

Risk Assessment of Tocotrienols from Annatto (*Bixa orellana* L.): Regulatory Contributions

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Abstract

Tocotrienols are found naturally in several plant sources, mainly in grains and oils, such as wheat germ, soybean oil, barley, oats, and olive oil. However, these sources have much lower concentrations of tocotrienols. Higher concentrations of tocotrienols are found in Annatto (*Bixa orellana* L.) seeds. The monograph Dietary Supplement Compendium (DSC) from the United States Pharmacopeia for Annatto Seed Oil Tocotrienols defines that tocotrienols fraction must contain at least 70% of total tocotrienols, calculated on an anhydrous basis of the total tocotrienol content, it must contain between 84% and 92% of δ -Tocotrienol and between 8% and 16% of γ -tocotrienol. The risk assessment with NaturalMax Annatto Tocotrienol™ Oil 70%, extracted from annatto seeds, provided 100% tocotrienols (gamma and delta-tocotrienol) less than 1% α -tocopherol in base anhydrous. NaturalMax Annatto Tocotrienol™ Oil 70% is an innovative ingredient that demonstrated to be according to regulatory requirements and can be applied in dietary supplements, cosmetics, and pharmaceutical products.

Keywords: *Bixa orellana* L. • Urucum • Annatto • Tocotrienols • Regulatory

Introduction

Annatto (*Bixa orellana* L.) is a native Brazilian plant recognized for containing a natural pigment, found in the seeds, that most food industries worldwide use. The plant was domesticated by the northern Brazilian Amazon population, being the world's largest producer of annatto seeds with an estimated harvest of 15.625 tons in 2020 [1].

Although it is primarily recognized as the raw material of the natural dye extensively used by the food industries and one of the main condiments in Brazilian cuisine, Urucum has long been used in folk medicine in the treatment of a large number of diseases. These pharmacological activities of different parts of the annatto are widely documented in the scientific literature. The annatto is cited as: analgesic, antacid, anti-inflammatory, antifebrile, antiparasitic (malaria, leishmaniasis), cicatrizant, antibacterial, antiallergic, in combating stomatological infections, in the treatment of heart disease, diabetes, aphrodisiac, antimicrobial, anticoagulant, in the treatment of cancer, antioxidant, among others [2-8].

Literature Review

Advances in research on isomers that make up the group of substances with vitamin E activity present tocotrienol, especially delta-tocotrienol, as the vitamin E isomer, has more significant antioxidant activity and attributes to tocotrienol a series of benefits to human health. Delta-tocotrienol has the highest antioxidant property among all forms of vitamin E. This property is credited to the lower methylation of these substances' chromanol ring, which allows for greater absorption of these molecules by cell membranes [9,10].

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According to Tan, the gamma and delta-tocotrienol forms were four times more active in eliminating peroxy radicals than the others.

The Food and Drug Administration (FDA) and European Medicine Agency (EMA) have proven safety parameters for tocotrienol consumption as dietary supplements. In these countries, tocotrienol extracted from Annatto seed's is marketed in bottles of about 60 to 125 gelatin capsules containing a mixture of gamma and delta-tocotrienol in concentrations ranging from 50 to 125 mg, with a proportion of approximately 10% of gamma-tocotrienol and 90% delta-tocotrienol (similar to the natural composition of these nutrients in annatto seeds). Clinical research advocates daily consumption in the range of 100 mg to 200 mg, which would make it necessary to eat huge portions of food [11-13].

In 2016, Shahid-UI-Islam et al. conducted a review on the biological activities and phytochemical composition of Annatto [14]. In this review, the authors sought to correlate the pharmacological activities with the presence of some substances that may explain these effects. According to the authors, most of the articles evaluated in the review confirmed that Annatto presents a wide range of biological activities that deserve to be explored to introduce this plant in pharmaceutical products [14]. Among these substances with biological activities, some deserve to be highlighted for presenting great importance for human health is tocotrienols. The presence of tocotrienols in Annatto grains may explain much of the pharmacological properties traditionally attributed to these seeds.

