Tocotrienols, tocopherols and tocomonoenols: Characterization in Costa Rican Palm Oils, and intracellular and tissue distribution as a function of the hepatic alpha-tocopherol transfer protein

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Dedicate to my family in Costa Rica.

To my parents Ligia and Roger, my sister Marisol, and my grandfather Mario.

“I know that sometimes I look back, but it is to know where I come from”

“Sé que a veces miro para atrás, pero es para saber de donde vengo”

Summary

Vitamin E is a generic term for a group of micronutrients exhibiting the biological activity of α-tocopherol (αT). Initially, four tocopherols (T; α-, β-, γ-, δ-) and four tocotrienols (T3; α-, β-, γ-, δ-) were recognized as the naturally occurring vitamin E compounds. The main difference among T and T3 is the 3-fold unsaturated 16-carbon side chain of the T3 compared to the saturated 16-carbon side chain of the T. Recently, a group of four vitamin E compounds with a single double bond at carbon 11' were discovered, namely tocomonoenols (T1). Edible oils are the major source of T, T3, and of T1.

As a fat-soluble vitamin, the vitamin E is absorbed after oral intake and transported in the circulation to the liver, where vitamin E undergoes sorting by the action of the α-hepatic-tocopherol transfer protein (TTP) and the cytochrome P_{450} (CYP) enzymes. αT is preferentially secreted into the bloodstream, while the non-αT congeners are metabolized by CYP to the carboxyethylhydroxychromanols (CEHC), which are excreted via urine and feces. The TTP has been recognized as necessary for the maintenance of normal αT concentrations in plasma and extrahepatic tissues. Interestingly, TTP might also protect the non-αT congeners from side-chain degradation, and therefore prevent their metabolic degradation.

The present thesis aimed at increasing our knowledge of the non-αT congeners of vitamin E with respect to their occurrence in food, their intracellular localization upon uptake into liver cells, and their tissue distribution in mammals. A potential role of the TTP in the intracellular and intra-organismic trafficking of the non-αT congeners was a second focus of the current investigations.

To this purpose, the vitamin E profiles and contents in oils of three *Elaeis Guineensis*, two *Elaeis Oleifera*, and one hybrid OxG palm fruit genotypes from Costa Rica were determined after mechanical extraction with a screw press and chemical extraction with hexane. Vitamin E profiles in the palm oils were similar, irrespective of the genotype and extraction procedure, and α- and γ-tocotrienols (αT3 and γT3) were the most abundant congeners. α-Tocomonoenol (αT1) was found in oils from five of the six varieties. Hexane extraction yielded up to 2.5-fold higher total vitamin E compared to screw press extraction.

The two most abundant tocotrienols (αT3 and γT3) in the oils were selected for further studies with respect to their cellular uptake and intracellular distribution in cultured liver cells with and without stable expression of TTP and compared to their respective tocopherol counterparts (αT and γT). After uptake, all four congeners were primarily associated with the lysosomes, endoplasmic reticulum and plasma membrane. Overall, the results conclude that neither the structural differences between the four congeners, nor the TTP-expression are important factors behind the intracellular trafficking (uptake and distribution) of the congeners in cultured liver cells.

Finally, an animal study was performed to examine the tissue distribution of αT1, which was found as a novel vitamin E congener during the characterization of the oils, in mice in comparison to αT. Besides was investigated the influence of TTP. Wild-type ($TTP^{+/+}$) and

TTP knockout ($TTP^{-/-}$) mice were fed a standard diet with either αT or αT1 for 2 weeks. Concentrations of αT and αT1 were measured in blood and several tissues. αT1 was only found in blood, not in tissues. Loss of TTP function in $TTP^{-/-}$ mice resulted in almost complete depletion of αT in all tissues. Interestingly, αT1, contrary to αT, was still present in blood of $TTP^{-/-}$ mice. In conclusion, αT1 reached the blood in mice with and without TTP function, suggesting that TTP may not, or only to a limited extent, be required for the secretion of αT1 into the systemic circulation.

Overall, the findings of this thesis show that the non-αT congeners αT3 and γT3, have similarities with αT regarding the intracellular trafficking, whereas αT1 has different tissue and blood distribution compared to αT. The TTP lacks a significant role mediating the intracellular trafficking of αT and non-αT congeners. TTP is, however, involved in the secretion of αT from the liver into the bloodstream and might, to a certain extent, also be involved in the secretion of αT1.

Based on the finding that the non-αT congeners represent the majority of vitamin E congeners in palm oils, the identification of additional food sources and a deeper understanding of the biological roles of these congeners is warranted. Since more is known about αT than the non-αT congeners, new opportunities for further research on the biological activities and consequent health benefits of the non-αT congeners have arisen based on the contributions of the present thesis.

Zussamenfassung

Vitamin E ist der Oberbegriff für eine Gruppe von Mikronährstoffen, welche die biologische Aktivität von α-Tocopherol (αT) aufweisen. Bisher wurden acht Kongenere, vier Tocopherole (T; α-, β-, γ-, δ-) und vier Tocotrienole (T3; α-, β-, γ-, γ-, δ-) als die natürlich vorkommenden Vitamin-E-Verbindungen klassifiziert. Die Unterscheidung zwischen T und T3 wird durch die dreifach ungesättigte 16-Kohlenstoff lange Seitenkette der T3´s im Vergleich zur gesättigten 16- Kohlenstoff langen Seitenkette der T´s definiert. Vor kurzem wurde eine Gruppe von vier Vitamin-E-Verbindungen mit einer Einfach-Doppelbindung am 11ten Kohlenstoffatom entdeckt und als Tocomonoenole (T1) bezeichnet. Speiseöle sind die Hauptquelle für T, T3 und T1.

Das fettlösliches Vitamin E wird oral aufgenommen und zur Leber transportiert. In der Leber durchlaufen die Vitamin E Kongenere eine Sortierung durch das α-hepatischen-Tocopherol-Transferprotein (TTP) und der Cytochrom P_{450} (CYP)-Enzyme. Das αT wird bevorzugt in den Blutkreislauf abgegeben, während die nicht-αT-Kongenere von den CYP-Enzymen zu den Carboxyethylhydroxychromanolen (CEHC) metabolisiert und über Urin und Stuhl ausgeschieden werden. Für die Aufrechterhaltung der normalen αT-Konzentration in Plasma und extrahepatischem Gewebe ist das TTP daher notwendig. Interessanterweise ist es möglich, das TTP auch die nicht-αT-Kongenere vor dem Abbau der Seitenkette schützen und so deren metabolischen Abbau verhindern kann.

Ziel dieser Arbeit war unser Wissen über die nicht-αT-Kongenere von Vitamin E in Bezug auf ihr Vorkommen in der Nahrung, ihre intrazelluläre Lokalisation bei der Aufnahme in Leberzellen und ihre Gewebeverteilung bei Säugetieren zu erweitern. Die potentielle Rolle des TTP bei der intrazellulären Verteilung und dem Transport im Organismus der nicht-αT-Kongenere war ein zweiter Schwerpunkt der aktuellen Untersuchungen.

Zu diesem Zweck wurden die Vitamin-E-Profile und -Gehalte in Ölen von drei *Elaeis Guineensis*, zwei *Elaeis Oleifera* und einem hybriden OxG-Palmenfrüchte-Genotyp aus Costa Rica ermittelt. Die Öle wurden entweder mechanisch mit einer Spindelpresse oder chemisch mit Hexan extrahiert. Die Vitamin-E-Profile in den Palmölen waren unabhängig vom Genotyp und Extraktionsverfahren ähnlich, α- und γ-Tocotrienole (αT3 und γT3) waren die häufigsten Kongenere. α-Tocomonoenol (αT1) wurde in fünf der sechs Sorten gefunden. Die Extraktion mit Hexan ergab im Vergleich zur mechanischen Spindelpresse einen bis zu 2,5-fach höheren Gesamt-Vitamin E-Gehalt.

Aufgrund der aus den Ölen gewonnen Erkenntnisse bezüglich der Häufigkeit der vorkommenden nicht- αT-Kongenere, wurden die beiden Tocotrienole, αT3 und γT3, für weitere Studien auf ihre zelluläre Aufnahme und intrazelluläre Verteilung in kultivierten Leberzellen mit und ohne stabiler TTP-Expression ausgewählt und mit ihren jeweiligen Tocopherol-Gegenstücken, dem αT und γT, verglichen. Nach Aufnahme der vier Kongenere waren diese hauptsächlich mit den Lysosomen, dem endoplasmatischen Retikulum und der Plasmamembran assoziiert. Zusammengefasst lassen diese Ergebnisse darauf schließen, dass

weder die strukturellen Unterschiede, noch die Expression des TTP ausschlaggebende Faktoren für den intrazellulären Transport (Aufnahme und Verteilung) der Kongenere in kultivierten Leberzellen sind.

Abschließend wurde eine Tierstudie durchgeführt, um die Gewebeverteilung des αT1, welches als neues Vitamin E Kongener bei der Charakterisierung der Öle festgestellt wurde, bei Mäusen im Vergleich zu αT untersuchen. Ebenfalls wurde hierbei der Einfluss von TTP mit berücksichtigt. Wildtyp ($TTP^{+/+}$) und TTP Knockout ($TTP^{-/-}$) Mäuse erhielten 2 Wochen lang eine Standarddiät mit αT oder αT1. Die Konzentrationen an αT und αT1 wurden im Blut und verschiedenen Geweben bestimmt. Das αT1 konnte ausschließlich im Blut, nicht in den Geweben, gefunden werden. Der Verlust der TTP-Funktion bei $TTP^{-/-}$ Mäusen führte zu einer fast vollständigen Abnahme der αT-Konzentrationen in allen Geweben. Interessanterweise konnte αT1, im Gegensatz zu αT, noch im Blut von $TTP^{-/-}$ Mäusen nachgewiesen werden. Das Vorhandensein von αT1 im Blut von Mäusen mit und ohne TTP-Funktion deutet darauf hin, dass TTP für die Sekretion von αT1 in den systemischen Kreislauf nicht oder nur eingeschränkt erforderlich sein könnte.

Insgesamt zeigen die Ergebnisse dieser Arbeit, dass die nicht-αT-Kongenere αT3 und γT3, Ähnlichkeiten mit αT in Bezug auf den intrazellulären Transport besitzen, wohingegen αT1 eine andere Gewebe-und Blutverteilungen aufzeigt. Das TTP scheint eine untergeordnete Rolle im intrazellulären Transport der Vitamin E Kongenere zu spielen. Es ist jedoch an der Sekretion von αT aus der Leber in den Blutkreislauf beteiligt und kann daher auch bis zu einem gewissen Grad an der Sekretion von αT1 beteiligt sein.

Basierend auf der Feststellung, dass die nicht-αT-Kongenere die Mehrheit der Vitamin-E-Kongenere in Palmölen repräsentieren, ist die Identifizierung zusätzlicher Nahrungsquellen und ein tieferes Verständnis der biologischen Rolle dieser Kongenere gerechtfertigt. Aufgrund des höheren Wissenstandes über αT im Vergleich zu den nicht-αT-Kongeneren, sind auf Grundlage der vorliegenden Arbeit neue Möglichkeiten für die weitere Erforschung der biologischen Aktivitäten und den daraus resultierenden gesundheitlichen Vorteile der nicht-αT-Kongenere entstanden.

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List of Abbreviations

ABC	ATP-binding cassette
ABCA1	ABC transporter subfamily A, member 1
Ala	alanine
Arg	arginine
αT	α-tocopherol
αT1	α-tocomonoenol
αT3	α-tocotrienol
α-TTP, TTP	α-tocopherol transfer protein
AVED	ataxia with vitamin E deficiency
βT	β-tocopherol
βT3	β-tocotrienol
13'-COOH, α-13'-COOH	13'-carboxychromanol and α-13'-carboxychromanol
CDMDHC or 11'-COOH	carboxydimethyldecylhydroxychromanol
CDMOHC or 9'-COOH	carboxymethyloctylhydroxychromanol
CDMHHC or 7'-COOH	carboxymethylhexylhydroxychromanol
CMBHC or 5'-COOH	carboxymethylbutylhydroxychromanol
CEHC or 3'-COOH	carboxyethylhydroxychromanols
$CDMD(en)_2HC$	carboxydimethyldecadienylhydroxychromanol
CDMOenHC	carbodimethyloctenylhydroxychromanol
$CDMO(en)_2HC$	carboxydimethyloctadienylhydroxychromanols
CMHenHC	carboxymethylhexenylhydroxychromanol
CMBenHC	carboxymethylbutadienylhydroxychromanol
CETP	cholesteryl ester transfer protein
CR	chylomicrons remnants
CD36	cluster of differentiation 36
CG	conjugates
COX-2	cyclooxygenase-2
CYP	cytochrome P-450
ERα	estrogen receptor α
δT	δ-tocopherol
δT3	δ-tocotrienol
HO-1	hemeoxygenase-1
HDL	high-density lipoproteins
13'-OH, α-13'-OH	13'-hydroxychromanol and 13'-hydroxychromanol
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzime A
12-HPETE	12-hydroperoxy-eicosatetraenoic acid
IL-1β	interleukin-1β
ICM	intermediate chain metabolites
Ile	isoleucine
LTB_4	leukotriene B4
Leu	leucine
LPS	lipopolysaccharide
LPL	lipoprotein lipase
5-LOX	5-lipoxygenase
12-LOX	12-lipoxygenase
LCM, α-LCM	long chain metabolites and α-long chain metabolites

LDL	low-density lipoproteins
LDLR	low-density lipoprotein receptor
LRP	low-density lipoprotein receptor-related proteins
Lys	lysine
MDT	marine-derived tocopherol
mRNA	messenger ribonucleic acid
Met	metionine
MDR3	multidrug resistance proteins
NADH	nicotinamide adenine dinucleotide
NPC1L1	niemann-pick C1-like protein 1
NF-κB	nuclear factor κB
PPARα, PPARγ, PPARδ	peroxisome proliferator-activated receptors α, γ and δ
Phe	phenylalanine
PIP_2	phosphatidylinositol 4,5-bisphosphate
PI3Kγ	phosphoinositide 3-kinase γ
PLTP	phospholipid transfer protein
PXR	pregnane X receptor
PGE_2	prostanglandin E2
PKC	protein kinase C
PP_2A	protein phosphatase 2A
SR-A	scavenger receptors class A
SR-B1	scavenger receptor class B type 1
SCM	short chain metabolites
SULT	sulfotransferases
T	tocopherols
TAP	tocopherol-associated proteins
T3	tocotrienols
T1	tocomonoenols
UGT	UDP glucuronosyltransferases
Val	valine
VLDL	very low-density lipoproteins
γT	γ-tocopherol
γT3	γ-tocotrienol

Chapter 1

Introduction

1. Vitamin E

In the year 1922, Herbert Evans and Katherine Bishop reported for the very first time the existence of an unrecognized dietary factor essential for reproduction of rats, present in foods such as green leafy vegetables and butterfat, and capable of restoring the fertility of rats with proven sterility [1]. Its designation as vitamin came in 1925, named alphabetically as vitamin E, the fifth vitamin of the small family of vitamins discovered at that moment [2]. Years later, in 1936, Herbert Evans and his co-workers isolated from wheat-germ oil an alcohol exhibiting vitamin E activity and they proposed the name α-tocopherol, in agreement with their biological activity and chemical feature of alcohols (tokos = childbirth; phero = to bear; -ol, indicating an alcohol); besides they reported that after treatment of the α-tocopherol with methyl alcoholic silver nitrate, they convert the compound in another substance that exhibited a reduced vitamin E activity, leading them to conclude that vitamin E activity was not the property of one single compound [3].

1.1 Structures and stereochemistry

Vitamin E is a generic term for a group of micronutrients exhibiting the biological activity of α-tocopherol (αT). The eight naturally occurring vitamin E compounds, four tocopherols (α-, β-, γ-, δ-) and four tocotrienols (α-, β-, γ-, δ-) consist of a chromanol head (with two rings; one phenolic and one heterocyclic) bound to a saturated phytyl (tocopherols) or threefold unsaturated isoprenoid (tocotrienols) 16-carbon sidechain. The assigned prefixes α, β, γ, or δ are based on the number and position of methyl groups substituted at the chromanol head (Figure 1).The phytyl side chain of the tocopherols (T) has three chiral centers at positions 2, 4’ and 8’, which can be either in the R- or S- configuration, permitting eight different stereoisomers (RRR, RSR, RRS, RSS, SRR, SSR, SRS and SSS). Tocotrienols (T3) only have one chiral center at position 2, so they can have R-or S- configuration. However, the presence of double bonds at positions 3’ and 7’ on the isoprenoid side chain allows for the existence of four *cis/trans* geometrical isomers, leading at the end of theoretically eight isomers (R, *cis-cis*; R, *cis-trans*; R, *trans-cis*; R, *trans-trans*; S, *cis-cis*; S, *cis-trans*; S, *trans-cis*; S, *trans-trans*) [4].

In addition to the T and T3, an emerging group of vitamin E compounds with the structural feature of a unique double bond at carbon 11' has been discovered, reported for the very first time in 1995 and named α-tocomonoenol (αT1) [5]. Years later, the congeners β-, γ- and δ-tocomonoenol where found and reported [6-8]. Besides, in 1999 an isomeric form of the αT1

with an unusual methylene unsaturation at the isoprenoid-chain terminus (carbon 12') was found in marine products and named marine-derived tocopherol (MDT) [9] (Figure 1).

1.2 Occurrence and dietary intake

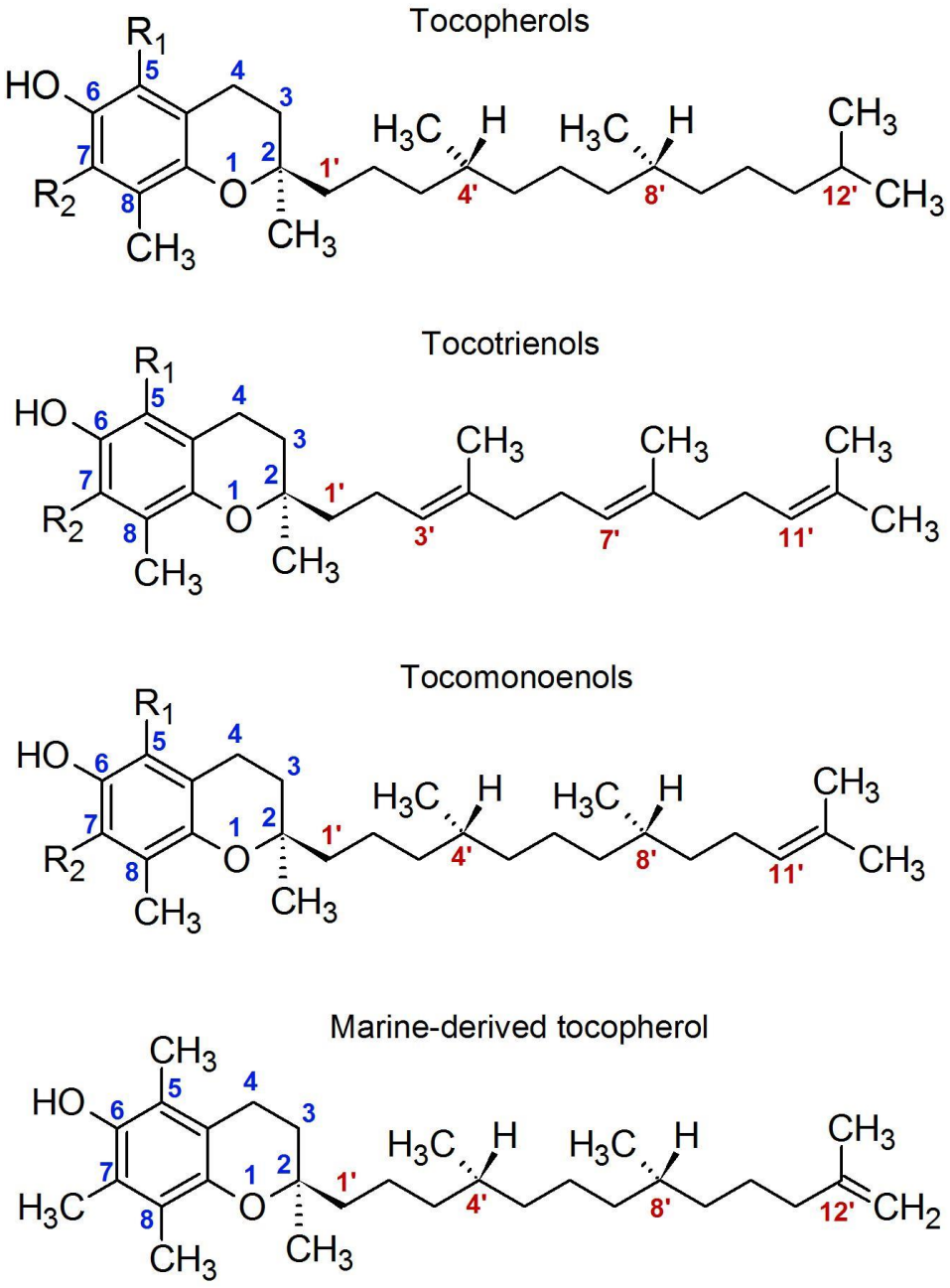
Vitamin E is exclusively synthesized by photosynthetic organisms. T are the predominant congeners in higher plants and found in oil seeds, leaves, roots, tubers, cotyledons, hypocotyls, stems and flowers. T3 on the other hand are not usually found in the green part of the plants, their accumulation is mostly in seeds, the pericarp and specialized cells like latex tuber [10].

