tocotrienols are GRAS in more recent peer reviewed manuscripts.^{15, 16}

In 21 CFR 101 subchapter B – “Food for Human Consumption” subsection 14, palm oil (which is reported to contain up to 50% tocotrienols), is referred to as a “food”, “vegetable oil”, and an “oil ingredient of a food intended for human consumption”. In the Federal Register Volume 63, No. 101, 1998 pp. 28893–28895, FDA states that sheanut oil (GRAS per 21 CFR 184.1702) is “similar in chemical composition to commonly used GRAS fats and oils, such as cocoa butter, cottonseed oil, soybean oil, corn oil and **palm oil**”. This further confirms FDA’s thoughts on the GRAS status of palm oil. Palm oil naturally contains approximately 69 ppm δ -tocotrienols.³⁵

In 2009, the Malaysian Palm Oil Board submitted GRAS notification (GRN 307) to FDA for palm oil-derived tocols, containing tocotrienols and α -tocopherol as

its principal components. FDA issued a no-objection letter on April 23, 2010. The products included in GRN 307 were Davos palm TRFs (tocotrienol rich-fractions) containing various levels of tocotrienols, reaching up to 61.5%. The conditions of use include addition to various foods at specified levels equivalent to 0.045–5.6 mg of tocotrienols per serving. The calculated maximum exposure level, at the 90th percentile intake, was estimated at 0.99 mg/kg/day for tocotrienols. This equates to 69.3 mg tocotrienols daily for a 70 kg human.

In 2008, the European Food Safety Authority (EFSA) evaluated the safety and bioavailability of three preparations of vitamin E: tocotrienols, tocotrienol plus tocopherol, and mixed tocopherols as nutritional substances in food supplements (EFSA, 2008). The committee found that the tocotrienol plus tocopherol product, containing 13.5 mg of tocotrienols (amounting to an intake of 0.23 mg tocotrienols/kg bw/day for a 60 kg person) is at least 500 times lower than the no observed adverse effect level (NOAEL) for the tocotrienols in a subchronic toxicity study in rats (which was 120 mg/kg bw/day for males, and 130 mg/kg bw/day for females). Hence it concluded that the proposed level of use is not of safety concern.

However, the authority determined that there was insufficient safety data to conclude that the tocotrienol preparation containing up to 1000 mg of tocotrienols per daily serving, was safe, as it would result in a daily intake of 16.7 mg tocotrienols/kg bw/day for a 60 kg person, which is “higher than the 5 mg/kg bw/day frequently demonstrated to be without adverse effects in human studies”.

The Daily Reference Intake for vitamin E (as determined by the Institute of Medicine) is currently set at 15 mg per day for adults, although is currently based only upon α -tocopherol activity. Tolerable Upper Intake Levels (ULs) for vitamin E have been set by the Food and Nutrition Board (FNB) based on the potential for vitamin E to have hemorrhagic effects, however, again, the recommendations are for α -tocopherol alone. The ULs apply to all forms of supplemental α -tocopherol, including the eight stereoisomers present in synthetic vitamin E. Doses up to 1,000 mg/day (1,500 IU/day of the natural form or 1,100 IU/day of the synthetic form) in adults appear to be safe and the ULs are currently set at this dose for males and females over 19 years of age, including those pregnant and lactating.

Table 6. References for Government Opinions Related to DeltaGold®

Reference	Ingredient	Description	Use	Limitation
21CFR 73.30	Annatto extract	Extract prepared from annatto seed, <i>Bixa orellana</i> L., using any one or an appropriate combination of the food-grade extractants that follows: Alkaline aqueous solution, alkaline propylene glycol, ethyl alcohol or alkaline solutions thereof, edible vegetable oils or fats, mono- and diglycerides from the glycerolysis of edible vegetable oils or fats. The alkaline alcohol or aqueous	Color additive	Amounts consistent with good manufacturing practice

		<p>extracts may be treated with food-grade acids to precipitate annatto pigments, which are separated from the liquid and dried, with or without intermediate recrystallization, using the solvents listed under paragraph (a)(1)(ii) of this section. Food-grade alkalis or carbonates may be added to adjust alkalinity.</p> <p>(a)(1)(ii) Acetone, ethylene dichloride, hexane, isopropyl alcohol, methyl alcohol, methylene chloride, trichloroethylene</p>		
21CFR 73.1030	Annatto extract	Extract shall conform in identity and specifications to the requirements of 73.30	Coloring <u>drugs</u> generally including those intended for use in the area of the eye	Amounts consistent with good manufacturing practice
21CFR 73.203	Annatto extract	Extract shall conform in identify and specification to the requirements for annatto extract in 73.30	Coloring <u>cosmetics</u> generally including cosmetics intended for use in the area of the eye	Amounts consistent with good manufacturing practice
European Food Safety Authority: The EFSA Journal (2008) 640, 1-34	Tocotrienols	Determined that a specific mixture that contains 13.5 mg of tocotrienols (amounting to an intake of 0.23 mg tocotrienols/kg bw / day for a 60 kg person) is safe because this is over 500 times below the subchronic rat toxicology study NOAEL of 120 mg/kg bw / day, and the fact that “5 mg/kg bw / day [is] frequently demonstrated to be without adverse effects in human studies”.		
21 CFR 182.3890	Tocopherols	Generally recognized as safe (GRAS). Considered to include tocotrienols as discussed previously.	Chemical preservatives	Amounts consistent with good manufacturing practice
21 CFR 182.8890	Tocopherols	Generally recognized as safe (GRAS). Considered to include tocotrienols as discussed previously.	Nutrients	Amounts consistent with good manufacturing practice
Food and Nutrition Board,	Vitamin E, defined as α -	Tolerable Upper Limit Levels defined as: 200 mg for children 1-3 years		