The monograph Dietary Supplement Compendium (DSC) from the United States Pharmacopeia (USP, 2020) determines that: [15]

"Annatto Seed Oil Tocotrienols is the tocotrienol fraction derived from the oil of the seeds of the annatto tree (*Bixa orellana* L.). It contains NLT 70% of total tocotrienols calculated on the anhydrous basis. Of the total tocotrienols content, NLT 84% and NMT 92% is δ -tocotrienol and NLT 8% and NMT 16% is γ -tocotrienol. It contains NMT 1% of α -tocopherol on the anhydrous basis. It may contain added vegetable edible oils."

Based on regulatory contributions, this study analyzed NaturalMax Annatto Tocotrienol™ Oil 70% to demonstrate the safety assessment of Tocotrienols from Annatto.

Preclinical Studies with Tocotrienol

Preclinical (*In vivo*) and clinical experiments showed potential health

benefits with tocotrienol supplementation, such as a lipid-lowering effect and anti-inflammatory and antioxidant activity.

In addition to these effects, in recent decades, hundreds of publications have reported the pharmacological activities of tocotrienols in preclinical studies using animal models (*In vivo*). In addition, several clinical studies have been carried out and confirmed the effects observed in pre-clinical studies, mainly the protection of tocotrienols against genotoxic effects [16]. For the most part, preclinical (mouse) and clinical (human) experiments have shown potential health benefits with supplementation of tocotrienols, mainly T3, including anti-inflammatory activity. This anti-inflammatory activity of tocotrienols (T3) has also been found to protect against neurodegenerative diseases such as Alzheimer's disease (AD) and alcohol-induced cognitive impairment in animals [17]. Studies in patients with cardiovascular disease have shown the lipid-lowering effect and anti-inflammatory and antioxidant activity of tocotrienols compared to tocopherols [18].

Nakamura and collaborators performed an oral toxicity study using a palm oil extract containing 70% tocotrienols for 13 weeks in rats, at concentrations of 0, 119, 474 and 2130 mg/kg in the diet of male rats, and 0.130, 491 and 2,047 mg/kg in the diet of females. The study verified that the dose where no adverse effect is observed, NOAEL (no observed adverse event level) was 1.9 g/kg in the diet, which corresponds to 120 mg/kg/day for male rats and 130 mg/kg/day for female rats. Adverse events, such as increased liver and adrenal gland weight in treated male rats and increased ovarian and uterine weight in female rats, were observed only in the two groups that received the highest doses of tocotrienols, 2130 mg/kg and 2,047 mg/kg, respectively [19]. Tasaki and collaborators performed a chronic study with tocotrienol supplementation (52 weeks) in Wistar Hannover rats of both sexes. The animals were treated with a pure fraction in tocotrienols at concentrations of 0, 0.08, 0.4 or 2% of the powdered basal diet. The rats (males and females) that received the dose of 0.4% or less did not show toxicological alterations in any of the examined parameters. However, nodular liver lesions were found in animals that received the higher dose diet (2%), indicating liver toxicity. Death of six male rats by hemorrhage occurred after administration of 2% of the powdered basal diet of tocotrienols. Based on these results, the authors concluded that the dose where no adverse effect is observed (NOAEL) is 0.4%, which corresponds to 303 mg/kg/day for male rats and 472 mg/kg/day for female rats. Shen and collaborators administered a high-fat diet in C57BL/6J mice, supplemented or not with tocotrienols (800 mg) extracted from the annatto seed, for 14 weeks. Results indicated that tocotrienol supplementation reduced inflammation and oxidative stress, accelerated macronutrient (carbohydrate) metabolism, and reduced obesity-associated hypercholesterolemia/hyperglycemia [18].

Materials and Methods

Tocotrienols is substances with vitamin E activity, one natural fat-soluble antioxidant. Historically, vitamin E is recognized for alpha-tocopherol's biological activity, but lately, it has been observed that the tocotrienol family has different functions than alpha-tocopherol. Although alpha-tocopherol was the first form of vitamin E to be recognized, eight chemically distinct forms have been identified, consisting of alpha (α), beta (β), gamma (γ) and delta (δ)-tocopherols and α , β , γ and δ -tocotrienols (T3), all of which are referred to as vitamin E [20]. Tocopherols and tocotrienols have a similar chemical structure, a chromanol ring with a hydroxyl group that can donate a hydrogen atom to reduce free radicals. The difference between tocopherols and tocotrienols is the presence of three double bonds in the hydrophobic side chain of tocotrienols, which provide greater antioxidant potency [21]. While tocopherols are present in most plants' seeds and leaves, tocotrienols are found in a small fraction of them [22].