Edible oils are the major source of T and T3. αT is the predominant vitamer in sunflower, safflower, wheat germ, peanut and olive oils, while soybean, canola, sesame, corn and linseed oils contain high amounts of γ-tocopherol (γT). Recently, genetically modified sunflower oils with high contents of β- and δ-tocopherols have been developed. Important amounts of αT were also detected in seed oils of lemon, orange and tangerine; meanwhile γT was the most abundant in grape, guava, melon, tomato and pumpkin seed oils [11].

Palm oil and rice bran oils are the first and second most abundant natural sources of T3, with γ-tocotrienol (γT3) as the most prominent vitamer. Wheat germ oils, cereal grains, such as oat, rye and barley, and tobacco seeds oil are also important sources of T3. α-Tocotrienol (αT3) is the predominant form in oat and barley. β-Tocotrienol (βT3) is the major vitamer in hulled and dehulled wheats [11-13].

Tocomonoenols (T1) have been detected in edible oils, fruits and leaves, such as αT1 in palm, pumpkin and sunflower oils [5, 6, 14]; γ-tocomonoenol in pumpkin oil [6]; δ-tocomonoenol in kiwi fruits [7]; and β-, γ- and δ-tocomonoenol in green leaves and etiolated beans of *Kalanchoe daigremontiana* and *Phaseolus coccineus* [8]. MDT was found in cold-water fishes, such as salmon and Antarctic notothenioid; as well as Antarctic krill (*Euphausia superba*) and phytoplankton [9, 15, 16].

Dietary intake recommendations for vitamin E from food, fortified food and multivitamins are based on the activity of the 2R-stereoisomeric forms of αT, because the RRR- is the form that occurs naturally in foods and is the most biologically active stereoisomer of all the naturally occurring vitamin E forms. Also the other three synthetic 2R-stereoisomers of αT exert a higher biological activity in comparison with the 2S-forms [17].



Congener	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 1. Chemical structures of tocopherols, tocotrienols, tocomonoenols and the marine-derived tocopherol.

The Institute of Medicine [17] in the US defined a Recommended Dietary Allowance for both men and women of 15 mg (35 μmol)/day of αT and for infants, children, and adolescents of 6-15 mg (14-35 μmol)/day of αT. Even though a vitamin E deficiency is very rare, for adults an Estimated Average Requirement of 12 mg (28 μmol)/day of αT was estimated to achieve a sufficient protective effect of the vitamin E as antioxidant, and a Tolerable Upper Intake Level of 1000 mg (2325 μmol)/day of αT was established to avoid adverse effect of increased tendency to hemorrhage [17]. The European Food Safety Authority (EFSA) defined an Adequate Intake of 13 mg/day of αT for men, 11 mg/day of αT for women and 5-13 mg/day of αT for infants and children [18].

1.3 Absorption, distribution and metabolism

As a fat-soluble vitamin, the route of vitamin E after oral intake follows in general the pathway of other lipophilic molecules and lipids. Even though the presence of lipid-rich foods is required for the absorption of vitamin E, some natural food ingredients as dietary fiber, plant sterols, eicosapentaenoic acid and retinoic acid, among others, can compete with the absorption of the vitamin E and limit is absorption rate, which varies from 20% to 80% [19].

The intestinal absorption starts with the secretion of pancreatic enzymes, bile acids, dietary lipids and vitamin E into the duodenum where all components interact to form mixed-micelles with the vitamin E embedded. Integrated in micelles, vitamin E is taken up into enterocytes by passive diffusion or receptor-mediated transport via the scavenger receptor class B type 1 (SR-B1) and the Niemann-pick C1-like protein 1 (NPC1L1) [19]. In the enterocytes, vitamin E is incorporated into chylomicrons which are secreted into the lymphatic system mediated by the ATP-binding cassette (ABC) transporter subfamily A, member 1 (ABCA1) [18] (Figure 2).

The chylomicrons in the lymphatic system pass via the portal vain into the blood system, where they are hydrolyzed by lipoprotein lipase (LPL) to chylomicron remnants (CR) that transport vitamin E to the liver, where vitamin E is imported into the liver cells via low-density lipoprotein receptor-related proteins (LRP) and low-density lipoprotein receptor (LDLR) (Figure 2). The extent of vitamin E absorption and transport to the liver is the same for all vitamers [19].

Once in the liver, vitamin E undergoes sorting mediated by the hepatic α-tocopherol transfer protein (α-TTP) (see section “α-tocopherol transfer protein”) or metabolic degradation via cytochrome P-450 (CYP) enzymes (see below), leading to almost exclusive secretion of αT into bloodstream [19]. So far, no specific plasma transport protein for vitamin E has been described, so the transport in the blood follows the lipoprotein transport route of other lipids that ends up distributing the vitamin E between the liver and extrahepatic tissues.

αT is incorporated into very low-density lipoproteins (VLDL) and exported through ABCA1 [20]. VLDL is enzymatically hydrolyzed by LPL resulting in the formation of low-density lipoproteins (LDL) that can incorporate αT back into liver or into extrahepatic tissues. From

extrahepatic tissues, αT is transported back to the liver in HDL, which are secreted involving ABCA1, where it can either be directly imported into the liver via SR-B1, or transferred to LDL mediated by the phospholipid transfer protein (PLTP) or cholesteryl ester transfer protein (CETP) [19] (Figure 2).

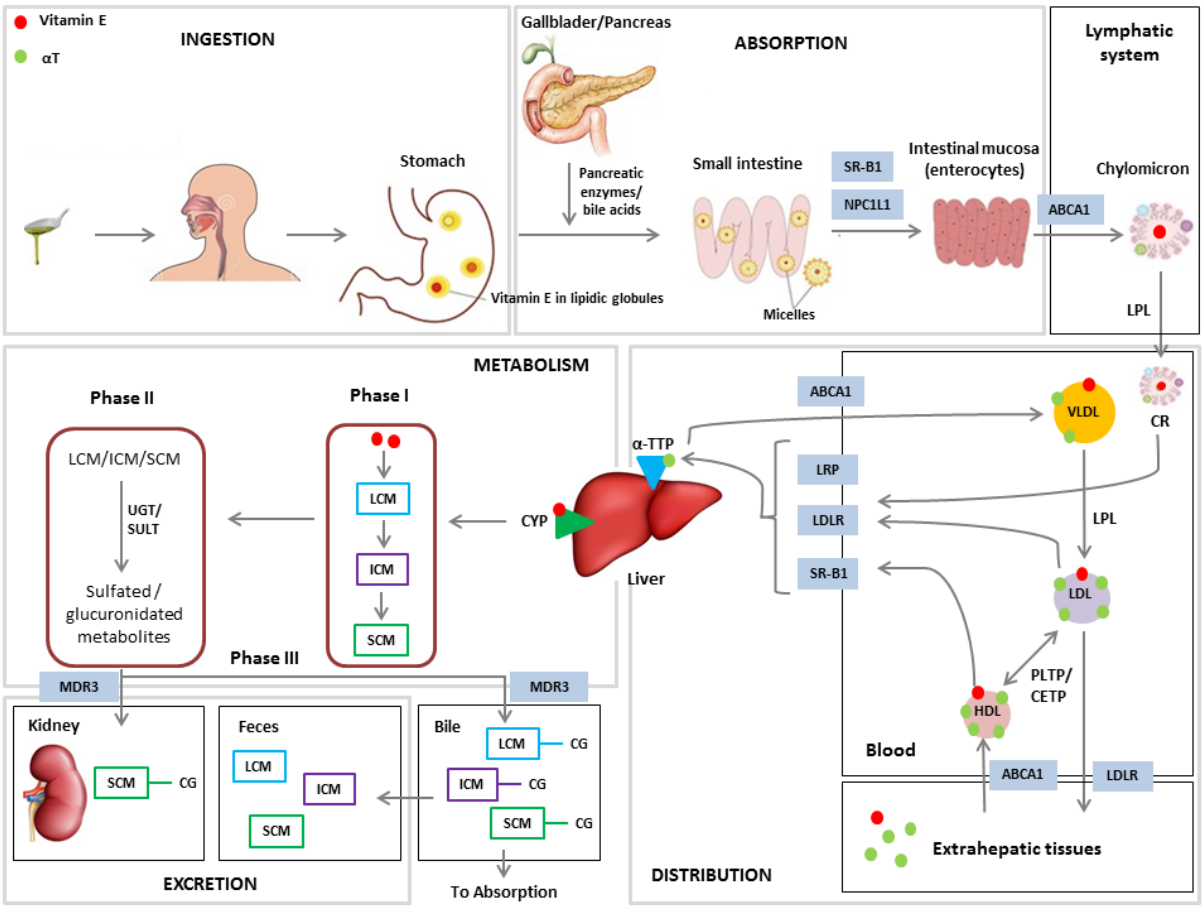


Figure 2. Ingestion, absorption, distribution, metabolism and excretion of vitamin E. After ingestion, vitamin E follows in general the pathway of other lipids. Intestinal absorption starts with the pack of vitamin E into micelles, which are taken up by the enterocytes via receptors. Then vitamin E is incorporated into chylomicrons through an ABCA1 transporter, and transported into the blood circulation for distribution to the liver and extrahepatic tissues. This distribution occurs via chylomicrons remnants and lipoproteins. The vitamin E that enters the liver via receptors undergoes sorting mediated by the α-TTP or metabolic degradation via CYP. α-TTP discriminates in favor of αT, which protects αT from excessive metabolism and excretion, and instead mediate its secretion through an ABCA1 transporter back to bloodstream. Non-αT congeners are preferentially degraded in three phases, following the path of xenobiotic metabolism. Phase I is mediated by the CYP enzymes and produce specific metabolites, which are sulfated and glucuronidated by phase II enzymes. Free and conjugated metabolites are transported to kidney and bile through a phase III transporter; and excreted free or conjugated in urine and feces, or secreted via bile into the intestine for absorption. The figure was modified from [18, 19].

In the liver, all vitamers are degraded following the same catabolic pathway that leads to specific metabolites with an intact chromanol ring and differences in the side-chain. However,

the rate of degradation differs among vitamers due to differences in the chemical structure and nature of vitamin E source [21].

Studies carried out by Sontag and Parker [21] using the CYP4F2 tocopherol ω-hydroxylase, one of the CYP enzymes associated with the initial reaction of vitamin E metabolism, showed that the rate of metabolic degradation of the vitamin E congeners is mediated by two substrate structural features influencing the CYP4F2 activity, first the saturation of the side-chain and, to a lesser extent, the methylation pattern of the chromanol ring, with the desmethyl forms and tocotrienols being preferentially metabolized.

The first step of vitamin E metabolism occurs in the endoplasmic reticulum of the hepatocytes. This ω-hydroxylation reaction, which is mediated by the CYP enzymes CYP3A4 and CYP4F2, results in the formation of the long chain metabolite (LCM) 13'-hydroxychromanol (13'-OH), which subsequently is ω-oxidized by alcohol and aldehyde dehydrogenase to the LCM 13'-carboxychromanol (13'-COOH). The LCM are transported into peroxisomes, probably via ABC transporters, as happens for very long-chain acyl-CoA molecules. Once in the peroxisomes, β-oxidation steps shorten the side-chain, with the elimination of propionyl-CoA or acetyl-CoA and formation of the LCM carboxydimethyldecylhydroxychromanol (CDMDHC or 11'-COOH) and carboxymethyloctylhydroxychromanol (CDMOHC or 9'-COOH). As the further side chain-shortening steps take place in the mitochondria, the import of the LCM into mitochondria might occur through carnithin-acyl-transferase. Three β-oxidation steps take place in the mitochondria, producing the intermediate chain metabolites (ICM) carboxymethylhexylhydroxychromanol (CDMHHC or 7'-COOH) and carboxymethylbutylhydroxychromanol (CMBHC or 5'-COOH), and the catabolic end-products or short-chain metabolites (SCM) carboxyethylhydroxychromanols (CEHC or 3'-COOH) [19]. T3 experience a comparable catabolism as T, even if different LCM and ICM are formed, the resulting end-product is also the CEHC, leading to the conclusion that the side-chain of T3 is saturated before shortening mediated by the reductase and isomerase type-enzymes [22] (Figure 3).

The LCM, ICM and SCM are conjugated by phase II enzymes of xenobiotic metabolism, mainly via UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT), and therefore the metabolites appear either sulfated or glucuronidated. Due to their hydrophilicity, the SCM are excreted via urine, mainly in glucuronidated form. In parallel, all the conjugated or non-conjugated metabolites of T and T3 are secreted via bile into the intestine and later eliminated via feces. In the feces, all the metabolites, T and T3 are excreted, and the LCM is the major metabolite present in percentages over 60%. So far, no specific transport proteins for conjugated or non-conjugated CEHC excretion have been reported, however, the phase III transporters multidrug resistance proteins (MDR3) are proposed as possible mediators in the elimination of the vitamin E metabolites [19] (Figure 2).

αT is the predominant form found in human plasma with concentrations of 22-32 μmol/L, which are much higher than the concentrations found for γT (1,4-4,8 μmol/L) [23-25], βT

(~0,4 µmol/L) [23], δT (~0,3 µmol/L) [23, 24], and αT3, γT3 and δT3 (nearly undetectable, raising concentrations < 1 µmol/L after 8 days supplementation with 250 mg T3) [26].

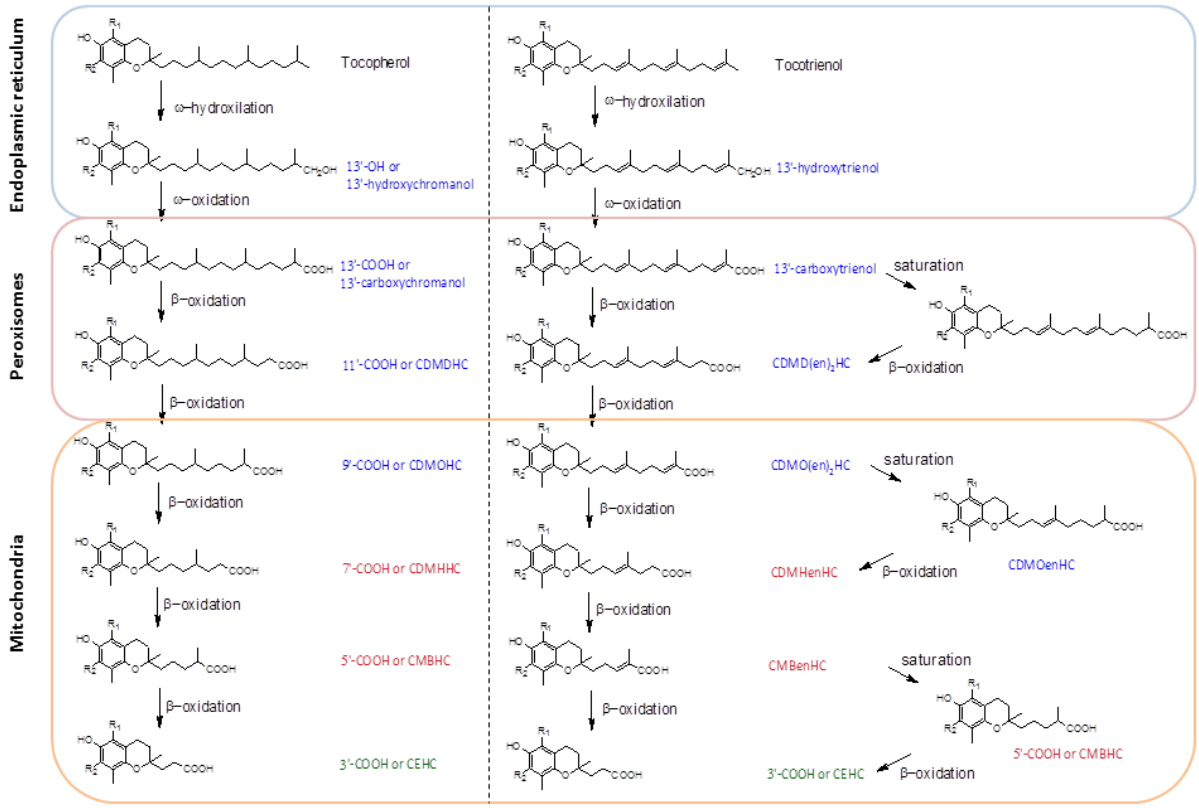


Figure 3. Metabolic pathway of tocopherols and tocotrienols and the intracellular compartmentation of the different reaction steps. The metabolism of vitamin E is initiated with a CYP4F2/CYP3A4-dependent ω-hydroxylation in the endoplasmic reticulum, resulting in the formation of the 13′ hydroxychromanol. Next, a ω-oxidation leads to the formation of 13′ carboxychromanol, which is transported to the peroxisomes. Next, five cycles of β-oxidation shortened the chain and finally results in the formation of the carboxyethylhydroxychromanols (CEHC) in the mitochondria. T3 experienced a comparable metabolism as T, although they differ in the intermediate metabolites, the end product is the same. The figure was modified from [19]. LCM are named in blue, ICM in red and SCM in green. For the meaning of R_1 and R_2, referred to the box displayed inside the Figure 1.

2. α-Tocopherol transfer protein

α-Tocopherol transfer protein (α-TTP) is a cytosolic 32-kDa protein first described in 1975 as a component of rat liver cytoplasm [27]. Years later, α-TTP was purified and characterized from rat liver [28, 29] and its cDNA isolated from human liver [30]. Hepatic α-TTP is required to maintain normal plasma and extrahepatic tissue concentrations of αT, its role starts after vitamin E is delivered into the liver, where α-TTP is able to preferentially binds and selectively retains αT over the others congeners, and furthermore mediates the intracellular

trafficking of αT until its secretion from the liver [31, 32]. Subsequent studies revealed the expression of α-TTP in other tissues as rat brain, spleen, lung, and kidney [33]; in rat uterus [34] and eye retina [35], suggesting that its expression in other organs regulates distinct tissue-specific accumulations of the vitamin E [32], and aims the maintenance of sufficient αT in sensitive tissues, *i.e.* the central nervous system and the developing embryo [20].

A key role of the α-TTP in regulation of vitamin E status was discovered after observation that humans suffering from ataxia with vitamin E deficiency (AVED) carry mutations in the *TTPA* gene that encodes α-TTP. AVED is a primary vitamin E deficiency where the lack of α-TTP leads to an inability to discriminate between different forms of vitamin E, reflected in lower αT plasma concentrations and increased metabolism of αT and excretion of αCEHC. Clinical symptoms of AVED are characterized by a progressive spinocerebellar ataxia, dysarthria, loss of tendon jerks, decreased visual acuity, and impaired vibration sense, among others. Similar phenomena are observed in *Ttpa* knockout mice, were the generated absence of α-TTP produces the same symptoms observed in AVED patients, including primary vitamin E deficiency and spinocerebellar ataxia. Hence, the α-TTP knockout mice is an animal model to study the role of the α-TTP in vitamin E homeostasis [19, 32], or to investigate the specific roles of vitamin E congeners in different tissues, identify genes related with α-T in various diseases and for dissociate the antioxidant functions of the α-T from its other functions [36].