Institute of Medicine, National Academies Established Daily Reference Intake Levels	tocopherol	300 mg for children 4–8 years 600 mg for humans 9–13 years 800 mg for humans 14–18 years 1000 mg for humans 19 years and older		
GRAS notification # 307	Palm oil derived tocots	Generally recognized as safe (GRAS) for intended conditions of use.	Food ingredient	Levels specified for individual foods, in the overall range of 0.045–5.6 mg T3 per serving

Intended Use and Estimated Daily Intake (EDI)

For the purpose of this GRAS notification, American River Nutrition’s DeltaGold® tocotrienol Annatto seed ingredient, manufactured in accordance with GMP, is intended to be used as an ingredient in food, where standards of identity allow. DeltaGold® is not intended for use in infant formula, meat, egg, catfish or any products that would require additional regulatory review by USDA.

DeltaGold® is intended to be used at an addition level up to a maximum of 100 mg tocotrienols per serving. In order to calculate an estimated daily exposure level for this ingredient, data reported in an article from the USDA Center for Nutrition Policy and Promotion was utilized.⁷⁶ USDA utilized data from Market Research Corporation of America Information Services, on 5,752 adults for the 1992–1994 period, as relates to their consumption of foods based on detailed 14-day food diaries. According to the data, males aged 51 or greater consumed the greatest total number of servings of food from all food groups (18.2 total servings per day). Women aged 19–24 consume the least number of servings of all food groups (12.5 total servings per day).

It is reasonable to assume that due to the high cost of this specialty ingredient, DeltaGold® would not be present in all servings of food consumed in a day. For this reason, the USDA’s estimated 18.2 daily servings of food can be divided by 10, leading to a conservative estimate of approximately 2 servings of food per day that may contain DeltaGold®. If 2 servings of a person’s daily food consumption contained DeltaGold® at the highest intended addition level of 100 mg per serving, the resulting exposure would be 200 mg per day. This level is still consistent with the GRAS standard of “reasonable certainty of no harm”,

based on scientific procedures described above and is below a dose shown to be non-toxic in animal toxicology studies (for example, using a 100-fold safety factor and a NOAEL of 303 mg/kg bw/day from the Tasaki one-year toxicity study⁴¹, a safe level of 212 mg tocotrienols per day for a 70 kg human can be calculated. More importantly and relevant, higher levels than this have been well tolerated in human studies. Furthermore, EFSA stated that 5 mg/kg (350 mg in a 70 kg human) has been shown to be without adverse effects.

The addition of DeltaGold® to food categories is intended to replace consumption of other forms of vitamin E added to foods in the same respective food categories. For example, if a consumer were to choose a nutritional bar containing DeltaGold®, this would likely replace consumption of a similar nutritional bar that contains another branded tocotrienol ingredient. Hence, for consumers who already purchase food products containing tocotrienols, DeltaGold® is not expected to add additional exposure to tocotrienols from the same categories of foods.

General Recognition

Toxicological safety assessments on tocotrienols, which are pivotal in demonstrating the safety for human consumption, have been published and are available in the public domain. These studies have not suggested toxicological concern with regard to consumption of tocotrienols and hence DeltaGold® under its intended conditions of use. The public availability of the safety information related to tocotrienols, that is the basis for this GRAS determination, meets the requirement for common knowledge and general recognition. It also demonstrates that there is consensus among qualified experts that the ingredient is generally recognized as safe for its intended use as stated in this notification. Citations for these assessments can be found in the reference section of this dossier.

Basis for the GRAS Determination

DeltaGold®, a tocotrienol-rich extract of *Bixa orellana* (annatto), has been the subject of a thorough safety assessment as described above. Batch analyses of DeltaGold® show production consistency that meets all product specifications. Animal and clinical scientific studies support the safety of tocotrienols, and these scientific studies are supported by a long history of safe consumption.

As discussed in the Safety Assessment section of this notification, acute oral toxicity, sub-chronic oral toxicity and chronic oral toxicity studies performed administering tocotrienols are pivotal for determining that DeltaGold® at its intended use level is safe for consumption. The long history of human exposure to tocotrienols, human clinical trials, as well as governmental and non-profit organizations' positions on the use of tocotrienols in food corroborates the fact that tocotrienols, and hence DeltaGold®, is considered safe for human consumption. Furthermore, tocotrienols are the subject of GRN 307, which was filed with FDA in 2010 without questions. With exception of rare reports of annatto allergies, no reports of adverse events associated with the long-term

consumption of tocotrienols have been found in the public domain. Based on the evidence provided in this notification, DeltaGold® is considered GRAS for its intended use in food.

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