Some of the main sources with the highest tocotrienols/tocopherols ratio are rice bran oil (50/50), palm oil (75/25), and Annatto (*Bixa orellana* L.) seeds (99.9/0.1), the latter being the plant source with the highest amount of tocotrienols [23-25].

Annatto (*Bixa orellana* L.) belonging to the botanical family Bixaceae, native to Tropical America. It is a hardy perennial plant that can reach up to 6 m in height. The word urucum originates from the Tupi-Guarani language

transliterated "uru-ku" and means "red". Its scientific name, "*Bixa orellana* L.", was given in honor of Francisco de Orellana (1490-1546), a member of the expedition of Francisco Pizarro, who was the first Spanish explorer to sail the Amazon River. The wide geographical distribution of this plant has made it known by several names [26]. In Brazil, it is known by names such as urucu, urucum, urucu-uva, urucu-bravo, açafão, and bixa, in addition to indigenous names such as ahitê, nukirê, bixe, and bixá. The urucum is also known as achiote, anoto, achote, onotto, onotillo, roekoe, schirabaeli, koessewee, koesowe, bija, cacicuto, uruca, achiotillo, arnotto, arnolta, roucou, chancaguarica, kuxub, achihuiti, achiti, shambu, huantura, atta, santo domingo, analto, and guajachote. In Spain, it is called bija. In France rocuyer. In Germany orlenasbaum. In Italy, England and the United States is called Annatto. In India is known as lathan or kolssewil.

Annatto seed is considered the main raw material for the extraction of tocotrienols, with a low level of tocopherols. This characteristic has been considered relevant because several studies have indicated that the presence of tocopherols interferes with the absorption of tocotrienols [27]. Besides the dye, the annatto oil is also extracted and separated during the dye extraction process. It is a natural source of gamma and delta-tocotrienol and has been regularly imported from Brazil by American companies that perform its fractionation, separating the tocotrienols [28]. In Brazil, the processing of this oil is recent and the technology for this is the result of studies conducted by the Institute of Food Technology (ITAL), in partnership with the Brazilian company New Max Industrial Ltda.

New Max Industrial is the first Brazilian company to separate Tocotrienol from Annatto (*Bixa orellana* L.). New Max Industrial manufactures the product NaturalMax Annatto Tocotrienol™ Oil 70%, extracted from annatto seeds, with greater antioxidant power (gamma and delta-tocotrienol).

Herein regulatory issues on safety aspects of tocotrienols from Annatto is reported. The Food and Drug Administration (FDA), European Medicine Agency (EMA), Brazilian Health Regulatory Agency (ANVISA) have proven safety parameters for Annatto that may be used in dietary supplements, foods, cosmetics, and pharmaceutical products.

In the FDA regulation, Annatto extract is part of the Listing of color additives exempt from certification of Code of Federal Regulations Title 21, which may be used in foods, drugs, and cosmetics [29]. Also, the FDA recognized the human security use of tocotrienols derived from annatto (*Bixa orellana* L.) seed oil, with δ -tocotrienol γ -tocotrienol, in Generally Recognized as Safe (GRAS).

The Commission Regulation (EU) 2020/771 of 11 June 2020 allows the use of Annatto as a food additive [30]. The Directive 2002/46/EC of the European Parliament and the Council of 10 June 2002 Tocotrienol is part of Annex II of Vitamin and mineral substances, which may be used on food supplements [31].

ANVISA allows the use of Annatto as a food additive in dietary supplements, as described on Resolution RDC n. 239 of 26 July 2018 [32]. In 2021, ANVISA included tocotrienol as Active Pharmaceutical Ingredient (API) on the Common Brazilian Denominations (DCB) list, Resolution RDC n. 480 of 15 March 2021 [33]. In parallel with that, New Max Industrial requested inclusion in the Dietary Supplement positive list (Table 1).