2.1 Structure, active site and affinity to vitamin E

α-TTP is classified as a member of the SEC14-like protein family, characterized for being involved in lipid regulation. This family of proteins possesses a typical CRAL_TRIO lipid-binding domain. The domain structure consists of five parallel β-strands constituting the floor of the binding pocket, a variable number of α-helices and a mobile helical gate at the carboxyl-terminal group that constitutes the entrance of the ligand to the binding pocket [37, 38] (Figure 4).

Crystallographic data showed the ligand-binding pocket of α-TTP mostly formed by hydrophobic amino acids, except for two serine residues and three molecules of water. When αT enters the binding pocket, the aromatic methyl group in 5-position of the chromanol ring fits perfect into a niche formed by the hydrophobic side-chains of the amino acids Ile194, Val191, Leu183, Ile154 and Ala156, establishing strong van der Waals interactions (Figure 4). The other aromatic methyl groups in the 7- and 8-position, as well as the methyl at the 2-position of the chromanol ring, make contacts to hydrophobic residues, Phe, Val, Leu and Ile, inside the cavity; and the phenolic hydroxyl group connects with the backbone carbonyl of a Val residue through a hydrogen bond. The prenyl side-chain folds into a U-shape, leading the methyl groups at 4'- and 8'-position in contact with Ile residues [37].

The relative affinities of the α-TTP for the different vitamin E congeners were reported as: RRR- αT, 100%; βT, 38%; γT, 9%; δT, 2%; SRR- αT, 11% and αT3, 12% [39].

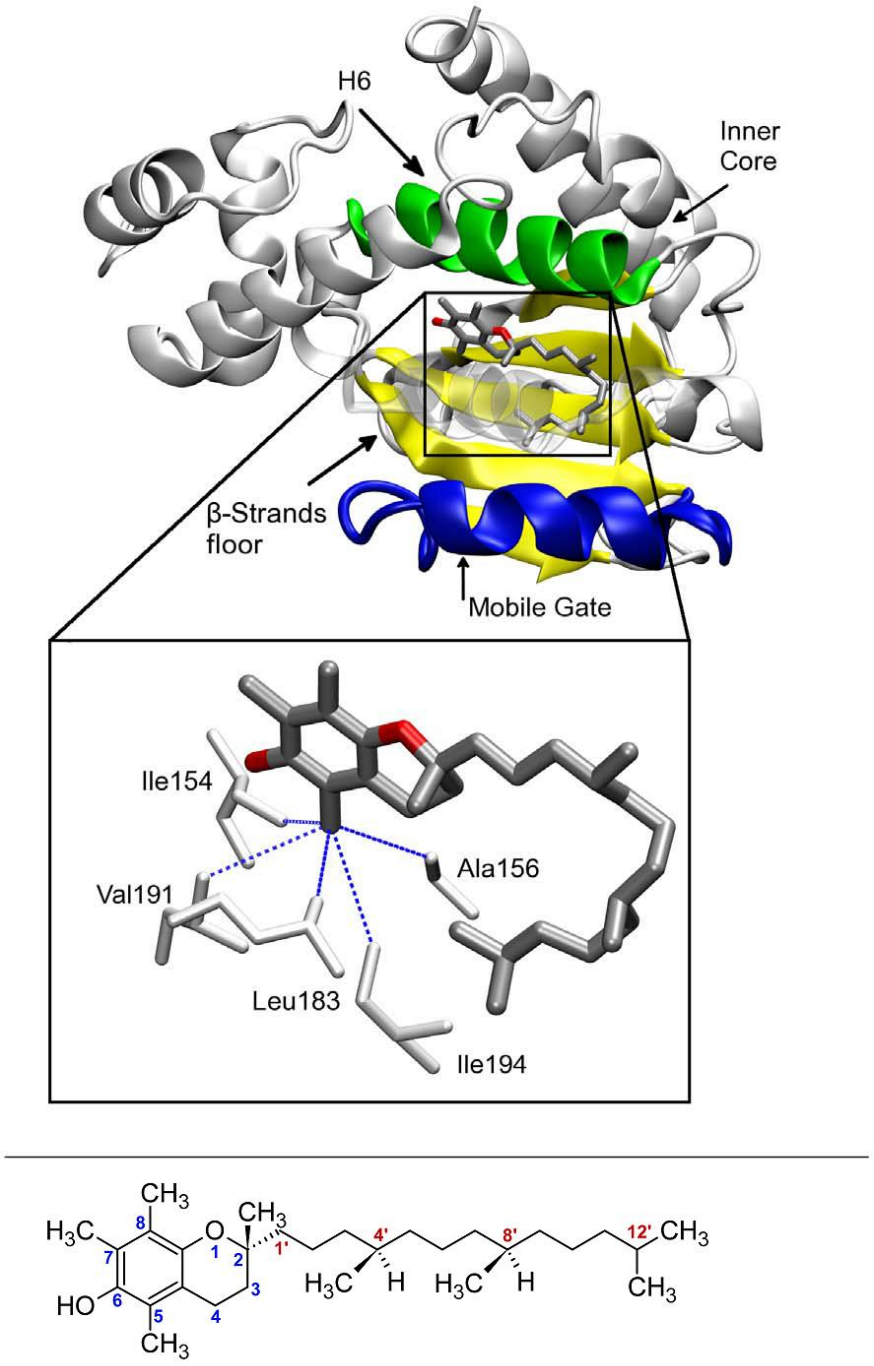


Figure 4. Structure of α-TTP (from X-ray data) bound to αT. *Top panel:* the four faces of the binding pocket are highlighted; the α-helix 6 (H6) in green (top), the β-strands floor of the cavity in yellow (bottom), the mobile helical gate in blue (front) and the inner core of the binding pocket (back). *Inset panel:* 3D rendering of αT bound to α-TTP, where the residues Ile194, Val191, Leu183, Ile154 and Ala156 in van der Waals contacts with the aromatic methyl group in 5-position of the chromanol ring are highlighted in white and the interaction in dashed blue lines. *Bottom panel:* chemical structure of αT. The figure was taken from [38].

The presence of the methyl group in 5-position of the chromanol ring was proposed as the main reason for the selectivity of the α-TTP for αT. It was initially postulated that the absence of one methyl group reduces the surface available for hydrophobic interactions and diminishes the packing density into the binding pocket, and that this may be the reason why

even if the other tocopherols fit into the cavity as well [37], their relative binding affinities for the α-TTP are lower compared to αT [39]. However, more recently was reported that the Ala156 residue, forming part of the hydrophobic niche that surrounds the 5-methyl group, is the critical position behind the selectivity of the α-TTP for αT (Figure 4), since a mutant α-TTP with modification in this residue preferentially binds to γT [38].

Another important feature determining α-TTP binding affinity is the R-configuration in the 2-position of the chromanol ring, as observed in an *in vivo* experiment [40]. This is in agreement with the lower relative binding affinities of the SRR-stereoisomers of αT compared to the RRR-stereoisomers [39]. The position and interactions of the methyl groups of the prenyl side-chain inside the binding pocket were reported not to be a key factor on the α-TTP binding selectivity, most probably due to the fewer packing restraints and higher flexibility of the chain [37].

For the binding and unbinding of αT, the α-TTP undergo conformational changes in its binding pocket. A mobile helical surface segment acts as a lid sealing and opening the binding pocket. In the closed conformation, the hydrophobic residues of the lid are in direct contact with the side-chain of the αT. When the protein get in contact with the membrane, the residues in the positively charged cleft interact with the phosphate groups of the phosphatidylinositol 4,5-bisphosphate (PIP_2). The interaction caused the open of the lid. The acyl chains of the PIP_2 point toward the hydrophobic binding pocket, promoting a lipid exchange reaction in which the PIP_2 in the membrane is exchange for αT. The α-TTP can therefore close the lid. The close conformation exposes a more polar face that facilitates its release from the membrane, followed by a transport inside the cells. This exchange of PIP_2 by αT is reversible and when the two lipids are present in two different liposomes, α-TTP can transfer them in opposite directions [37, 41, 42].

2.2 Role on vitamin E transport and metabolism

In vitro experiments with cultured hepatocytes expressing α-TTP revealed that the protein is not essential for the αT uptake from either HDL or LDL, leading to the conclusion that α-TTP action in the trafficking of αT inside the liver takes place in a later step [43].

Evidence was found that once αT enter the intracellular organelles; it is internalized into vesicles that end up in the endocytic compartment (late endosomes and lysosomes) where α-TTP is localized, before being transported to the plasma membrane [43]. Later was reported that even though α-TTP is normally localized in late endosomes/lysosomes, its intracellular distribution changes in the presence of αT, which induces the protein to re-distribute throughout the cytosol. With the use of α-TTP mutants, was studied how the interactions of the protein with membrane lipids influence its role on trafficking. Results revealed that the hydrophobic surface on α-TTP (residues of Phe, Ile, Val and Met) are required for association of the protein with all membranes; whereas the 5-phosphoinositides in the plasma membrane selectively associate with the α-TTP binding pocket by contact with a positive patch of

residues on α-TTP surface (Lys and Arg), essentials for releasing of αT from the protein binding cavity to the membrane. According with the mutants used, the key residues for both interactions are the Phe169 and the Lys217, respectively [20].

These findings allowed the proposal of a mechanism for the role of α-TTP on αT trafficking in hepatocytes. Lipoproteins containing αT are endocytosed and reach the late endosomes/lysosomes, where the lipoprotein is degraded and αT is released [43]. The α-TTP binds to the recycling endosomes using the hydrophobic residues including Phe169. These endosomes are characterized by the presence of the CD71/transferrin and GTPase Rab8/transferrin receptors, important in the binding of αT in the recycling endosomes. α-TTP binds the αT in its hydrophobic pocket and together with the recycling endosome then translocate to the plasma membrane. α-TTP binds with the PIP_2 through its positively charged residues including Lys217. This interaction causes a conformational change in α-TTP binding space that produces the exchange of αT by PIP_2 (see section 2.1). αT in the plasma membrane exits through an ABCA1 transporter and associates with lipoproteins that deliver it to extrahepatic tissues. α-TTP travels back inside the cell to repeat the cycle. The directionality of the process is leading by the opposite concentration gradients of PIP_2 (high at the plasma membrane) and αT (high at the endocytic vesicles) [20] (Figure 5).

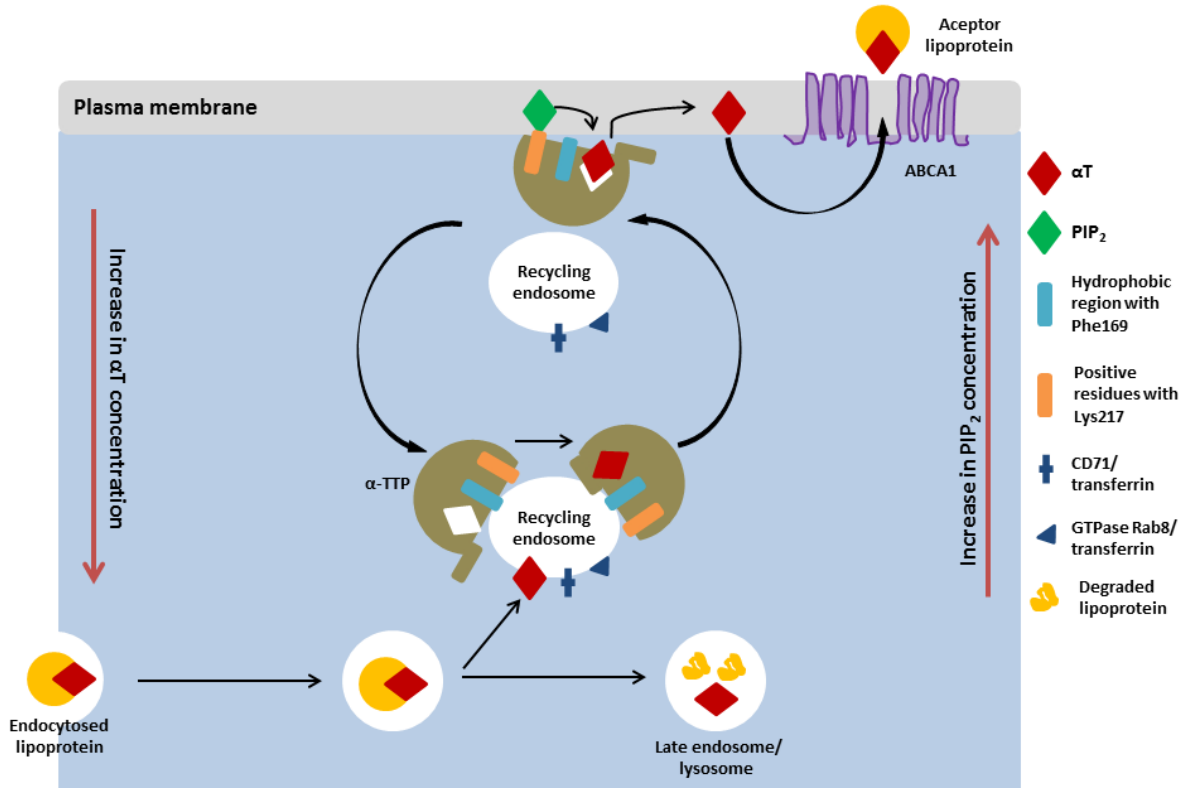


Figure 5. Proposed mechanism underlying α-TTP role on αT trafficking in hepatocytes. αT enters the cell by endocytosis and reaches the recycling endosome and/or is storage in the late endosomes/lysosomes. In the presence of αT, the α-TTP associates with the recycling endosome through its hydrophobic region with Phe169, and then binds αT. The recycling endosome together with the α-TTP and αT then travel to the plasma membrane. α-TTP then associates with the PIP_2 through its positively charged residues with Lys217, and αT is exchange by the PIP_2. Release αT exits the plasma membrane through an ABCA1 transporter and associates with lipoproteins. α-TTP translocate back inside the cell to repeat the cycle. Binding of αT is regulated by the CD71/transferrin and GTPase Rab8/transferrin receptors of the recycling endosome. Directionality of the process is provided by the opposite concentration gradients of PIP_2 and αT. The figure was modified from [20, 41].

In addition to α-TTP acting as a mediator of αT-trafficking inside the liver, previous research suggests that α-TTP might prevent the metabolic degradation of vitamin E congeners. *In vitro* experiments with hepatic α-TTP-expressing cells incubated with αT and/or γT, revealed a reduced γ-CEHC formation in α-TTP-cells, whereas an increase of the metabolite was observed with increasing αT concentration. Furthermore, *in vivo* experiments in wild-type and α-TTP knockout mice fed with equal doses of αT and γT in the absence or presence of sesamin (an inhibitor of vitamin E metabolism), revealed that the inhibition of metabolism increases γT concentrations in tissues, whereas the knockout of α-TTP reduces them [44].

The proposed model, based on these previous findings, suggests that the non-αT congeners, once they enter the liver, might be partly protected from side chain-degradation when bound to α-TTP. However, as the binding affinities of the non-αT congeners are lower than that of αT, the non-αT congeners are displaced from α-TTP binding in the presence of αT and become available for ω-hydroxylation again [44]. However, the above described protective

role of vitamin E from metabolic degradation is unlikely to be the primary role of α-TTP, but more likely a side effect of the binding affinities for several ligands. Furthermore, the metabolism and not α-TTP is primarily responsible for the discrimination between the vitamin E congeners [44], and the primary role of α-TTP seems to be the maintenance of adequate tissue concentrations by facilitating vitamin E secretion from the liver [18].

Curiously, the ability of the different vitamin E congeners to act as ω-hydroxylase substrates are for most of them the inverse of their binding affinities for the α-TTP, suggesting a collaborative role of the two proteins in the vitamin E homeostasis [21]. Besides, experiments using *Drosophila melanogaster*, an insect that naturally lacks of α-TTP expression, showed that it can selectively accumulates αT over the others vitamers by a mechanism involving CYP tocopherol ω-hydroxylase, respalding the crucial role of metabolism for the discrimination between vitamers and a separate evolution of the two hepatic mechanisms involved in vitamin E homeostasis [45].

This differences in rate degradation of the vitamin E congeners are supported by *in vivo* results, where after supplementation with a mixture of the different congeners, they appear in human plasma with half-lives in an increasing order of δT3 (2,3 h) < αT3 (4,3 h) ≤ γT3 (4,4 h) < γT (8-16 h) < αT (45-60 h) [19]. Besides, a study on the cytotoxicities of the different vitamin E congeners in murine macrophages and the elimination rates on hepatocytes, reported an inverse correlation between both parameters, underlying a physiological reason behind the different rates and degradation of the congeners [46].

3. Functions and health implications of vitamin E

Soon after its discovery, in 1931 the antioxidant activity of αT was discovered and proposed as its major biological function [47]. Later on, other biological functions of the vitamin E, non-related with its antioxidant capacity, were discovered. The role of the vitamin E has been recognized on cellular signaling, gene regulation and catalytic activity of several enzymes [48, 49].

3.1 Antioxidant function of vitamin E: lipid-soluble radical scavenger

Radical scavenging is one of the important functions of antioxidants. Free radicals are chemical species that contain one or more unpaired electrons. Several free radicals are constantly produced in the body as a result of physiological processes, as examples, reactive oxygen species (superoxide, hydroperoxide, peroxyl, hydroxyl, alcoxyl, aryloxyl), reactive nitrogen species (nitric oxide, nitrogen dioxide, peroxynitrites) and carbon centered radicals. Free radicals attack macromolecules such as lipids, proteins, carbohydrates and DNA; and start self-propagating radical chain reactions, which eventually damage the cells. Besides the macromolecules, these free radicals can react with other free radicals or antioxidants, to form

stable or much less reactive species. When the antioxidants in the body are unable to contain the free radicals produced, thus oxidative stress is induced [50].

There is well-documented evidence *in vitro* and some evidence *in vivo* that support the function of vitamin E as a peroxyl radical scavenger capable of preventing lipid peroxidation in cellular systems. In the presence of lipid peroxyl radicals, all vitamin E congeners break chain reactions, forming relatively stable lipid peroxides and tocopheroxyl radicals. Tocopheroxyl radicals are reduced back to the original vitamin E congeners by ascorbate (vitamin C) and by this means, prevent any potential pro-oxidant effect of the tocopheroxyl radicals on e.g. lipoproteins. Ascorbyl radical is regenerated with the support of the nicotinamide adenine dinucleotide (NADH)-dependent reductases (e.g. glutathione, dihydrolipoic acid, thioredoxin) [18, 48-50].

The peroxyl radical scavenger characteristic of the vitamin E congeners is due to their ability to donate hydrogen from the phenolic group on the chromanol ring. Since the phenolic moiety is similar among the vitamin E congeners, all are considered potent antioxidants [51]. However, *in vitro*, the reactivity of the congeners has been reported in the order: $\alpha T > \gamma T \approx \beta T > \delta T$. These differences have been attributed to the hydrogen-donating ability of the congeners, which increases with the methylation of the chromanol ring [52].

T3 are reported to be more efficient peroxyl radical scavengers *in vitro* than T in liposomal membranes; presumably because the unsaturated side chain allows for a more uniform penetration, distribution and mobility in the lipid layers of the cell membranes. Thus, lead to a more efficient interaction with peroxyl radicals and a subsequent faster recycling by the co-antioxidants in the membranes [12, 13, 51]. However, *in vivo*, the few studies conducted concluded that T3 are equally potent as T in terms of their antioxidant capacity. The lower bioavailability of T3 compared to the T, would lead to a reduced antioxidant activity *in vivo*, mostly because of their lower plasma and tissue concentrations [12].

In addition to its function quenching reactive oxygen species, the vitamin E congeners can also trap reactive nitrogen species, which are mostly formed endogenously by phagocytes and macrophages during inflammatory processes. δT and γT have been reported to be superior to αT in detoxifying nitrite oxide and peroxynitrites from membranes. This higher capacity of δT and γT seems to be related with the un-methylated 5-position at the chromanol ring that allows the trapping of the nitrogen species. αT possesses a methyl group at the 5-position and can therefore not efficiently trap reactive nitrogen species [49, 51, 53].

3.2 Non-antioxidant functions of vitamin E

Roles of the vitamin E in processes of cellular signaling, gene regulation and enzyme activity have been recognized of importance in the past years. Recently, their long chain metabolites also appeared as molecules with specific and distinct biological activities. In the next paragraphs are summarized some non-antioxidants functions documented for the tocopherols, tocotrienols and long chain metabolites.

3.2.1 Non-antioxidant function of tocopherols

αT, specifically, inhibits smooth muscle cells proliferation by regulation of the protein kinase C (PKC) activity. αT inhibits the PKC activity by inducing its dephosphorylation through the activation of protein phosphatase 2A (PP_2A) [18, 48, 49]. The PKC inhibition by αT have been linked with improve in pathophysiological conditions such as inflammation, lipid deposition in aorta, diabetic vascular complications and platelet aggregation [48].

αT also decreases the release of the pro-inflammatory cytokine interleukin-1β (IL-1β) via the inhibition of the 5-lipoxygenase (5-LOX) catalyzed reaction [49]. Same function was reported for δT and γT in lung epithelial cells. αT, δT and γT also inhibited the production of the pro-inflammatory prostanglandin E2 (PGE_2) via suppression of the cyclooxygenase-2 (COX-2) activity in macrophages. Supplementation with γT or γT-rich mixed T was reported to decrease the formation of PGE_2 via COX-2, and leukotriene B4 (LTB_4) via 5-LOX inhibitions in models of acute allergic and colitis inflammation. Since pro-inflammatory mediators from COX-2 and 5-LOX pathways are recognized to contribute to cancer development, tocopherols have been proposed as potentially chemoprevention agents [51].

The regulation of gene transcription by vitamin E has been reported for various proteins. One of the earliest evidences was the inhibition of xanthine oxidase activity and of its production of reactive oxygen species [48]. αT up-regulated the expression of α-tropomyosin leading to reduced cell proliferation. Furthermore, αT inhibited the age-dependent increase of collagenase in human skin fibroblasts; and the proliferation of rat A7r5 smooth muscle cells. At a transcriptional level, αT down-regulated the expression of the scavenger receptors class A (SR-A) in macrophages and cluster of differentiation 36 (CD36) in smooth muscle cells [48, 49]. The down-regulatory effect on CD36 was also observed in the liver, where αT prevented diet-induced lipid accumulation, since the hepatic CD36 act as the major fatty acid transporter [54]. αT can bind to the family of tocopherol-associated proteins (TAP) and forms a complex capable of inhibiting the expression of the phosphoinositide 3-kinase γ (PI3Kγ). In *in vitro* studies, this inhibition has been shown to suppress prostate cancer cell growth [48].

αT and δT equally induce the expression of the hepatic messenger ribonucleic acid (mRNA) for the α-tocopherol transfer protein (α-TTP) [49]. Supplementation with a sufficient dose of αT/γT-mix indirectly down-regulated the expression of the mRNA for the hemeoxygenase-1 (HO-1), contributing to a decrease in the oxidative stress in cells [55]. δT and γT up-regulated the expression of the peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor that have been demonstrated as important for inhibition of cell proliferation and induction of apoptosis in breast cancer. Besides, δT and γT down-regulated estrogen receptor α (ERα) signaling, which may play a role in the inhibition of mammary tumorigenesis [53].

3.2.2 Non-antioxidant function of tocotrienols

T3, especially αT3, have been associated with neuroprotective properties, among other reasons, due to their role as inhibitor of the 12-lipoxygenase (12-LOX) pathway and the signaling of the nuclear factor κB (NF-κB) [12]. 12-LOX predominates in the brain and catalyzes the oxidation of arachidonic acid to 12-hydroperoxy-eicosatetraenoic acid (12-

HPETE), a precursor in the synthesis of pro-inflammatory leukotrienes. 12-HPETE is thought to be neurotoxic and has been also implicated in the pathogenesis of Alzheimer's disease due to its role as retrograde messenger in learning and memory [12, 13].

αT3, γT3 and δT3 up-regulated the peroxisome proliferator-activated receptors α, γ and δ (PPARα, PPARγ and PPARδ). The activation of these receptors have been associated with improved body glucose utilization and insulin sensitivity of diabetic mice, highlighting a role of T3 as mediators of target genes against diabetes [11].

As well, the effectivity of T3 lowering serum total and LDL-cholesterol levels has been reported. T3 reduces the endogenous synthesis of cholesterol by inhibition of enzymatic activity of the 3-hydroxy-3-methylglutaryl-coenzime A (HMG-CoA) reductase through a post-transcriptional mechanism. This reduction in the cholesterol biosynthesis may thus protects against hypercholesterolemia and metabolic syndrome, which are considered risk factors for neurodegenerative diseases [12, 13].

3.2.3 Non-antioxidant function of long chain metabolites

Recently, the long chain metabolites (LCM) of vitamin E metabolism, particularly α-13'-hydroxychromanol (α-13'-OH) and α-13'-carboxychromanol (α-13'-COOH) from the catabolism of αT, have been proposed as a new class of regulatory metabolites with specific and distinct activity compared to the precursor αT. In human macrophages, α-LCMs decreased the uptake of oxidized low-density lipoproteins (LDL) and oxidized LDL-induced lipid accumulation, due to a decreasing in phagocytic activity. Besides, α-LCMs act as anti-inflammatory agents by blocking the lipopolysaccharide (LPS)-induced up-regulation of the expression of nitric oxide synthase, COX-2 and IL-1β [18].

Recently has been reported a biological activity of α-13'-COOH that is not shared by αT, nor by γT, αT3 or the corresponding SCM. α-13'-COOH activated the nuclear receptor pregnane X receptor (PXR), which induces the protein expression and transport activity of the P-glycoprotein on LS 180 intestinal cells [56].

4. Aims of the doctoral dissertation

Since its discovery as an essential nutrient for female fertility in rodents in 1922, most studies have focused on αT and comparably little is known about the intracellular trafficking and tissue distribution of non-αT congeners and novel vitamin E derivatives, such as the T1, and their sources.

The first aim of the present thesis was to characterize food sources of non-αT forms of vitamin E. To this end, the vitamin E composition of Costa Rican palm oil from six varieties (three *Elaeis Guineensis*, two *Elaeis Oleifera* and one hybrid OxG genotype) and the impact of oil processing (mechanical vs. chemical extraction) on the vitamin E profiles were investigated for the first time. The hypothesis that tocotrienols are the major compounds in the palm oils and that the vitamin E concentrations differ between genotypes and extraction methods was tested **(Chapter 2)**.

The second aim of the thesis was to elucidate, for the first time, structure-activity relationships in the cellular uptake and intracellular distribution of αT, αT3, γT, and γT3 in cultured hepatocytes expressing α-TTP or not. The hypothesis that differences on the chemical structure between T and T3 and the presence of α-TTP are the key factors behind the intracellular trafficking was tested **(Chapter 3)**.

Finally, because α-tocomonoenol (αT1) was detected in palm oil, and encouraged by the novelty of this congener and the lack of information regarding its behavior in vivo, the third aim of the thesis was to study, for the first time, the tissue distribution of αT1 compared to αT in a rodent model with homozygous α-TTP knockout ($TTP^{-/-}$) and α-TTP-expressing wild type ($TTP^{+/+}$) mice. Using this knockout-mouse model, it was possible to explore the impact of α-TTP on the tissue distribution of αT1. The hypothesis that αT1 differs in tissue distribution from αT was tested **(Chapter 4)**.

Chapter 2

Tocopherols, Tocomonoenols, and Tocotrienols in Oils of Costa Rican Palm Fruits: A comparison between Six Varieties and Chemical versus Mechanical Extraction

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Tocopherols, Tocomonoenols, and Tocotrienols in Oils of Costa Rican Palm Fruits: A Comparison between Six Varieties and Chemical versus Mechanical Extraction

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Supporting Information

ABSTRACT: Palm oil is one of the richest sources of tocotrienols and may contain other non-tocopherol vitamin E congeners. The vitamin E profiles of fully ripened fruit mesocarp of three *Elaeis guineensis*, two *Elaeis oleifera*, and one hybrid O × G palm fruit genotypes from Costa Rica were analyzed by high-performance liquid chromatography with fluorescence detection and gas chromatography−mass spectrometry after mechanical extraction by a screw press and chemical extraction with hexane. γ-Tocotrienol, α-tocotrienol, and α-tocopherol were the most abundant tocochromanols, while other tocopherols (β-tocopherol, γ-tocopherol, and δ-tocopherol) and α-tocomonoenol were detected at minor concentrations. Significant differences in vitamin E profiles between genotypes were observed, and the variety *E. oleifera* Quepos (CB9204) had by far the highest content of total tocotrienols (890 μg/g of oil) and total vitamin E (892 μg/g of oil). Chemical extraction with hexane afforded up to 2.5-fold higher vitamin E yields than screw press extraction. α-Tocomonoenol co-eluted with γ-tocopherol in reversed-phase high-performance liquid chromatography analyses and is a possible source of error in the quantification of γ-tocopherol in foods.

KEYWORDS: *Elaeis guineensis, Elaeis oleifera, hybrid O × G, oil extraction, chromatography, palm fruit oil, pumpkin seed oil, palm oil extraction byproducts, vitamin E*

INTRODUCTION

Vitamin E is a generic term for a group of micronutrients comprising eight naturally occurring lipid-soluble compounds with a chromanol ring structure bound to a saturated (tocopherols) or 3-fold unsaturated (tocotrienols) 16-carbon side chain (Figure 1). The four tocopherols and four tocotrienols are assigned the prefixes α, β, γ, or δ based on the number and position of methyl groups substituted at the chromanol ring (Figure 1). Vitamin E is exclusively synthesized by photosynthetic organisms and, thus, present in plant foods, particularly vegetable oils.[1]

In addition to the well-known and -studied tocopherols (T) and tocotrienols (T3), a group of minor vitamin E compounds with a single double bond at carbon 11′ [tocomonoenols (T1)] has been detected in plants and plant foods, such as α-tocomonoenol in palm oil (*Elaeis* sp.),[2−7] pumpkin seed oil (*Cucurbita pepo* L.),[8] and sunflower oil (*Helianthus annuus*),[9] γ-tocomonoenol in pumpkin seed oil,[8] δ-tocomonoenol in kiwi (*Actinidia chinensis*),[10] and β-, γ-, and δ-tocomonoenol in leaves of *Kalanchoe daigremontiana* and *Phaseolus coccineus*.[11]

In Costa Rica, two *Elaeis* species have industrial importance in the palm oil market, *Elaeis guineensis* from Central and West Africa and *Elaeis oleifera* from Central and South America, and in the past few years, hybrid varieties have been developed. Interspecific hybridization between *E. guineensis* and *E. oleifera* aimed at achieving oil yields as high as the oil from *E. guineensis* combined with the reduced tree height, resistance to diseases, and superior nutritional value (higher content of vitamin E, carotenoids, and unsaturated fatty acids) of the oil from *E. oleifera*.[4,12,13]

Palm oil is extracted from the ripened mesocarp of the fruits of the palm oil tree (family Arecaceae, genus *Elaeis*)[13] and a rich source for tocotrienols, which make up ca. 80% of its total vitamin E content,[1] and a source for tocomonoenols.[2−7] The two most common extraction procedures involve either mechanical pressing with a screw or hydraulic press or the extraction of the oil with organic solvents, such as hexane, petroleum ether, or pentane.[13,14] It is not known how these two different extraction processes differ regarding their total vitamin E yield and if the extraction yields of individual vitamin E congeners, particularly the minor compounds, such as T1, may differ from the major congeners.

We thus aimed to determine the complete vitamin E profiles of palm oils extracted mechanically or with hexane from three *E. guineensis*, two *E. oleifera*, and one hybrid O × G genotypes.

MATERIALS AND METHODS

Plant Material. Fully ripe fruits of three *E. guineensis*, two *E. oleifera*, and one hybrid O × G genotypes were obtained from ASD Costa Rica, Grupo Numar (Coto 47, Puntarenas, Costa Rica; latitude, longitude, and altitude, 8.60088025, −82.94484615, and 20 m over sea

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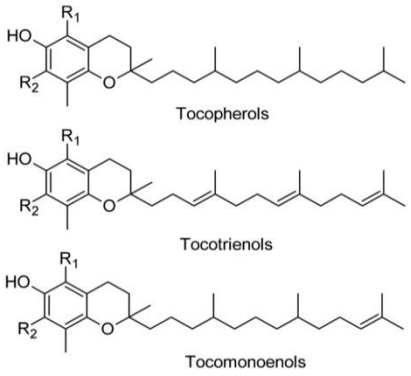

Congener	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 1. Chemical structures and methylation patterns of tocopherols, tocotrienols, and tocomonoenols.

level, respectively). *E. guineensis* genotypes were two mother lines 'Deli Dami' from Papua New Guinea (age of plant, 12 years) and 'Tanzania' from Tanzania (age of plant, 13 years) and the commercial line 'Deli × Nigeria' that is a cross-link of a *E. guineensis* mother 'Deli Dami' from Papua New Guinea with a *E. guineensis* father 'Nigeria' from Ghana (age of plant, 11 years). *E. oleifera* genotypes were two mother lines 'Quepos' from Costa Rica (age of plant, 25 years) and 'Manaos' from Brasil (age of plant, 8 years). The hybrid genotype O × G is a cross of an *E. oleifera* mother 'Manaos' from Brasil with a composed hybrid father (age of plant, 8 years). The composed hybrid father is a mixture of 6% genes of *E. oleifera* 'Quepos' from Costa Rica with 94% genes of different *E. guineensis* genotypes. In the hybrid O × G, each parent provided 50% genes, where the composed hybrid father strictly provides 3% genes of *E. oleifera* 'Quepos' from Costa Rica and 47% genes of different *E. guineensis* genotypes. The genotypes have a six-digit experiment code designated by the breeders of ASD Costa Rica to facilitate exact identification. For the purpose of this study, *Elaeis* genotypes were identified using their name followed by their experiment code (e.g., Deli Dami CB087F; see the Supporting Information).

Palm Oil Extraction. Palm oil was extracted from palm fruits that were previously removed by hand from freshly harvested palm fruit bunches. Palm fruits were sterilized with an autoclave (Steam Steriliser Varioklav HP Medizintechnik, Oberschleißheim, Germany) at 130 °C for 1 h, allowed to cool, and stored at 4 °C until the extraction processes. As far as possible, the temperatures during the extraction processes were maintained below 40 °C to avoid thermal degradation of vitamin E.

Screw Press Extraction. The palm oil extraction with a screw press was based on work published by Poku[14] and Adetola et al.[15] Palm fruits were digested by magnetically stirring the fruit with water at 40 °C for 10 min. Then, the seed was manually removed from the peel and mesocarp and discarded. The pre-digested peel and pulp (100–120 g) were pressed with a screw press (CA59G, IBG Monfort Oekotec, Mönchengladbach, Germany), and the nozzle was preheated for 5 min with an electric ring before the first and third extractions. Three consecutive exhaustive extractions at screw speeds of 40.0, 34.6, and 28.0 rpm and with decreasing nozzle diameters (6, 5, and 4 mm, respectively) were performed, and the residual cake was recirculated during the whole extraction. To separate the extracted oil from fibrous material and non-oily solids, the crude oil in a centrifugation tube was warmed in a water bath at 40 °C for 10 min and then centrifuged twice at 1690*g* at 20 °C for 5 min. The upper oil layers were transferred to and combined in a fresh flask. The extracted oils were stored at 4 °C in a glass bottle wrapped with aluminum foil to prevent light exposure.

Additional experiments with two representative genotypes (Deli Dami CB087F and Quepos CB9204) were performed to investigate the vitamin E profiles in the resulting palm oils in comparison to the residual press cake and centrifugation byproduct to better understand the differences in extraction efficiencies.

Chemical Extraction. The chemical extraction protocol was based on Prada et al.[16] and Morcillo et al.[17] The seed and peel of each fruit were removed by hand from the mesocarp, which was dried in an oven at 70 °C for 6 h and then ground to a powder with mortar and pestle. The ground mesocarp was mixed with hexane (10:100 by vol) and stirred for 30 min at room temperature. The solid residue was removed by vacuum filtration, and the supernatant was dried in a vacuum rotary evaporator (Rotavapor R-100, Büchi Labortechnik, Essen, Germany) at a maximum temperature of 30 °C to remove hexane. The crude extracted oils were stored at 4 °C in a glass bottle wrapped with aluminum foil to prevent light exposure. Additional experiments with two representative genotypes (Deli Dami CB087F and Quepos CB9204) were performed to investigate the vitamin E profiles in the resulting palm oils in comparison to the residual peel to better understand the differences in extraction efficiencies.

Chemicals and Stock Solutions. All chemicals used were of the highest purity and purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany) or Merck (Darmstadt, Germany). Methanol was high-performance liquid chromatography (HPLC) grade, and all water used was deionized and filtered water (Millipore, Billerica, MA, U.S.A.). Individual stock solutions (10 mmol/L) of authentic *α*-, *β*-, *γ*-, or *δ*-tocopherol and -tocotrienol (Sigma-Aldrich, minimum of ≥95.5% pure) were prepared in ethanol, and concentrations were confirmed photometrically. Identical aliquots of the eight individual stock solutions were combined to prepare a standard solution containing 125 μmol/L of each compound. Pumpkin seed oil (100% pure, PureNature, Austria) was purchased from a local supermarket in Stuttgart (Germany).

HPLC Analysis of Vitamin E. Tocopherols and tocotrienols were extracted and quantified as previously described.[18] In brief, six replicate samples were taken from each palm or pumpkin oil sample, and three replicates were taken from the palm oil extraction byproduct experiments. A total of 20 mg was weighed into a glass tube with a screw cap, and 2 mL of 1% ascorbic acid in ethanol (w/v), 900 μL of deionized water, and 600 μL of saturated potassium hydroxide were added. Samples were saponified at 70 °C in a shaking water bath for 30 min and then cooled on ice. Ethanolic butylated hydroxytoluene (25 μL, 1 mg of butylated hydroxytoluene/mL of ethanol), 1 mL of deionized water, 600 μL of glacial acetic acid, and 2 mL of *n*-hexane were added, and the glass tubes were closed and mixed for 1 min by mechanical inversion (Bio RS-24 mini-rotator) and centrifuged (188*g* at 4 °C for 3 min) to facilitate phase separation. An aliquot of the supernatant (1.5 mL) was transferred to a fresh glass tube. The extraction was repeated with 2 mL of *n*-hexane and transfer of 1.5 mL of supernatant, and a third extraction was performed with 2 mL of *n*-hexane and a transfer of 2 mL of supernatant. All supernatants were combined in a glass tube and dried under vacuum with a centrifugal evaporator (RVC 2-25 CD Plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). The dried residues were resuspended in 250 μL of ethanol, vortex-mixed, transferred to 0.5 mL microcentrifuge tubes, and centrifuged at 17000*g* for 1 min, and

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Table 1. Mean Concentrations (±SD; n = 6; All in μg/g) of Tocotrienols, Tocopherols, and α-Tocomonoenol in Palm Oils from *Elaeis* sp. Genotypes Extracted with Screw Press Extraction[a,b]

	Concentration in palm oil (μg/g)					
Compound	Tanzania CB063B	Deli Dami CB087F	Deli × Nigeria CB082C	Quepos CB9204	Manaos CB1201	Hybrid O × G CB127B
αT	7.7 ± 0.4 a	12.1 ± 2.0 b	9.4 ± 1.3 a	0.91 ± 0.12 c	0.090 ± 0.079 c	5.1 ± 1.1 d
βT	1.0 ± 0.1 a	0.57 ± 0.11 b	0.52 ± 0.04 b	0.047 ± 0.035 c	nd[c]	0.15 ± 0.02 c
γT	0.58 ± 0.02 a	0.36 ± 0.11 b	0.34 ± 0.10 b	0.16 ± 0.09 c	nd	0.27 ± 0.08 bc
δT	0.23 ± 0.25	nd	nd	nd	nd	nd
αT1[d]	2.7 ± 0.2 a	0.078 ± 0.079 b	0.677 ± 0.099 c	0.20 ± 0.12 b	0.18 ± 0.25 b	0.59 ± 0.11 c
αT3	46.9 ± 2.7 ab	26.5 ± 4.6 cd	61.7 ± 8.3 e	35.8 ± 5.5 ac	21.1 ± 3.6 d	59.4 ± 14.0 be
βT3	5.8 ± 0.4 a	1.9 ± 0.4 b	8.6 ± 0.5 c	0.23 ± 0.15 d	0.22 ± 0.12 d	1.1 ± 0.2 e
γT3	70.8 ± 6.5 a	98.1 ± 13.4 a	90.5 ± 20.0 a	559.0 ± 37.3 b	211.7 ± 26.9 c	146.0 ± 19.4 d
δT3	27.5 ± 0.7 a	24.1 ± 1.9 b	38.3 ± 1.5 c	27.1 ± 1.1 a	20.7 ± 1.5 d	9.4 ± 0.8 e
total T	9.5 ± 0.5 a	13.0 ± 2.0 b	10.3 ± 1.3 a	1.1 ± 0.2 c	0.090 ± 0.079 c	5.5 ± 1.2 d
total T3	151.1 ± 9.2 a	150.5 ± 19.2 a	199.0 ± 28.5 ab	622.2 ± 43.2 c	253.7 ± 28.1 d	215.9 ± 33.2 bd
total VE	163.3 ± 9.7 a	163.6 ± 20.6 a	210.0 ± 29.7 ab	623.5 ± 43.5 c	253.9 ± 28.2 b	222.0 ± 34.5 b

[a]Comparison of means using Tukey's multiple comparison test. Values within a row not sharing a common letter are significantly different at $p < 0.05$. [b]Vitamin E congeners differ in molecular weight, and thus, similar weights do not represent similar numbers of molecules. [c]nd = not detected. [d]Expressed as α-tocopherol equivalents.