According to the monograph of Dietary Supplement Compendium (DSC)

Table 1. Regulatory requirements for tocotrienol.

Health Authority	Brazilian Health Regulatory Agency (ANVISA)	European Medicine Agency (EMA)	Food and Drug Administration (FDA)
Tocotrienol	(<i>Bixa orellana</i> L.) Annatto	<i>Bixa orellana</i> L.) δ -tocotrienol γ -tocotrienol	(<i>Bixa orellana</i> L.) seed oil δ -tocotrienol γ -tocotrienol
Regulatory Requirements	Resolution RDC n. 239 of 26 July 2018 (ANVISA, 2018)	Commission Regulation (EU) 2020/771 of 11 June 2020	Code of Federal Regulations Title 21
Products can be applied	food additive in dietary supplements	foods, drugs, and cosmetics	foods, drugs, and cosmetics

for Annatto Seed Oil Tocotrienols, defines tocotrienols fraction must contain at least 70% of total tocotrienols, calculated on an anhydrous basis [15]. The total tocotrienol content must have between 84% and 92% of δ -Tocotrienol and between 8% and 16% of γ -tocotrienol. It contains NMT 1% of α -tocopherol on the anhydrous basis. It may have added vegetable edible oils.

Tocopherols have a longer saturated tail, which make them stationary and consequently protect only a limited surface area of the cell membrane. Unlike tocopherols, the tocotrienol molecule has a shorter tail, with unsaturated side chains (farnesyl). This chemical structure allows tocotrienols to move through the body 50 times faster than tocopherols and penetrate into the tissues more effectively, promoting a big difference in various functional effects of the body, such as a much higher antioxidant activity.

In addition, studies have shown that tocopherol interferes with the benefits of tocotrienol. The cholesterol-lowering action of tocotrienols was compromised by the presence of alpha-tocopherol and it was concluded that effective tocotrienol preparations should contain less than 15-20% of alpha-tocopherol and more than 60% of gamma and delta-tocotrienol [27]. Delta-tocotrienol is considered the form with the highest antioxidant power among all compounds with vitamin E activity (Figures 1-3).

According to Certificate of Annatto Seed Tocotrienols Extract RS (2020b)

for assay of delta-Tocotrienol prepare as described in Standard solution 1 (Graph 1). For assay of gamma-Tocotrienol prepare as described in Standard solution 2 (Graph 2).

Results

NaturalMax Annatto Tocotrienol™ Oil 70% is a natural product obtained from annatto seeds (*Bixa orellana* L.) It is manufactured by ecofriendly green chemistry procedures from Annatto seeds being fractionated without use of organic solvents, containing its isoform with the highest antioxidant activity (δ -tocotrienol) at a ratio of approximately 90% (Table 2).

Based on the monograph of Dietary Supplement Compendium (DSC) was analyzed three different batches of NaturalMax Annatto Tocotrienol™ Oil 70% [15]. This analysis's objective was to determine the total tocotrienol content, the proportion of δ - and γ -tocotrienol isomers content in tocotrienol fraction derived from annatto seed oil.

The method consisted on the detection and quantification using HPLC coupled to the detector UV / Visible, using the standard USP Annatto Seed Tocotrienols Extract R as a reference [34]. The retention times of the

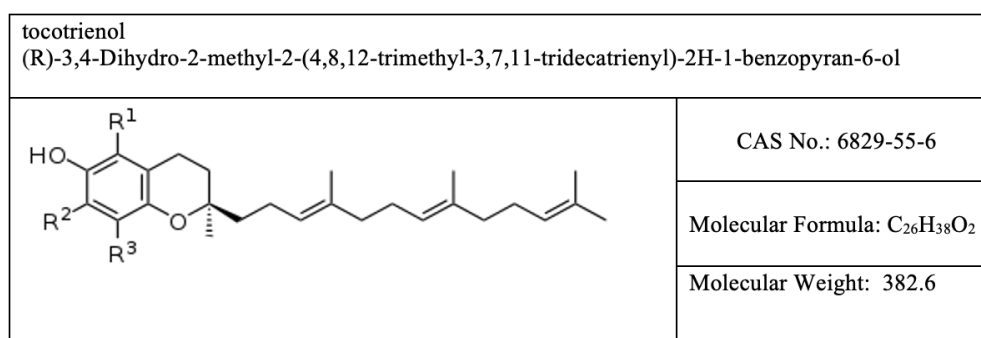


Figure 1. Tocotrienol.