Table 2. Mean Concentrations (±SD; n = 6; All in μg/g) of Tocotrienols, Tocopherols, and α-Tocomonoenol in Palm Oils from *Elaeis* sp. Genotypes Extracted with Chemical Extraction[a,b]

	Concentration in palm oil (μg/g)					
Compound	Tanzania CB063B	Deli Dami CB087F	Deli × Nigeria CB082C	Quepos CB9204	Manaos CB1201	Hybrid O × G CB127B
αT	12.2 ± 8.2 a	16.7 ± 4.6 a	19.1 ± 3.1 a	1.4 ± 1.0 b	1.5 ± 0.9 b	4.2 ± 1.2 b
βT	0.56 ± 0.28 a	0.30 ± 0.07 bc	0.51 ± 0.08 ab	nd[c]	0.11 ± 0.09 c	nd
γT	nd	0.086 ± 0.099 a	0.48 ± 0.13 b	nd	0.012 ± 0.029 a	nd
δT	0.08 ± 0.12 a	0.26 ± 0.20 a	0.16 ± 0.25 a	0.30 ± 0.24 a	0.11 ± 0.18 a	0.17 ± 0.27 a
αT1[d]	3.0 ± 2.6 a	nd	0.85 ± 0.68 b	0.42 ± 0.22 b	0.48 ± 0.33 b	1.4 ± 0.6 ab
αT3	85.7 ± 36.5 a	41.2 ± 10.1 b	126.1 ± 12.0 c	29.2 ± 10.3 b	37.1 ± 11.8 b	101.9 ± 22.2 ac
βT3	12.0 ± 2.3 a	3.5 ± 0.7 b	19.4 ± 0.8 c	0.15 ± 0.37 d	1.1 ± 0.3 d	3.0 ± 0.3 b
γT3	201.7 ± 37.9 a	237.4 ± 102.5 ab	269.6 ± 11.6 ab	822.2 ± 207.0 c	444.2 ± 178.5 b	343.3 ± 139.4 ab
δT3	54.5 ± 4.9 a	33.1 ± 7.5 b	89.0 ± 3.6 c	38.7 ± 7.7 b	42.1 ± 4.9 b	17.9 ± 1.4 d
total T	12.8 ± 8.0 a	17.4 ± 4.7 a	20.3 ± 3.1 a	1.7 ± 1.1 b	1.8 ± 0.8 b	4.4 ± 1.3 b
total T3	354.0 ± 77.6 a	315.2 ± 116.2 a	504.2 ± 16.9 a	890.3 ± 219.6 b	524.6 ± 180.8 a	466.1 ± 138.4 a
total VE	369.8 ± 83.0 a	332.6 ± 119.6 a	525.3 ± 18.8 a	892.4 ± 220.4 b	526.9 ± 181.1 a	471.9 ± 139.1 a

[a]Comparison of means using Tukey's multiple comparison test. Values within a row not sharing a common letter are significantly different at $p < 0.05$. [b]Vitamin E congeners differ in molecular weight, and thus, similar weights do not represent similar numbers of molecules. [c]nd = not detected. [d]Expressed as α-tocopherol equivalents.

the liquid phase was transferred to amber HPLC vials. A total of 15 μL of sample was injected into Shimadzu HPLC (system controller SCL-10A$_{VP}$, two pumps LC-10AT, autoinjector SIL-10AD$_{VP}$, column oven CTO-10AS$_{VP}$, and fluorescence detector RF-10A$_{XL}$, Shimadzu, Japan). Tocopherols and tocotrienols were separated on a Phenomenex Kinetex PFP column (2.6 μm particle size, 150 × 4.6 mm) maintained at 40 °C, using methanol/water (80:20, v/v, eluent A) and methanol (eluent B) at a flow rate of 0.8 mL/min. The elution gradient was as follows: isocratic 100% A for 15 min, from 100 to 50% A in 29.5 min, from 50 to 0% A up to 30 min, isocratic 0% A until 34 min, going back to 100% A in 0.01 min, and running isocratic at 100% A until 60 min total run. The fluorescence detector was operated at excitation/emission wavelengths of 296/325 nm, and peaks were recorded and integrated using LabSolutions software (version 5.82, Shimadzu Corporation) and identified and quantified using authentic tocopherol and tocotrienol standards (Sigma-Aldrich). Unknown peaks were collected manually from the detector immediately after visualization of the peak on the chromatogram and analyzed by gas chromatography–mass spectrometry (GC–MS) (see below).

Trimethylsilylation and GC–MS Analysis of Tocomonoenols. Crude palm oil or pumpkin seed oil (2 mg) and isolated fractions of unknown peaks collected from HPLC (see above) were dried under a gentle stream of nitrogen in a heating block. Then, 25 μL of pyridine and 50 μL of silylating reagent [*N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)/trimethylchlorosilane (TMCS), 99:1, v/v] were added. The vial was closed and heated at 70 °C for 30 min. Afterward, the silylating agent was removed by means of a gentle stream of nitrogen. The residue was redissolved in *n*-hexane (palm and pumpkin seed oil in 1 mL, isolated peak fractions in 200 μL of *n*-hexane) and subjected to GC–MS analysis.

Crude palm oil and pumpkin oil (after trimethylsilylation) were analyzed using a 6890/5973 GC–MS system (Hewlett-Packard/Agilent, Waldbronn, Germany, GC–MS system 1) equipped with a cool-on-column inlet as described recently in more detail.[19] A slightly different temperature program was used: After 1 min at 60 °C, the temperature was raised at 10 °C/min to 250 °C (hold time of 5 min), then at 5 °C/min to 300 °C, and finally at 30 °C/min to 350 °C (hold time of 7 min). The temperature of the transfer line, ion source, and quadrupole was set at 350, 230, and 150 °C, respectively. The ions m/z 209.1, 217.1, 223.1, 237.1, 357.3, 468.5, 472.5, 474.5, 482.5, 486.5, 488.5, 496.5, 500.5, and 502.5 were recorded in selected ion monitoring (SIM) mode after a solvent delay of 15 min with a dwell time of 30 ms.

Isolated HPLC fractions (after trimethylsilylation) were analyzed using another 6890/5973N GC–MS system (Hewlett-Packard/Agilent, Waldbronn, Germany, GC–MS system 2) with conditions

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identical to those previously described for the analysis of trimethylsilylated sterols.[19] Data was recorded in full-scan mode from m/z 50 to 650, and in SIM mode, the ions m/z 209.2, 223.2, 237.2, 468.4, 472.4, 474.4, 482.5, 486.5, 488.5, 496.5, 500.5, and 502.5 were recorded with a dwell time of 35 ms after a solvent delay of 6 min.

Two different GC−MS systems were used as a result of differences in the characteristics of the samples. The oils were analyzed on a short column with a nonpolar stationary phase (15 m, capillary column coated with 0.1 μm film thickness 100% dimethyl polysiloxane) to avoid interference of triacylglycerols that elute after the analytes on this column. The isolated fractions were analyzed on a longer column with a more polar stationary phase (30 m, 0.25 μm film thickness HP-5MS column) and better resolution that is capable of separating all congeners.

Statistical Analysis. All reported values are means with standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, U.S.A.). One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to determine significant differences between means ($p < 0.05$).

RESULTS AND DISCUSSION

The present work aimed to determine if palm oils from six different Costa Rican palm fruit varieties differ in their profiles and total contents of vitamin E compounds and to investigate if oil extraction by a screw press versus organic solvent gives different yields and profiles of vitamin E congeners in the extracted oils.

All eight known vitamin E forms α-, β-, γ-, and δ-tocopherol and -tocotrienol were detected in the extracted palm oils (Tables 1 and 2). A ninth vitamin E derivative, α-tocomonoenol, was found in all tested oils and was present in appreciable concentrations in the oils extracted from the varieties Tanzania (CB063B) and the hybrid O × G (CB127B) (see the discussion below). γT3 was the most abundant tocochromanol in all samples, irrespective of genotype and extraction procedure, followed by αT3 and δT3. αT was the most abundant tocopherol, and its concentrations were in a range similar to those of βT3 (Tables 1 and 2). Of the palm fruit genotypes investigated, the oils extracted from Quepos had by far the highest total vitamin E content, which was almost entirely made up of tocotrienols. The tocopherol contents of Quepos and also Manaos oils were negligible ($\leq$1.8 μg/g) and the lowest among all tested genotypes (Tables 1 and 2). γT, βT, and δT are minor compounds in palm oil and may have been present at concentrations close to or below the detection limit of our method. Therefore, dependent upon the oil and extraction method used, these congeners were not detectable in all samples. The concentrations and relative abundances of tocopherols and tocotrienols in the present palm oil samples were in general agreement with previous publications, even though the reported numbers were subject to large variability.[2,4−6,20−22] The total vitamin E contents in palm oils extracted with a screw press or hexane varied from 163 to 624 μg/g (Table 1) and from 333 to 892 μg/g (Table 2), respectively, in the present investigation and were somewhat lower than previously published values, which are in the range of 400−1300 μg/g for *E. guineensis*,[5,6,20−22] 500−1500 μg/g for *E. oleifera*, and 600−1600 μg/g for hybrid O × G.[20] However, lower total vitamin E contents were reported for *E. guineensis* (262 μg/g) and hybrid O × G (259 μg/g).[4] These large variations in reported values can be explained by differences in the geographical origin of the palm fruits, resulting differences in the climate conditions and ripeness at harvest, genetic differences in the sampled fruits, and different storage and postharvest processing.[12,13,21]

We observed significant differences in the vitamin E extraction efficiencies of the two extraction procedures tested ($p < 0.05$; Figure 3 and the Supporting Information). Chemical extraction with hexane yielded up to 2.5-fold higher total vitamin E compared to screw press extraction. Additional experiments were performed with Deli Dami and Quepos to investigate the reasons for the observed differences between the extraction methods. To this end, palm oils were produced and the processing byproducts [residual peel (hexane extraction) respectively residual press cake and centrifugation byproduct (screw press extraction)] were analyzed for their vitamin E contents and profiles and compared to the crude oils (Figure 4 and Supporting Table 1). If we look at the sum of the vitamin E

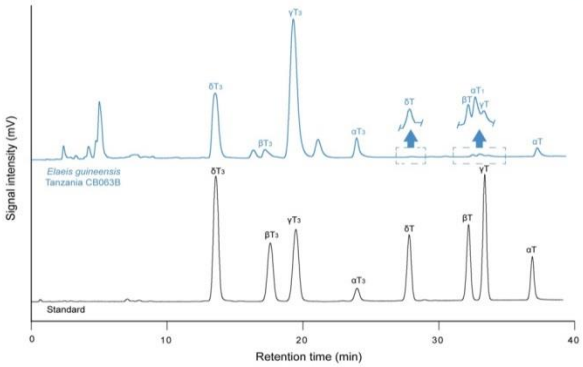

Figure 2. Representative high-performance liquid chromatography with fluorescence detection (HPLC−FD) chromatograms of the separation of tocopherols and tocotrienols in a calibration standard (black) and a palm oil sample of the genotype *E. guineensis* Tanzania CB063B obtained by screw press extraction (blue). The identity of α-tocomonoenol (αT1) was confirmed by GC−MS (see the Supporting Information).

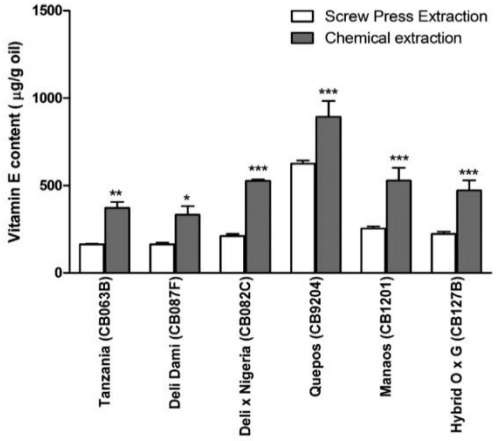

Figure 3. Mean (error bars reflect standard deviations; $n = 6$) total vitamin E contents (μg/g of oil) of palm oils extracted from *Elaeis* sp. genotypes using a screw press (white bars) or chemical extraction with hexane (black bars). The total vitamin E yield differed significantly between extraction methods [indicated by asterisks, (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$].

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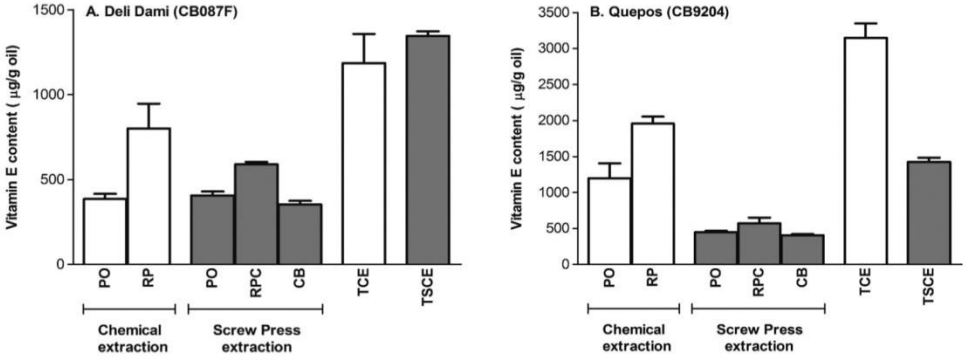

Figure 4. Mean (error bars reflect standard deviations; n = 3) total vitamin E contents (μg/g of oil) of palm oils (PO) and their processing byproducts [residual peel (RP), residual press cake (RPC), and centrifugation byproduct (CB)] from *Elaeis* sp. Deli Dami CB087F and Quepos CB9204 obtained by chemical extraction and screw press extraction. The bars TCE (total chemical extraction) and TSCE (total screw press extraction) represent the sum of all vitamin E congeners in the palm oils and their respective byproducts for each extraction method.

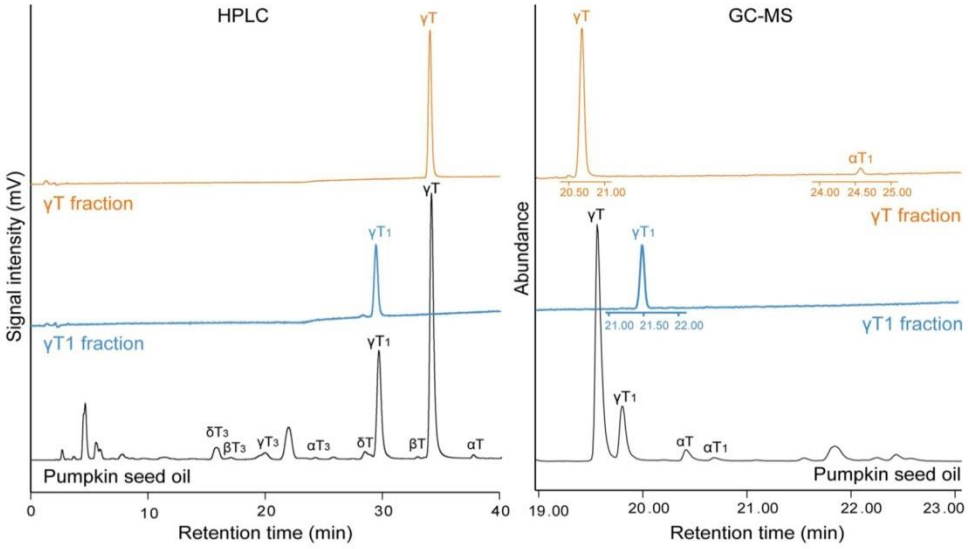

Figure 5. HPLC and GC−MS chromatograms of vitamin E compounds in pumpkin seed oil (black, GC−MS system 1) and the isolated fractions of γT1 (blue) and γT (orange, both GC−MS system 2). GC−MS analysis allowed for the identification of γT_1 (blue, m/z 486.5) and confirmed the identity of γT (orange, m/z 488.5) in the isolated fraction but also showed the presence of αT_1 (orange, m/z 500.5), which was not visible in the HPLC chromatogram, because it was masked by the γT peak. The GC−MS chromatograms for the collected fractions (shown in orange and blue) were obtained on different GC instruments and columns than that for the pumpkin seed oil (black trace), and therefore, the time scales and retention times differ between chromatograms.

forms (total vitamin E) of the crude palm oil and the byproducts for each extraction technique, we find similar total vitamin E for both extraction techniques for Deli Dami (Figure 4 and Supporting Table 1), explaining that the differences in the vitamin E extraction efficiencies among the studied techniques are mostly because of the vitamin E losses in the byproducts. Even though the variety Quepos contains more vitamin E, the differences between the vitamin E yields of both methods were larger for this variety than Deli Dami, and the screw press extraction had a much lower yield than the solvent extraction. This may be explained by the thickness of the mesocarp, which is inherently thinner in the genotype *E. oleifera*,[12] making the handling in the screw press, where fruit material may get stuck in the equipment, more difficult.

In both varieties, total vitamin E was higher in the residual peel from the chemical extraction and the residual press cake from the screw press extraction than in the crude palm oils. This is in agreement with previous findings that vitamin E retained in the palm fruit fiber can actually be 2-fold higher than in the crude oil.[5,23,24] Further loss of vitamin E during the screw press processing was caused by entrapment in the centrifugation byproduct of the clarification step (Figure 4). In agreement with our results, it was reported that vitamin E forms are more concentrated in the peel than in the inner mesocarp, which is a more fibrous material from which it is more difficult to release the oil.[14] This explains why the screw press extraction is less efficient than the solvent extraction. The vitamin E yield of the solvent extraction may be further improved by extracting rather than removing the peel during processing.

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As mentioned above, we detected a ninth vitamin E congener, α-tocomonoenol (αT1), in most oil samples at concentrations ranging from 0.08 to 3.0 μg/g and confirmed its identity by GC−MS analysis (see the Supporting Information). In previous publications, αT1 ranged from traces to 40 μg/g in *E guineensis* palm oils[2−7] and was 4 μg/g in the hybrid O × G.[4] Because there is currently no αT1 available as a reference standard, the αT1 concentrations in Tables 1 and 2 were given as α-tocopherol equivalents, assuming the same response factor as for αT. The αT1 contents of the analyzed oils were, irrespective of the extraction method, in the order of *E. guineensis* Tanzania (3.0−2.7 μg/g) > hybrid O × G (0.6−1.4 μg/g) = *E. guineensis* Deli × Nigeria (0.7−0.9 μg/g) > *E. oleifera* Quepos (0.2−0.4 μg/g) = Manaos (0.2−0.5 μg/g) > *E. guineensis* Deli Dami (from not detected to 0.08 μg/g).

We modified our previously developed reverse-phase HPLC method for the baseline separation of the eight standard vitamin E congeners[18] by changing from an isocratic to a gradient elution and prolonging the total run time to achieve better separation in the palm oil matrix (Figure 2). αT1, in agreement with the literature,[25,26] eluted between βT and γT and partly overlapped with the latter. Because of this overlap, we suspected that samples high in γT might contain αT1 that does not show up in standard reversed-phase methods. Indeed, HPLC analysis of a pumpkin seed oil sample did not reveal the presence of αT1 (left side of Figure 5) We then collected the γT peak from the HPLC detector and analyzed it by GC−MS, which revealed a low content of αT1 and a high content of γT (right side of Figure 5). The large γT peak thus masked the presence of αT1 in pumpkin seed oil. The co-elution of αT1 and γT, if not detected and accounted for, may result in the overestimation of the γT content of food samples, which may be even more pronounced than in this example, where the collected γT fraction contained 9% αT1. In addition, we detected yet another tocomonoenol in pumpkin seed oil, namely, γT1, that was clearly visible in the HPLC chromatogram. Its content was 79 μg/g (also calculated as γT equivalent), which is in agreement with a previous publication reporting the presence of 119 μg/g of γT1.[8] γT was predominant (63.5%) in pumpkin seed oil, and γT1 was the second most abundant tocochromanol (20.9%) in pumpkin seed oil (see the Supporting Information).

In conclusion, the profiles and total vitamin E contents of palm oils extracted from the six studied Costa Rican palm tree genotypes differed significantly. Even though hexane extraction yielded higher contents of tocochromanols than screw press extraction, the ranking of the samples according to the total vitamin E content was nearly unaffected by the extraction type. By far the highest vitamin E content was found in oils extracted from the genotype *E. oleifera* Quepos (99.8% tocotrienols, 0.2% tocopherols, and α-tocomonoenol), and the genotype Tanzania had the highest content of α-tocomonoenol. The latter compound co-eluted with γ-tocopherol using standard HPLC conditions and may thus, particularly when present at appreciable concentrations, lead to an overestimation of the γ-tocopherol content of foods.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02230.

Genetic relationship between palm fruit genotypes used in the study according to information given by ASD Costa Rica (Supporting Figure 1), HPLC and GC−MS chromatograms of vitamin E compounds in palm oil *E. guineensis* Tanzania CB063B and the isolated fraction of αT_1 (Supporting Figure 2), mean contents (μg/g of oil) of each individual vitamin E of palm oils extracted from *Elaeis* sp. genotypes using screw press extraction or chemical extraction with hexane (Supporting Figure 3), mean concentrations (μg/g) of tocotrienols, tocopherols, and α-tocomonoenol in palm oils and their processing byproducts in *Elaeis* sp. Deli Dami CB087F and Quepos CB9204 extracted with chemical extraction and screw press extraction (Supporting Table 1), and mean concentrations (μg/g) of tocotrienols, tocopherols, and γ-tocomonoenol in pumpkin seed oil (*C. pepo* L.) (Supporting Table 2) (PDF)

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Notes

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■ ABBREVIATIONS USED

T, tocopherol; T1, tocomonoenol; T3, tocotrienol; BSTFA/TMCS, *N,O*-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane

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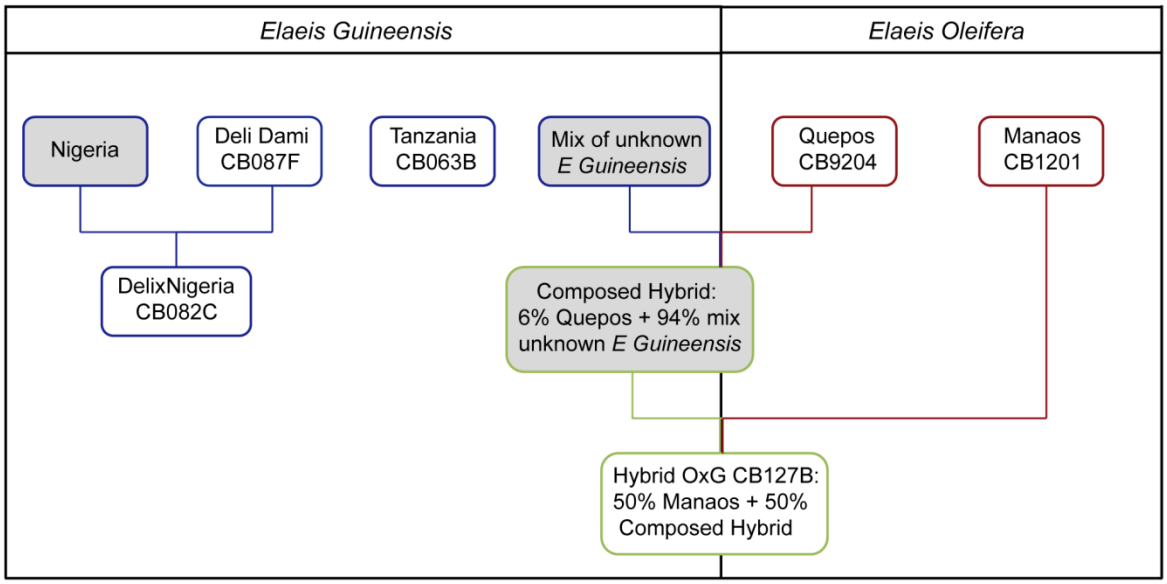
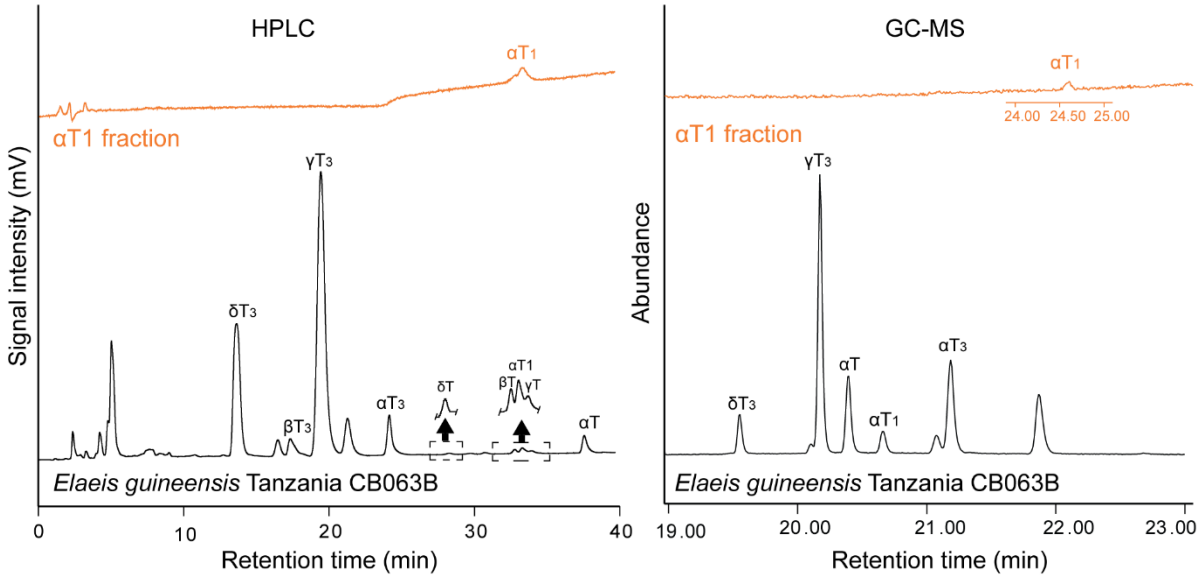
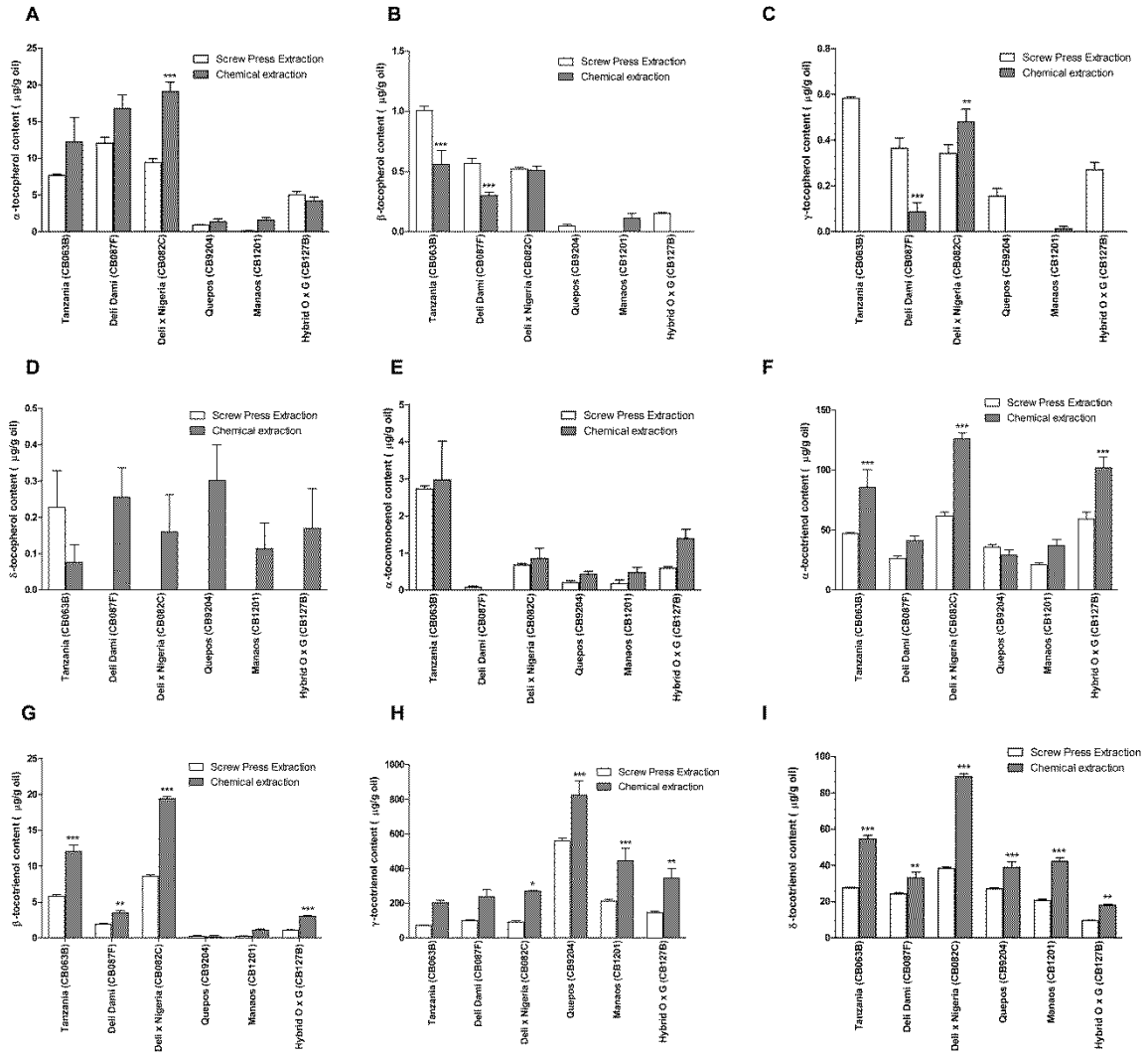


Supporting Figure 1. Genetic relationship between palm fruit genotypes used in the study according with information given by ASD Costa Rica. Genotypes studied are displayed in white boxes and their parents genotypes are in grey boxes. Each genotype is named using their name follow by their experiment number.



Supporting Figure 2. HPLC and GC-MS chromatograms of vitamin E compounds in palm oil *Elaeis Guineensis* Tanzania CB063B (black, GC-MS system 1), and the isolated fraction of αT_1 (orange, GC-MS system 2). GC-MS analysis (on GC-MS system 2) confirmed the identity of αT_1 (m/z 500.5). The GC-MS chromatograms for the collected fractions (shown in orange, GC-MS system 2) were obtained on a different GC and column than that for the palm oil (black trace, GC-MS system 1) and therefore the time scales and retention times differ between chromatograms.



Supporting Figure 3. Mean (error bars reflect standard deviations, n=6) α-tocopherol (A), β-tocopherol (B), γ-tocopherol (C), δ-tocopherol (D), α-tocomonoenol (E), α-tocotrienol (F), β-tocotrienol (G), γ-tocotrienol (H) and δ-tocotrienol (I) contents (µg/g oil) of palm oils extracted from *Elaeis sp* genotypes using screw press extraction (white bars) or chemical extraction with hexane (black bars). Significant differences between extraction methods are indicated by asterisks (*P < 0.05; **P < 0.01; ***P < 0.001).

Supporting Table 1. Mean concentrations (± standard deviation; n=3; all in μg/g) of tocotrienols, tocopherols and α-tocomonoenol in palm oils (PO) and their processing byproducts; residual peel (RP), residual press cake (RPC) and centrifugation byproduct; in *Elaeis* sp Deli Dami CB087F and Quepos CB9204 extracted with chemical extraction and screw press extraction.[1]

Compound	Deli Dami CB087F					Quepos CB9204				
	Chemical extraction		Screw press extraction			Chemical extraction		Screw press extraction		
	PO	RP	PO	RPC	CB	PO	RP	PO	RPC	CB
αT	89.4 ± 5.2	341.4 ± 65.9	122.7 ± 9.4	230.0 ± 5.4	107.2 ± 5.5	4.0 ± 1.4	9.6 ± 2.0	5.9 ± 0.5	17.9 ± 1.8	10.0 ± 2.0
βT	0.63 ± 0.05	4.1 ± 0.5	0.72 ± 0.04	2.65 ± 0.05	0.61 ± 0.05	nd[2]	nd	0.04 ± 0.01	0.11 ± 0.02	0.09 ± 0.01
γT	0.73 ± 0.06	8.2 ± 1.3	0.93 ± 0.04	2.5 ± 0.1	0.75 ± 0.03	0.15 ± 0.03	0.57 ± 0.09	0.48 ± 0.03	1.7 ± 0.2	0.74 ± 0.07
δT	nd	0.7 ± 0.1	0.09 ± 0.01	0.23 ± 0.01	0.08 ± 0.01	nd	nd	0.03 ± 0.01	0.08 ± 0.01	0.05 ± 0.01
αT1[3]	1.4 ± 0.1	6.8 ± 1.2	1.57 ± 0.07	nd	1.2 ± 0.2	1.7 ± 0.6	2.6 ± 0.6	0.95 ± 0.07	2.7 ± 0.4	1.8 ± 0.3
αT3	59.1 ± 3.7	135.6 ± 25.2	66.8 ± 4.5	88.8 ± 2.1	57.2 ± 5.0	48.0 ± 20.8	146.3 ± 19.3	19.3 ± 2.1	38.9 ± 5.9	27.8 ± 4.7
βT3	3.79 ± 0.55	7.8 ± 0.9	3.8 ± 0.8	4.3 ± 0.1	2.4 ± 0.4	0.5 ± 0.2	1.6 ± 0.3	0.32 ± 0.10	0.35 ± 0.02	1.8 ± 0.3
γT3	195.1 ±18.2	249.6 ± 44.4	184.4 ± 11.6	223.9 ± 5.7	161.6 ± 14.6	1090.4 ± 170.7	1742.0 ± 75.1	410.6 ± 17.6	489.3 ± 69.8	349.6 ± 8.7
δT3	34.5 ± 3.7	46.0 ± 6.4	23.7 ± 0.8	36.6 ± 0.8	20.5 ± 1.8	48.0 ± 18.2	54.7 ± 6.4	10.7 ± 0.8	18.5 ± 1.0	14.2 ± 2.0
Total T	90.8 ± 5.1	354.4 ± 67.7	124.4 ± 9.5	235.4 ± 5.5	108.6 ± 5.5	4.2 ± 1.4	10.2 ± 2.1	6.4 ± 0.6	19.8 ± 2.0	10.8 ± 2.0
Total T3	292.5 ± 26.1	439.1 ± 76.8	278.7 ± 15.9	353.7 ± 8.4	241.7 ± 21.4	1186.9 ± 209.3	1944.6 ± 93.8	440.9 ± 18.8	547.1 ± 76.7	393.3 ± 13.2
Total VE	384.7 ± 31.2	800.3 ± 145.1	404.7 ± 25.2	589.0 ± 13.8	351.6 ± 23.2	1192.7 ± 211.0	1957.3 ± 95.9	448.2 ± 18.9	569.5 ± 79.0	406.0 ± 15.1

[1]Vitamin E congeners differ in molecular weight and thus similar weights do not represent similar numbers of molecules.

[2]nd=not detected.

[3]Expressed as α-tocopherol equivalents.

Supporting Table 2. Mean concentrations (± standard deviation; n=6; all in μg/g) of tocotrienols, tocopherols and γ -tocomonoenol in pumpkin seed oil (*Cucurbita pep L.*)[1]

Compound	Concentration in pumpkin seed oil [μg/g]
αT	3.1 ± 0.5
βT	1.8 ± 0.4
γT	239.4 ± 9.7
δT	6.8 ± 0.3
γT1[2]	78.8 ± 2.5
αT3	3.6 ± 0.3
βT3	1.0 ± 0.2
γT3	42.0 ± 6.4
δT3	0.61 ± 0.04
Total T	251.0 ± 10.0
Total T3	47.1 ± 6.4
Total VE	377.0 ± 16.0

[1]Vitamin E congeners differ in molecular weight and thus similar weights do not represent similar numbers of molecules.

[2]Expressed as γ-tocopherol equivalents.

Chapter 3

α-Tocopherol transfer protein does not regulate the cellular uptake and intracellular distribution of α- and γ-tocopherols and -tocotrienols in cultured liver cells

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Research Paper

α-Tocopherol transfer protein does not regulate the cellular uptake and intracellular distribution of α- and γ-tocopherols and -tocotrienols in cultured liver cells

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ABSTRACT

Liver cells express a cytosolic α-tocopherol transfer protein (αTTP) with high binding affinity for α-tocopherol (αT) and much lower affinities for the non-αT congeners. The role of αTTP in the intracellular distribution of the different vitamin E forms is currently unknown. We therefore investigated the intracellular localization of αT, γ-tocopherol (γT), α-tocotrienol (αT3), and γ-tocotrienol (γT3) in cultured hepatic cells with and without stable expression of αTTP. We first determined cellular uptake of the four congeners and found the methylation of the chromanol ring and saturation of the sidechain to be important factors, with tocotrienols being taken up more efficiently than tocopherols and the γ-congeners more than the α-congeners, irrespective of the expression of αTTP. This, however, could perhaps also be due to an observed higher stability of tocotrienols, compared to tocopherols, in culture media rather than a higher absorption. We then incubated HepG2 cells and αTTP-expressing HepG2 cells with αT, γT, αT3, or γT3, isolated organelle fractions by density gradient centrifugation, and determined the concentrations of the congeners in the subcellular fractions. All four congeners were primarily associated with the lysosomes, endoplasmic reticulum, and plasma membrane, whereas only αT correlated with mitochondria. Neither the chromanol ring methylation or sidechain saturation, nor the expression of αTTP were important factors for the intracellular distribution of vitamin E. In conclusion, αTTP does not appear to regulate the uptake and intracellular localization of different vitamin E congeners in cultured liver cells.

1. Introduction

Vitamin E, discovered in 1922 as an "unknown factor X" required for fertility in female rats [1], is now known to comprise eight structurally related lipid-soluble compounds composed of a saturated (tocopherols (T)) or threefold unsaturated (tocotrienols (T3)) 16-carbon sidechain bound to a chromanol ring; the Greek letters α, β, γ, or δ are used as prefixes to designate the number and positions of methyl groups attached to the chromanol ring [2,3].