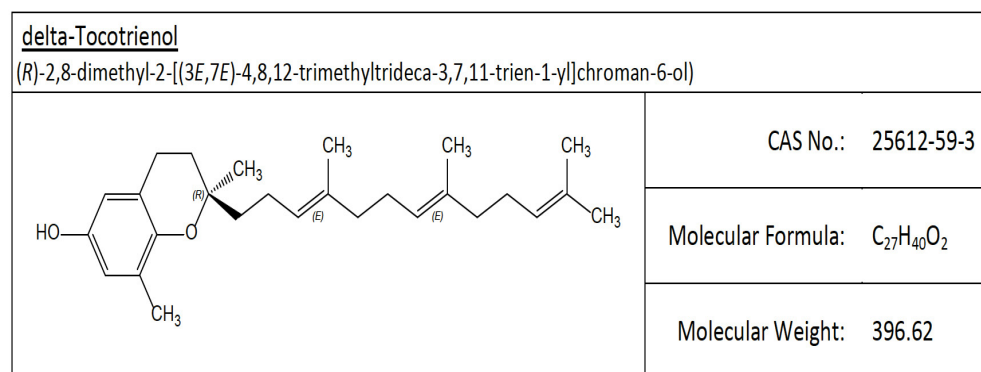


Figure 2. Delta-Tocotrienol.

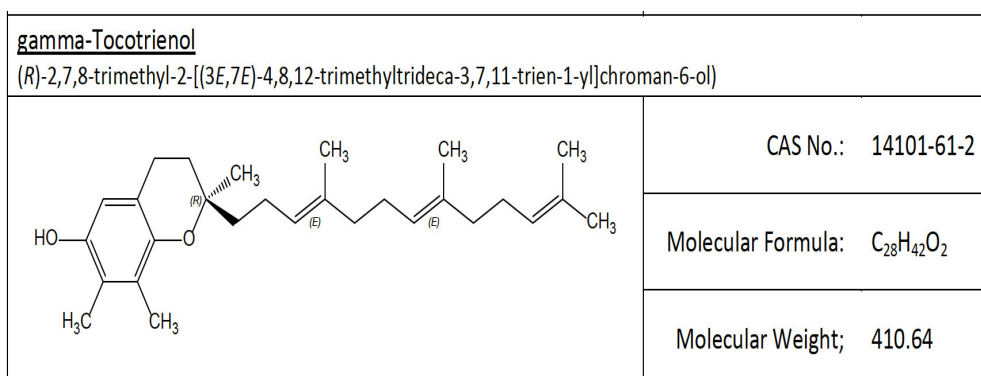


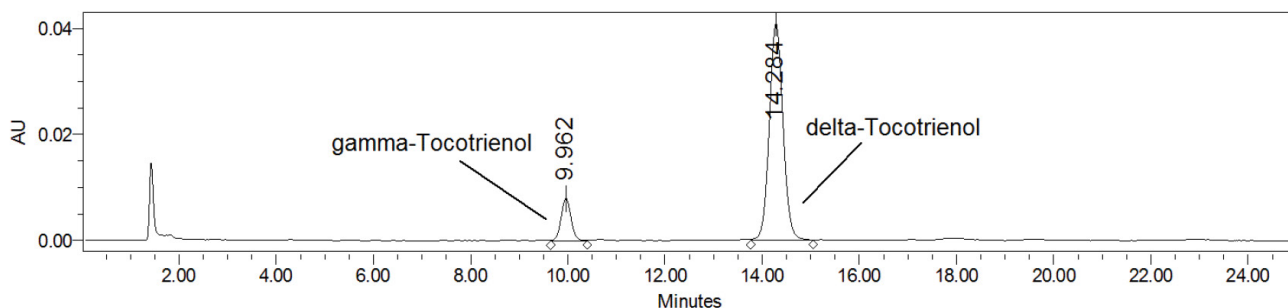
Figure 3. Gamma-Tocotrienol.