Upon oral intake, the lipid-soluble tocopherols and tocotrienols are incorporated into mixed micelles and absorbed in the small intestine, following the general path of dietary lipids. The extent of absorption and transport to the liver is nearly similar for all eight vitamin E congeners, but the liver then selectively releases α-tocopherol (αT) into the systemic circulation, while the non-αT congeners are preferentially metabolized to the sidechain shortened carboxyethyl hydroxychromanols via a cytochrome P_{450}-dependent pathway [4,5]. The selective retention of αT in the organism appears to be the result of an interaction of this catabolic pathway with the hepatic α-tocopherol transfer protein (αTTP) [6], a cytosolic protein that specifically binds αT [7,8] and has much lower affinities for β-tocopherol (38%), γ-tocopherol (9%), and δ-tocopherol (2%) [9].

Our current understanding of the involvement of αTTP in intracellular trafficking of αT in the liver is as follows: αT enters hepatocytes by endocytosis and reaches the late endosomal compartment, from where it is transported to the plasma membrane and secreted with

Abbreviations: αT, α-tocopherol; αT3, α-tocotrienol; γT, γ-tocopherol; γT3, γ-tocotrienol; αTTP, α-tocopherol transfer protein; AUC, are under the concentration-time curve; HepG2, human hepatoma cultured cell; HepG2-TTP, HepG2 cell line expressing the human TTP cDNA; HepG2-EV, HepG2 cell line transfected with the antibiotic resistance gene

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lipoproteins into the circulation. αTTP binds αT (and probably to a lesser extent the non-αT congeners) in the outer leaflet of the endosomal membrane and facilitates its transport to the plasma membrane, where the binding of resident phosphatidylinositol 4,5-bisphosphate induces a conformational change and results in the release of αT and its incorporation into the membrane [10–12]. αT then exits the cell involving the ATP-binding cassette transporter A1, is incorporated into lipoproteins and delivered to extrahepatic tissues. αTTP then translocates to the endosomal compartment to repeat the cycle [10].

In addition to lysosomal and plasma membranes, αT is also present in the endoplasmic reticulum, mitochondria, and peroxisomes [13–17]; organelles that are involved in its metabolism [14]. Although the role of αTTP in intracellular trafficking of αT is partly understood, its importance for the intracellular localization of the non-αT congeners in liver cells has not yet been studied. We therefore investigated the uptake and intracellular distribution of αT, γT, αT3, and γT3 in cultured hepatic cells as a function of the expression of αTTP. These four congeners were specifically chosen to allow conclusions regarding the importance of the methylation pattern and sidechain saturation for intracellular trafficking of vitamin E.

2. Materials and methods

2.1. Test compounds

RRR-α-tocopherol and RRR-γ-tocopherol (αT, ≥ 95%, CAS number 59-02-9, cat#KP5101; γT, ≥ 95%, CAS number 54–28-4, cat#KP5103) were from Calbiochem/Merck Millipore (Darmstadt, Germany), R-α-tocotrienol (αT3; ≥ 97% pure, CAS number 58864-81-6, #07205) was from Sigma-Aldrich (Taufkirchen, Germany) and R-γ-tocotrienol (γT3; ≥ 98% pure, extracted from vitamin E capsules as previously described [18]) was a kind gift from Professor Walter Vetter (Institute of Food Chemistry, University of Hohenheim, Germany).

αT (100 mmol/L), αT3 (50 mmol/L), γT (50 mmol/L) and γT3 (20 mmol/L) stock solutions were prepared in ethanol (Carl Roth, Karlsruhe, Germany). Substances were diluted in growth medium (see below) prior to experiments and ethanol concentrations did not exceed 0.1% (v/v).

2.2. HepG2 cell lines and cultivation

The authenticity of the human hepatoma cell line HepG2 and all transfected cell lines (see below) was confirmed by Multiplex Human Cell Line Authentication Test (Multiplexion; Immenstaad; Germany). Cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, Taufkirchen, Germany) with 10% (v/v) foetal calf serum (FCS; Life Technologies Corporation, Darmstadt, Germany) and 1% (w/v) penicillin/streptomycin (P/S; Biochrom AG, Berlin, Germany). HepG2 cells expressing the human TTP cDNA (HepG2-TTP) were generated by transfection with a pcDNA3 vector containing the TTP cDNA using the FuGENE HD Transfection Reagent (Roche, Grenzach-Wyhlen, Germany) according to the manufacturer's protocol. The empty vector control HepG2 cell line (HepG2-EV) not expressing the human TTP cDNA, but transfected with the antibiotic resistance gene, was generated using the same method as for HepG2-TTP. Stable transfectants were selected with geneticin 1% (w/v) (G418; Biochrom AG, Berlin, Germany) and cells were cultivated in DMEM with 10% (v/v) FCS and 0.5% (w/v) G418. All cell lines were cultivated at 5% CO_2, 37 °C, and used between passages 8 and 43.

2.3. Cell viability

Cell viability was measured using the neutral red uptake [19] and MTT assays [20] to determine the maximum concentrations of the test compounds to be used in the subsequent experiments. Cells were treated with the test substances (10–100 μmol/L), solvent control (ethanol 0.1% v/v), positive control (Triton X-100, 0.1%, v/v; Merck, Darmstadt, Germany), and culture medium controls. Incubation with up to 50 μmol/L αT or αT3, and up to 30 μmol/L γT or γT3 resulted in ≥ 90% viable cells (data not shown) and these concentrations were therefore used for the subsequent experiments.

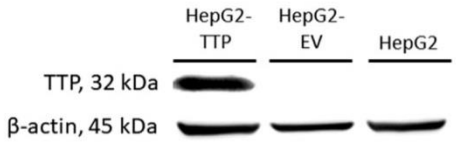

Fig. 1. Representative Western blots of α-tocopherol transfer protein expression in HepG2, empty vector-control HepG2-EV, and α-tocopherol transfer protein-transfected HepG2-TTP cells.

2.4. Time dependent-cellular uptake experiment

Cells were seeded in 12-well plates at a density of 3×10^5 cells and incubated for 24 h to reach 50–60% confluence. Cells were then treated with the respective test compound and control substances for 0.5, 1, 2, 4, 6, 24, 48 and 72 h. αT and αT3 were tested at 50 μmol/L, and γT and γT3 at 30 μmol/L in cultured medium. Cells incubated with culture medium alone and culture medium containing the test compounds incubated without cells were used as negative controls and stability controls, respectively.

After each incubation time, supernatants were collected and cells detached, washed, and resuspended in 20 μL lysis buffer (150 mmol/L NaCl; 50 mmol/L Tris(hydroxymethyl)-aminomethan hydrochloride (Tris-HCl; Carl Roth, Karlsruhe, Germany), pH 8.0; 1% (v/v) Nonidet P-40 (NP-40; Roche, Mannheim, Germany); 4% protease inhibitor cocktail (Roche, Mannheim, Germany)). After 20 min incubation at 4 °C, lysed cells were sonicated for 1 min and centrifuged. Four microliter lysed cell suspension were used for protein determination via Bradford assay [21]. The remaining cell lysis suspension and supernatants were stored at − 80 °C for vitamin E determination.

To determine the stability of the test compounds in the two culture media used in the experiments, 50 μmol/L of each of the four compounds were added to DMEM with penicillium/streptomycin 25 and DMEM with geneticin, respectively, and incubated at 25 and 37 °C, respectively, for up to 0.5 h in the absence of cells. Samples (n = 6) for vitamin E quantification were collected at 0 and 0.5 h.

2.5. Subcellular fractionation by density gradient centrifugation

An optimized density gradient centrifugation method was developed based on previously described protocols [22,23]. Confluent cells were sub-cultivated in T75 flasks at a ratio of 1:2 and incubated to reach confluence. Cells were then incubated with culture medium containing the test compounds (αT and αT3, 50 μmol/L; γT and γT3, 30 μmol/L) during 24 h. Afterwards cells were washed twice with 10 mL PBS, detached with 1 mL trypsin/EDTA after 10 min incubation at 37 °C and re-suspended in 10 mL culture medium. Cells were counted with a CASY cell counter (Innovatis, Darmstadt, Germany) and 1×10^8 cells were centrifuged (163 × *g*, 4 °C, 5 min). Cells were washed with 5 mL PBS and 5 mL homogenization buffer (0.25 mol/L sucrose (Carl Roth, Karlsruhe, Germany), 1 mmol/L EDTA, 0.1% (v/v) ethanol, 10 mmol/L morpholinopropane sulfonic acid (Mops; Serva Biochemica, Heidelberg, Germany) in double distilled water (H_2O_{dd}), pH adjusted to 7.4 with 1 mol/L sodium hydroxide (Carl Roth, Karlsruhe, Germany); 4% protease inhibitor cocktail (Roche, Mannheim, Germany)), with centrifugation in between. Cells were re-suspended in 3 mL homogenization buffer, transferred to Miltenyi tubes and disrupted with a tissue dissociator for 1 min (Miltenyi, Bergisch Gladbach, Germany). Cell breakage of 90% was monitored via trypan blue staining (50 μL PBS, 10 μL trypan blue 0.4% (v/v)) (Serva Biochemica, Heidelberg,

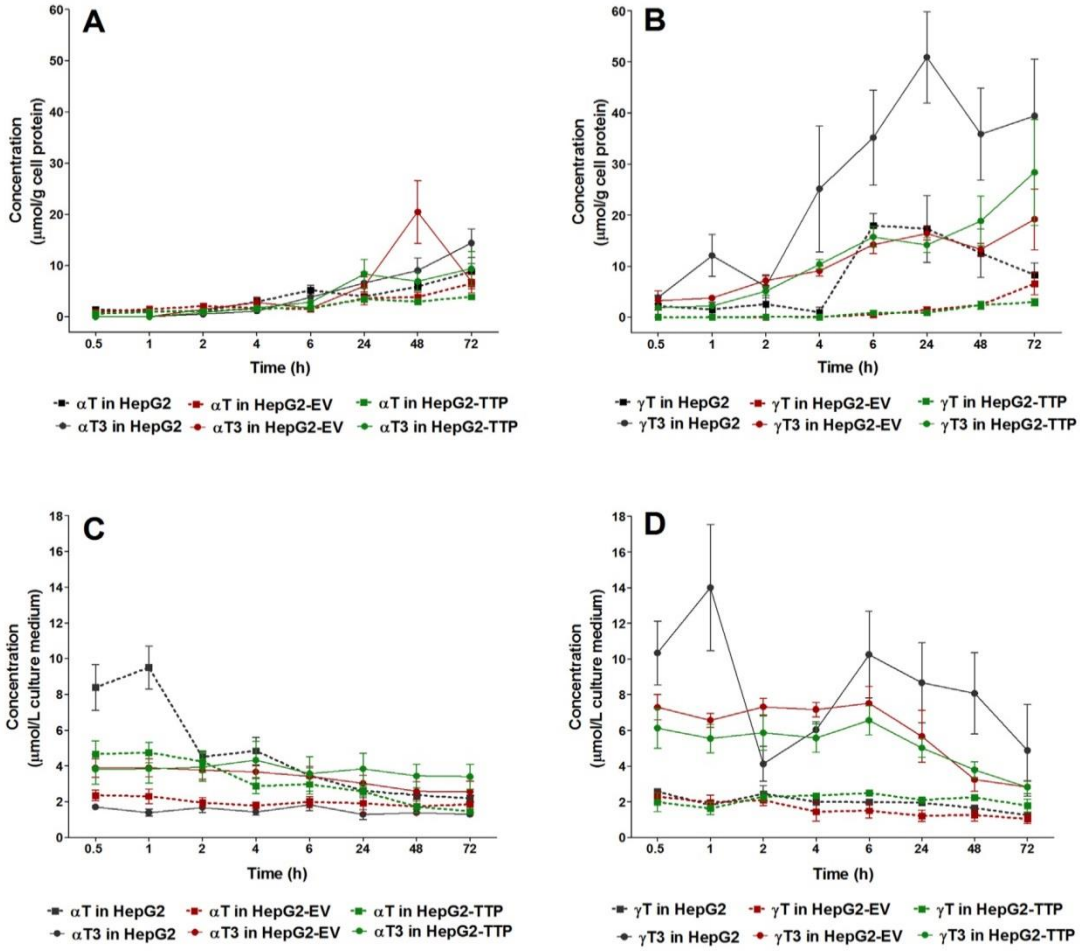

Fig. 2. Time course of the mean concentrations (error bars represent standard error of the mean; n = 3) of αT, γT (squares, dotted lines), αT3, and γT3 (circles, solid lines) in cell lysates (A, B) and cell culture medium (C, D) of HepG2, HepG2-EV (empty vector control) and αTTP-expressing HepG2-TTP cells incubated with 50 µmol/L of αT or αT3 or 30 µmol/L γT or γT3 for up to 72 h.

Germany). The cell suspension was then centrifuged (1000 × *g*, 4 °C, 10 min, without brake), the supernatant transferred, cells re-suspended in 4 mL homogenization buffer, centrifuged, and supernatants were combined. The cell pellet was discarded and post-nuclear supernatants homogenized with a dounce homogenizer using three strokes with a loose-fitting pestle. Post-nuclear supernatants were mixed with Optiprep™ working solution 50% (v/v), iodixanol (83.3% (v/v) OptiPrep™ (Axis-Shield PoC AS, Oslo, Norway), 16.7% (v/v) dilution medium (0.25 mol/L sucrose, 6 mmol/L EDTA, 0.6% (v/v) ethanol, 60 mmol/L Mops in H_2O_{dd}, pH adjusted to 7.4 with 1 mol/L NaOH)) for a final concentration of 24% iodixanol in 11 mL total volume. The solution was transferred to a centrifugation tube (OptiSeal™ tubes; Beckman Coulter Inc., Fullerton, CA, USA), overlaid with homogenization buffer and ultra-centrifuged (318,600 × *g*, 4 °C, 2 h, without brake from 773 × *g* on; Optima L-80 XP Ultracentrifuge with VTi 65.1 rotor, Beckman Coulter Inc., Fullerton, CA, USA). The generated gradient was unloaded carefully in 0.95 mL fractions, dense-end first. Three aliquots of 30 µL of each fraction were stored at − 80 °C for further Western blot analyses. The density of each fraction was measured in °Brix with a refractometer. Samples were stored at − 80 °C until vitamin E determination.

2.6. Western blot analysis

Fractions (30 µL) were mixed with 10 µL loading buffer (4 × SDS protein sample buffer: 250 mmol/L Tris-HCl (pH 6.8), 8% (w/v) SDS, 40% (v/v) glycerol (Sigma-Aldrich), 0.03% (v/v) bromphenol blue (Serva Biochemica, Heidelberg, Germany), 20% (v/v) beta-mercaptoethanol (Merck)), and proteins were separated by 8% and 15% SDS gel electrophoresis and transferred to polyvinylidenefluoride membranes, blocked for 1 h at room temperature in blocking buffer (5% bovine serum albumin (BSA; Sigma-Aldrich) in tris-buffered saline Tween-20 (TBST: 0.8% (w/v) NaCl, 0.24% (w/v) Tris-HCl (pH 7.6), 0.05% (v/v) Tween 20 in H_2O_{dd}; Sigma-Aldrich)) and incubated with the primary antibodies (Abcam, Cambridge, UK). LAMP1 (lysosomes-associated membrane glycoprotein 1, 1:1000, ab24170), Na^+-K^+-ATPase (1:20000, ab76020) and calnexin (1:1000, ab22595) were incubated with the membrane from the 8% SDS gel, and catalase (1:10000, ab76024) and COX IV (cyclooxygenase IV, 1:1000, ab33985) with the membrane from the 15% SDS gel. The primary antibodies were diluted in 5% BSA in TBST and incubated for 1 h at room temperature or overnight at 4 °C (for catalase). Membranes were washed in TBST and incubated for 1 h at room temperature with the secondary antibodies (rabbit anti-mouse peroxidase conjugated for COX IV (1:10000, cat#402335); and goat anti-rabbit peroxidase conjugated for the others (1:10000, cat#401353); Calbiochem/Merck Millipore). Membranes

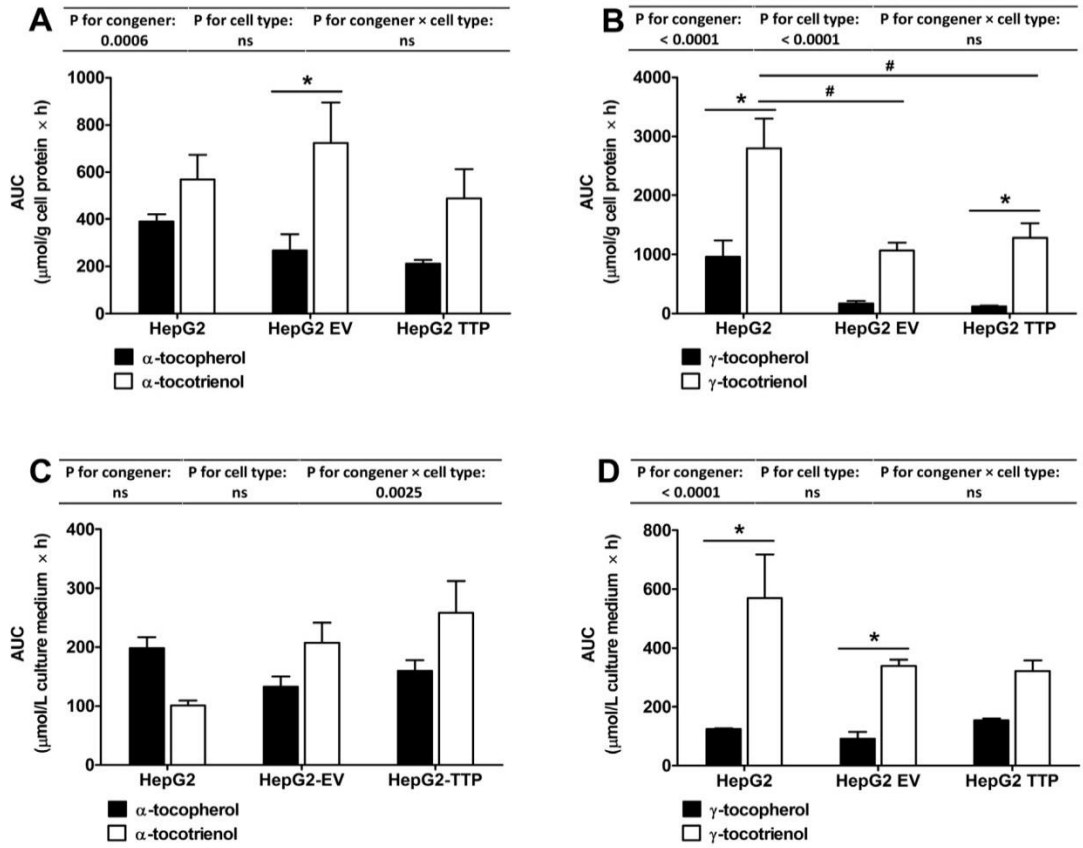

Fig. 3. Area under the concentration-time curve (AUC) (error bars represent standard error of the mean; n = 3) of αT, γT, αT3 and γT3 in cell lysates (A, B) and cell culture medium (C, D) of HepG2, HepG2-EV (empty vector control) and αTTP-expressing HepG2-TTP cells incubated with 50 μmol/L of αT or αT3 or 30 μmol/L γT or γT3 for up to 72 h. AUC was calculated with GraphPad Prism 5. Two-way ANOVA with Bonferroni post-hoc test were calculated to detect significant differences ($P < 0.05$). P-values for the effects of congener, cell type and congener x cell type interactions are reported above each graph. Significant differences between congeners are marked with asterisks (*) and between cell types with the number symbol (#).

were washed with TBST and bands were visualized using AceGlow™ Essential chemiluminescence solutions A and B (Peqlab Biotechnologie GmbH, Erlangen, Germany) and WesternBright™ Sirius; WesternBright™ Peroxide (Advansta, Menlo Park, CA, USA). Intensities were recorded on a Fusion FX and processed using the FusionCapt Advance software (Vilber Lourmat, Eberhardzell, Germany).