Table 2. Specification of naturalmax annatto tocotrienol™ Oil (70%).

504827004XX Naturalmax Annatto Tocotrienol (oil) (70%) (5380)							
Description	Naturalmax Annatto Tocotrienol (Oil) (70%) is a natural product obtained from annatto seeds (<i>Bixa orellana</i> L.)						
Origin	Vegetable						
Composition	Tocotrienols extracted from annatto oil.						
Description or production	Extraction, distillation and others						
Analytical parameters	Brazilian Pharmacopeia 6a ed. 2019; USP- NF (United States Pharmacopeia- National Formulary)						
Physico-chemical analysis							
Parameters	Limit		Evaluation Unit	Frequency	Methodology	Present in the report	
	Min.	Max.					
Aspect	Oily liquid, Slightly viscous		Internal	Every Batch	MA. 10 Internal Methodology	Yes	
Colour	Orange to red		Internal	Every Batch	MA. 10 Internal Methodology	Yes	
Total tocotrienols contents	70	100	% (m/m)	Internal	Every Batch	MA. 10 Internal Methodology	Yes
Delta-tocotrienols*	84	92	% (m/m)	Internal	Every Batch	MA. 10 Internal Methodology	Yes
Gamma-tocotrienols*	8	6	% (m/m)	Internal	Every Batch	MA. 10 Internal Methodology	Yes
Tocopherols	-	1	% (m/m)	Internal	Every Batch	USP41-NF36 Pg4331	Yes
Peroxide Value	-	10	meqO ₂ /kg	External	Six-Monthly	USP-NF (401)/Farm. Bras. 6 ed. 2019-5.2.29.11	Yes
Residual Solvent	Absent		External	Annual	USP-NF (467)/Farm. Bras. 6 ed. 2019-5.2.29	Yes	

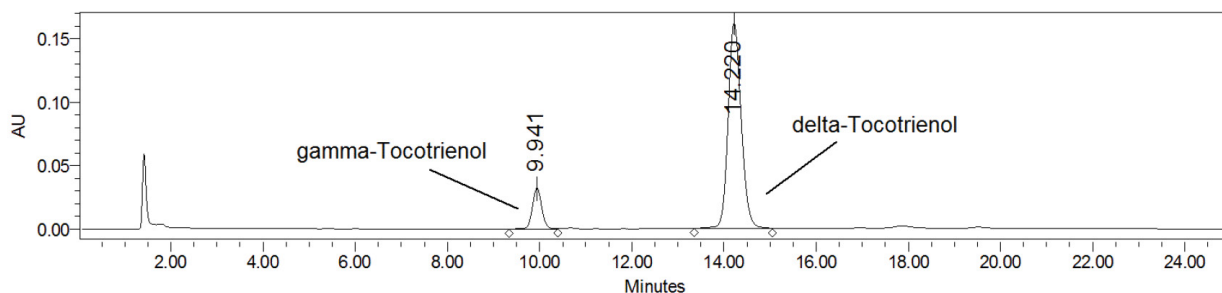
*Isomer content referring to total tocotrienols content.

Typical Chromatogram - Standard solution 1



Graph 1. Chromatogram of delta-Tocotrienol.

Typical Chromatogram - Standard solution 2



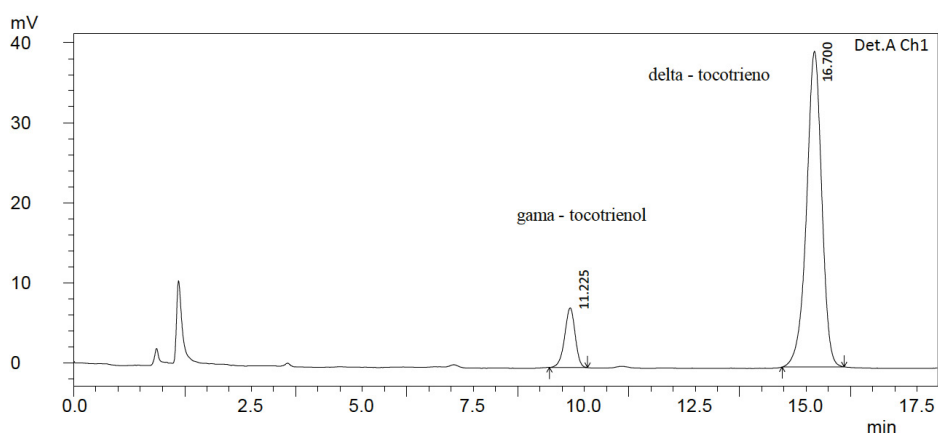
Graph 2. Chromatogram of gamma-Tocotrienol.

δ-tocotrienol and γ-tocotrienol peaks in the solution of sample must match those of standard solutions (Graph 3).

This chromatogram describes that NaturalMax Annatto Tocotrienol™ Oil 70% has a total of 92% of δ-Tocotrienol (Table 3) (Graphs 4 and 5).

The results of analysis of NaturalMax Annatto Tocotrienol™ Oil 70%, extracted from annatto seeds, provides 100% tocotrienols (gamma-Tocotrienol and delta-Tocotrienol), with less than 1% α-tocopherol in base anhydrous.

<Chromatogram>



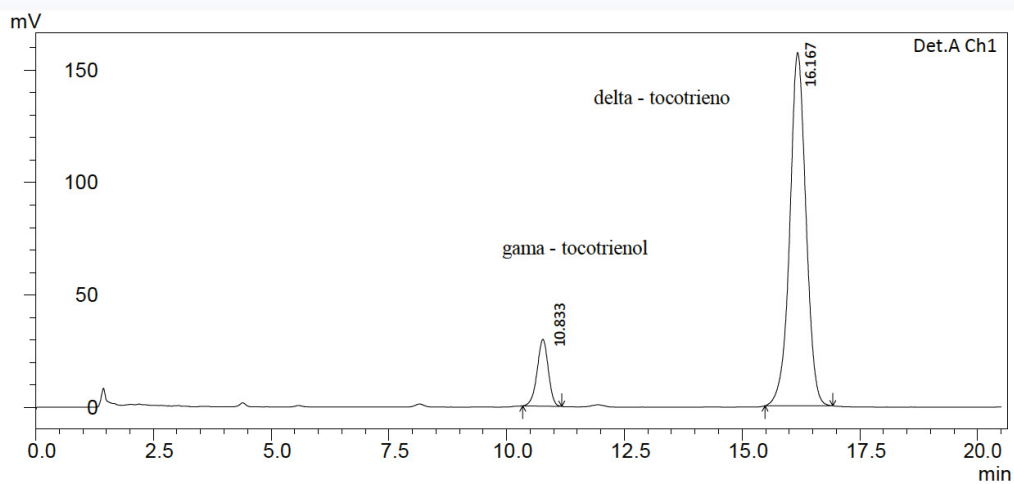
Peak#	Ret. Time	Area	Height	Area %	Height %
1	11.225	122851	6537	11.551	14.414
2	16.700	940675	38814	88.449	85.586
Total		1063526	45350	100.000	100.000

Graph 3. Chromatogram of delta-Tocotrienol in NaturalMax Annatto Tocotrienol™ Oil 70%.

Table 3. Naturalmax Annatto Tocotrienol™ Oil 70%.

Variables	Concentration (mg/g)	Concentration (g/100 g)	Concentration (% tocotrienol total)
-tocotrienol	667,08	66,71	88,80
-tocotrienol	84,10	8,41	11,20
-tocopherol	2,47	0,25	-

<Chromatogram>

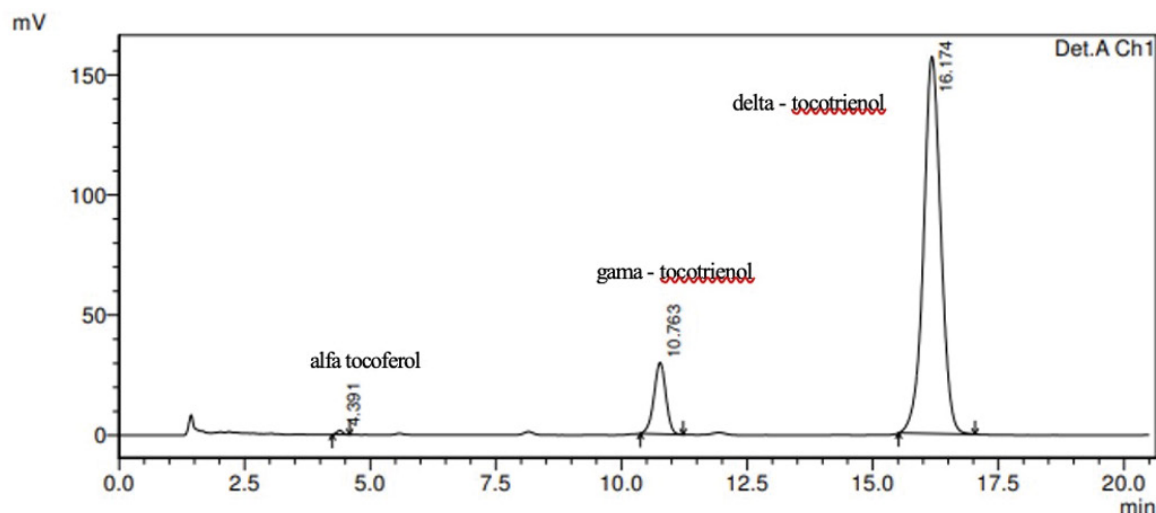


PeakTable

Detector A
Ch1 290nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	10.833	490580	23225	11.542	12.918
2	16.167	3759636	156567	88.458	87.082
Total		4250216	179792	100.000	100.000

Graph 4. Chromatogram of gamma-Tocotrienol in NaturalMax Annatto Tocotrienol™ Oil 70%.

<Chromatogram>

Detector A Ch1 290nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.391	14906	1764	0.343	0.931
2	10.763	516360	30098	11.874	15.892
3	16.174	3817259	157531	87.783	83.177
Total		4348524	189393	100.000	100.000

Graph 5. Naturalmax Annatto Tocotrienol™ Oil 70%.

Discussion

Given the pharmacokinetics of tocotrienols, including their short half-life, consumption of 3-5 mg/kg/day should not cause adverse effects, which is in line with conclusions reached by the EFSA (European Food Safety Authority) expert panel and another for GRAS (Generally Recognized as Safe). Tocotrienols are initially beta-oxidized by cytochrome P450 enzymes, then conjugated and excreted. Since all of these mechanisms are tightly regulated, the potential for adverse effects is limited [35].

In addition to medicinal use, tocotrienols' safety profile allows for the development of foods with functional and healthy properties. In this regard, the "Food and Drug Administration" (FDA) recently recognized the status of "Generally Recognized as Safe (GRAS)" for the addition of up to 40 mg tocotrienols per kilogram of food. The study conducted by Pandya et al. demonstrated that tocotrienols is safe to be used in food supplement products [36-38].

The chromatograms of the NaturalMax Annatto Tocotrienol™ Oil 70% have proven safety parameters for Annatto with the highest amount of tocotrienols (gamma-Tocotrienol and delta-Tocotrienol), according to DSC monograph.

Conclusion

New Max Industrial Ltda controls from planting the Annatto seeds to the manufacturing of the tocotrienol ingredient. The whole process of planting and manufacturing takes place in the national territory. The raw material production starts with the planting of the seeds on New Max's farm, harvesting and drying the seeds for the production of the raw material. The tocotrienol is produced from the manufacture of the natural urucum dye. Thus, after centrifuging and heating the dye, the tocotrienol oil is extracted. The product is distilled so that it contains at least 70% total tocotrienols, 84-92% delta-tocotrienol, 8-16% gamma-tocotrienol, and a maximum of 1% tocopherols.

NaturalMax Annatto Tocotrienol™ Oil 70%, tocotrienol from annatto seeds, is an innovative ingredient that is demonstrated according to regulatory requirements and can be applied in dietary supplements, cosmetics, and pharmaceutical products.

Author Contributions

Substantial contributions to the conception or design of the work. Analysis and interpretation of data for the work (TDSF, MAF, JEC, PRNC, ANC). Drafting the work or revising it critically for important intellectual content (TDSF, MAF, JEC, PRNC, ANC). Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy are appropriately investigated and resolved (TDSF, MAF, JEC, PRNC, ANC).

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