To verify the αTTP expression, 20 μL aliquots of cell suspensions of HepG2, HepG2-TTP and HepG2-EV, adjusted to 40 μg protein, were separated by 10% SDS gel, blotted and imaged as described using α-TTP (1:1000, ab155323) and goat anti-rabbit peroxidase conjugated as primary and secondary antibodies, respectively. The housekeeping protein β-actin was used as loading control, using β-actin (1:1000, Cell signaling Technology, Danvers, MA, USA) and goat anti-rabbit peroxidase conjugated as primary and secondary antibodies, respectively.

To correlate the association between the αTTP expression and the organelle markers in the subcellular fractions, HepG2-TTP cells incubated for 72 h were treated as described in Section 2.5 and the expression of αTTP and the different organelle markers was analyzed in the isolated subcellular fractions by Western blotting, as described above.

2.7. HPLC analysis of vitamin E congeners

All chemicals used were of highest purity and purchased from Sigma-Aldrich or Merck. Methanol was HPLC grade and all water used was deionized and filtered (Millipore, Billerica, MA, USA). αT, αT3, γT and γT3 in lysed cells and supernatants from uptake assays, and in subcellular fractions were extracted and quantified as previously described [24]. Briefly, for lysed cells and fractions, the complete cells and 800 μL samples were used, respectively, and the saponification was performed, followed by a liquid-liquid extraction with hexane. Supernatants were processed using 600 μL sample without saponification using the same extraction procedure. For all cases, the hexane extraction was performed twice, first with 2 mL *n*-hexane and transfer of 1 mL organic layer, and then with 1 mL *n*-hexane and a transfer of 1 mL organic layer. Prior to HPLC analysis, extracts were re-suspended in 100 μL methanol/water (85:15, v/v) and transferred to amber HPLC vials.

Twenty microliter sample was injected into a Jasco HPLC (system controller LC-Net II/ADC, two pumps X-LC™ 3185PU, mixing unit X-LC™ 3180MX, degasser X-LC™ 3080DG, autoinjector X-LC™ 3159AS, column oven X-LC™ 3067CO, fluorescence detector FP-2020 Plus; Jasco, Germany). Test compounds were separated on a Phenomenex Kinetex™ PFP column (2.6 μm particle size, 150 × 4.6 mm) maintained at 40 °C, using methanol/water (85:15, v/v) at a flow rate of 1.7 mL/min, for a total run time of 15 min. The fluorescence detector was operated at excitation/emission wavelengths of 296/325 nm, peaks were recorded and integrated using Chrompass software (version 1.9. 302.1124, Jasco), and quantified against external standard curves using the authentic compounds (Sigma-Aldrich, minimum ≥ 95.5% pure).

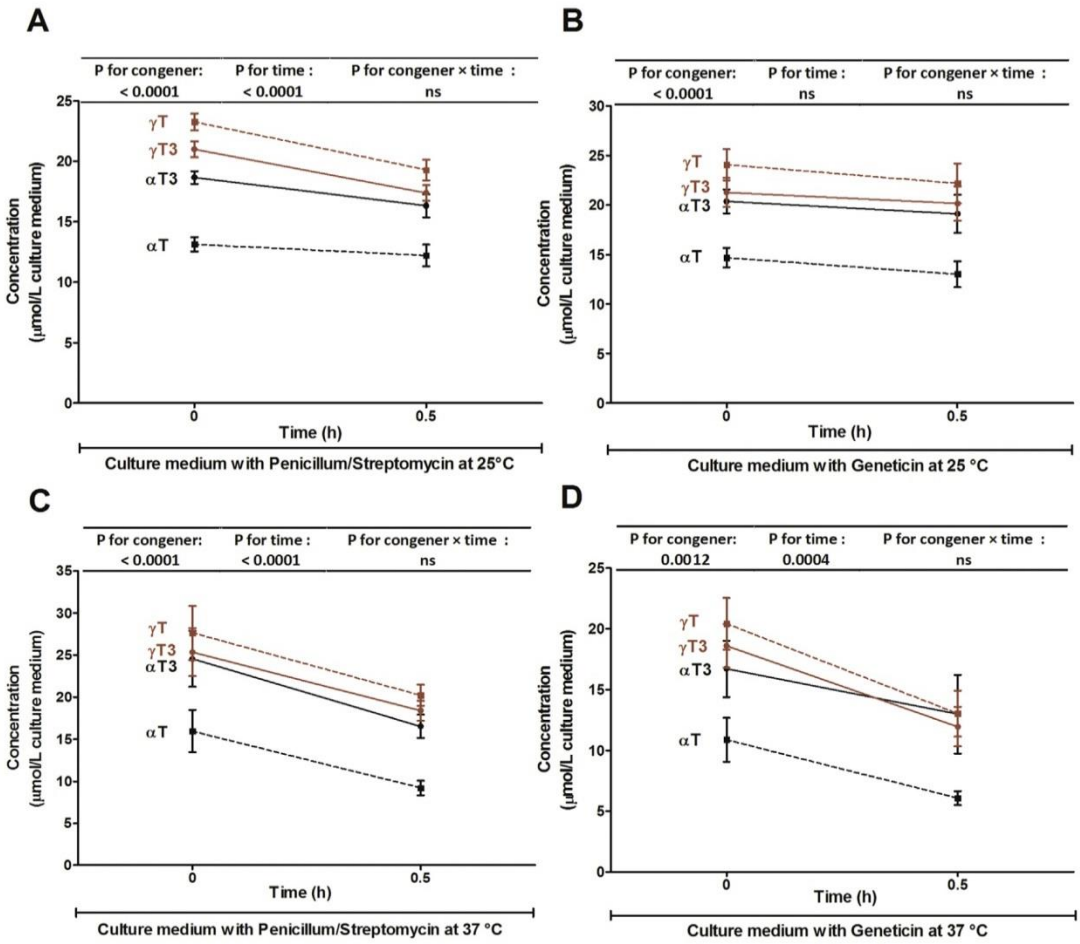

Fig. 4. Time course of the mean concentrations (error bars represent standard error of the mean; n = 6) of αT (black dotted lines), γT (brown dotted lines), αT3 (black solid lines) and γT3 (brown solid lines) in two cell culture media and two temperatures [DMEM/ 10% FCS/1% penicillum/streptomycin at 25 °C (A) and 37 °C (C) and DMEM/ 10% FCS/0.5% geneticin at 25 °C (B) and 37 °C (D)] incubated with 50 µmol/L of αT, γT, αT3 and γT3 for up to 0.5 h. Two-way ANOVA were calculated to detect significant differences ($P < 0.05$). P-values for the effects of congener, time and congener x time interactions are reported above each graph.

2.8. Statistical analyses

Cellular uptake experiments were performed in biological triplicates (n = 3), each consisting of three technical replicates, and subcellular fractionation experiments in biological triplicates (n = 3), each consisting of one experiment. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Areas under the concentration-time curve (AUC) were calculated for the cellular uptake experiments and two-way ANOVA was used to test for effects of congener, cell type and congener × cell type interactions. A Bonferroni post-hoc test was used to calculate significant differences between groups.

Stability tests for αT, γT, αT3, and γT3 in culture media were performed in six replicates (n = 6) and significant differences assessed by two-way ANOVA with congener and time as factors.

Non-parametric Spearman's rank order correlation tests were performed to determine significant correlation between the percentage of test compounds or band intensity of the αTTP expression and band intensity of cell organelle markers in the subcellular fractions. Differences and correlations were considered significant at $P < 0.05$.

3. Results and discussion

Although the human hepatoma cell line HepG2 is commonly used to investigate vitamin E uptake, trafficking and metabolism, the fact that it does not express αTTP brings about certain limitations to its predictive power for these processes in vivo. We therefore transfected HepG2 cells with a pcDNA3 vector containing the αTTP cDNA to generate HepG2 clones stably expressing αTTP (HepG2-TTP) or an empty vector (HepG2-EV; Fig. 1) [25], which allowed us to study the potential role of αTTP in the cellular uptake and intracellular distribution of αT, γT, αT3, and γT3 and to determine potential structure-dependent differences between these four congeners.

We first investigated the time-dependency of the uptake of αT, γT, αT3, and γT3 into the three hepatic cell lines by monitoring their concentrations in the culture medium and cell pellets for 72 h. We found a steady uptake into the cells over time (Fig. 2A and B), which was mirrored by decreasing concentrations in the incubation medium (Fig. 2C and D), albeit to somewhat different extents between the four compounds (see also Supplementary Tables 1 and 2). Sidechain saturation apparently affected cellular uptake, with tocotrienol (T3) concentrations being somewhat higher than those of the corresponding tocopherols (T). The differences were small within the first 4–6 h and became more apparent from 24 to 72 h.

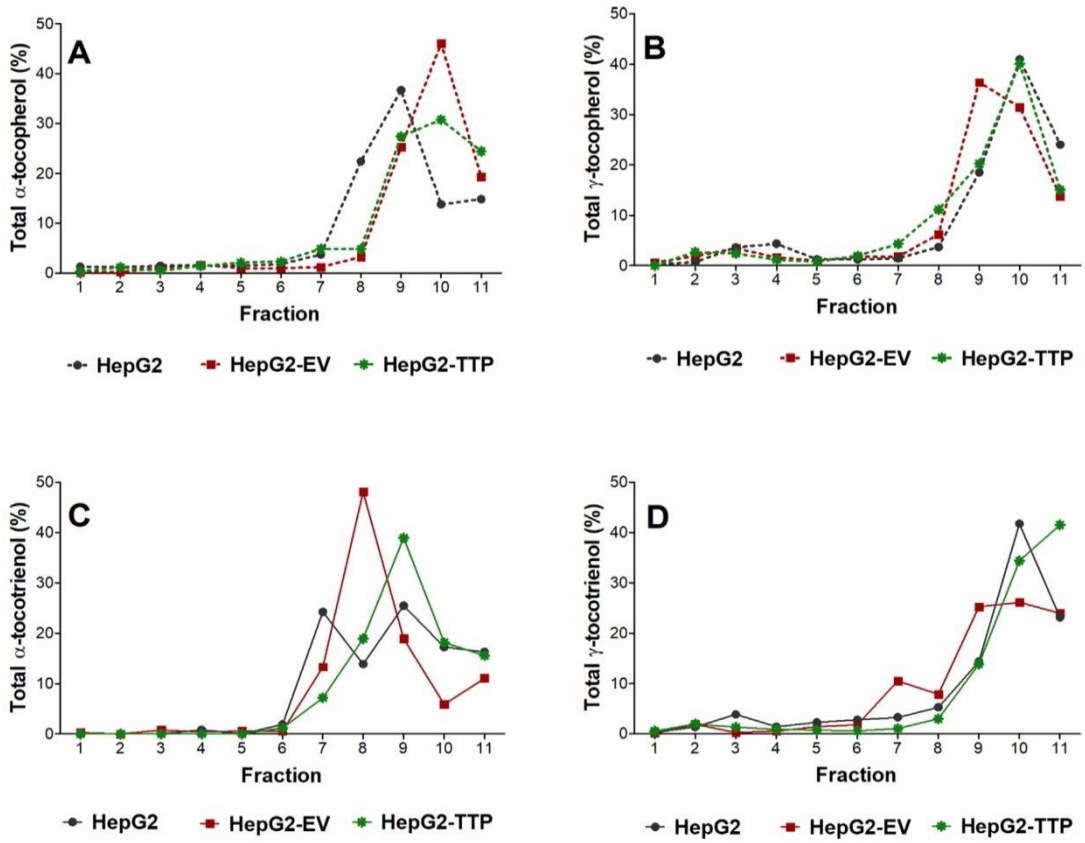

Fig. 5. Mean percentage (n = 3) of αT (A), γT (B), αT3 (C), and γT3 (D) in eleven fractions separated by density gradient centrifugation prepared from HepG2, HepG2-EV (empty vector control) and αTTP-expressing HepG2-TTP cells incubated with 50 μmol/L αT or αT3 or 30 μmol/L γT or γT3 for 24 h.

The observed higher uptake of T3 compared to T is in agreement with previous observations in hepatocytes [4], in human T-leukemia cells [16], in primary cortical neuron cells [26] and in human lung carcinoma cells [27], and has been attributed to the higher intermembrane mobility of the T3 [16]. The absence (HepG2-EV) or presence (HepG2-TTP) of αTTP in the cells did not significantly affect intracellular or medium concentrations of the congeners (Fig. 3A–D).

The better cellular uptake of the tocotrienols compared to their corresponding tocopherol congeners in all cell types was also confirmed by comparing the area under the concentration-time curves (AUC), which were higher for T3 than T (Fig. 3A and B). This was, however, not accompanied by a faster decrease in tocotrienol concentrations in the culture media. On the contrary, AUC in culture media were also higher for tocotrienols (Fig. 3C and D), which may suggest potentially lower stability of tocopherols in culture media rather than a better cellular uptake.

We therefore tested the recovery of all four congeners from cell culture media in the absence of cells at 0 and 0.5 h. Directly upon addition of all four congeners to cell culture media, significant losses in the order of αT > αT3 > γT3 > γT were evident and further declines, which were of similar extent for all congeners, were observed after 30 min (Fig. 4). This raises the question if the higher cellular uptake of tocotrienols observed here (Fig. 2) and previously [4,16,26,27] may in fact be a result of the lower stability and resulting greater loss of tocopherols, in particular αT, which often served as the reference tocopherol, in the culture media. In agreement, considerable losses of αT and γT in cultured macrophages and culture medium were reported and had been attributed to catabolism and/or in vitro oxidation during incubation [28]. The notion of lower oxidative stability of αT is consistent with the reduction-oxidation potentials and antioxidant activities of tocopherols and tocotrienols, which are higher for αT than for γT [29,30]. αT3, however, depending on the test system, is a similarly potent (in homogenous solutions) or even more potent (in membrane systems) antioxidant than αT and oxidative losses would therefore be expected to be similar. In support of the hypothesis that oxidative instability may at least partly explain the differences in the recovery from culture media, the recovery of the more reactive α-congeners was lower than that of the less reactive γ-congeners (Fig. 4). Another explanation could be that the congeners bind to serum proteins in culture media, perhaps with different affinity, and thereby escape detection. Although the reason for the observed loss of vitamin E upon addition to culture medium is not known and warrants further investigation, the subsequent intracellular distribution experiments were based on a qualitative, rather than a quantitative comparison between the congeners and are thus not affected by differences in the concentrations of the congeners in culture medium.

Based on the uptake experiments, an incubation time of 24 h was chosen for the intracellular distribution experiments to ensure sufficient intracellular accumulation of the test compounds, while limiting their enzymatic degradation to their respective short-chain metabolites, which becomes more pronounced after 48 h (data not shown). We employed a density gradient centrifugation technique for the separation into eleven fractions representing five different organelles, which were identified using organelle-specific marker proteins (Supplementary Fig. 1). All four congeners accumulated mainly in fractions 9–11 (Fig. 5). Because organelles overlapped over several fractions, that is each marker protein was present in more than one fraction (Supplementary Fig. 1 and Supplementary Tables 3–8), a non-

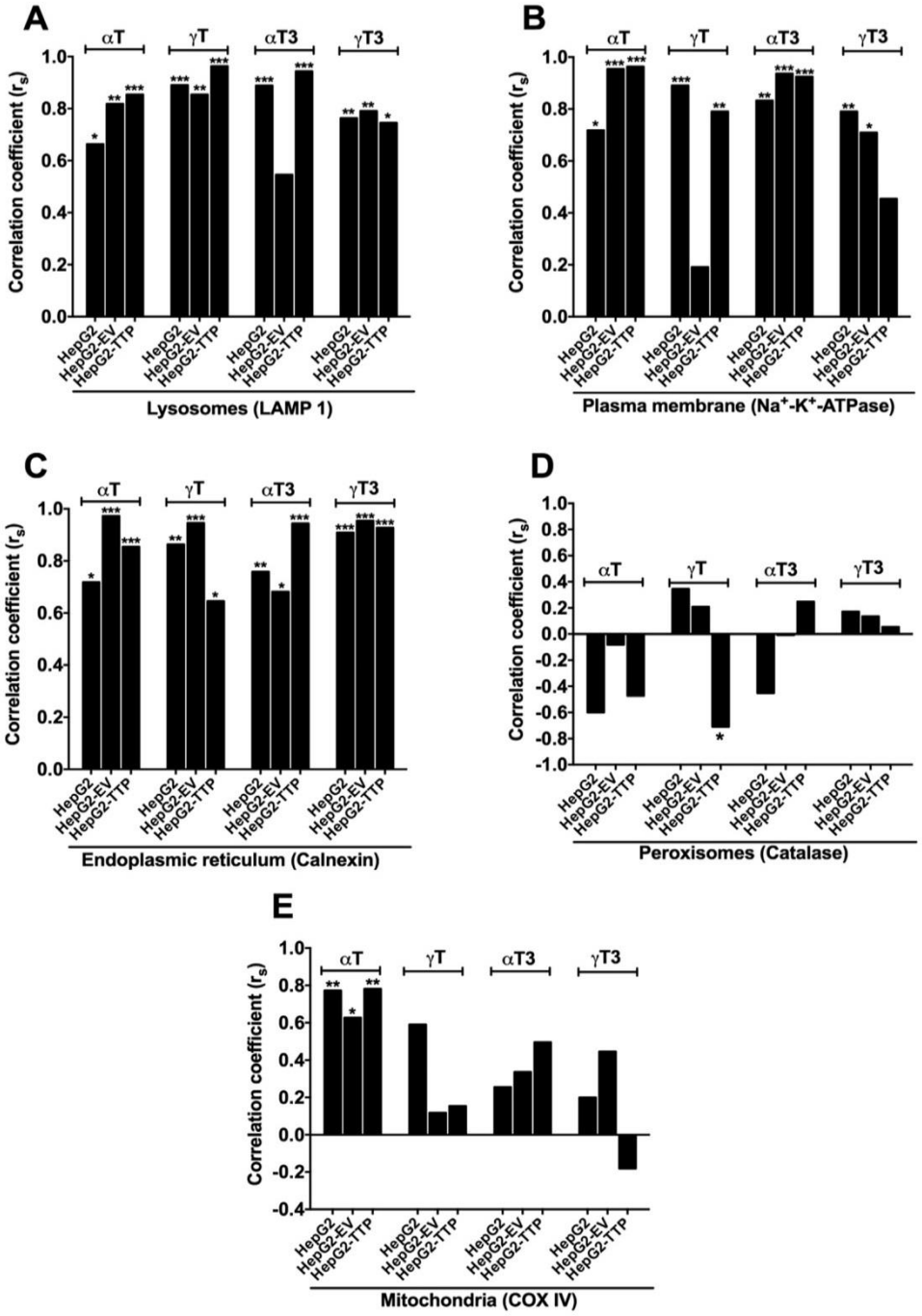

Fig. 6. Spearman's correlation coefficients (r_s) for the correlation between the percentage of αT, γT, αT3, and γT3 and the band intensities of the cell organelle markers for the lysosomes (LAMP1, **A**), plasma membrane (Na^+-K^+-ATPase, **B**), endoplasmic reticulum (calnexin, **C**), peroxisomes (catalase, **D**) and mitochondria (COX IV, **E**) in HepG2, HepG2-EV (empty vector control) and αTTP-expressing HepG2-TTP cells incubated with 50 μmol/L of αT or αT3 or 30 μmol/L γT or γT3 for 24 h. Significant correlations are indicated by asterisks; *P < 0.05; **P < 0.01; ***P < 0.001.

parametric Spearman's correlation test was computed to identify the organelles associated with high concentrations of αT, γT, αT3, or γT3. The concentrations of all four vitamin E forms correlated mainly with the organelle markers for the endoplasmic reticulum, plasma membrane, and lysosomes, but not with those for the peroxisomes (Fig. 6, Supplementary Tables 9 and 10), which is in line with the current understanding of the intracellular trafficking of αT (see introduction and literature cited there) and data from αT-injected rats, for which ca. 75% of hepatic αT was found in the microsomal (endoplasmic reticulum) fraction [14], wild type and NPC1 mice, for which the highest concentrations of αT in the liver were found in lysosomal membranes [17], as well as data from rat pheochromocytoma PC12 and human T-

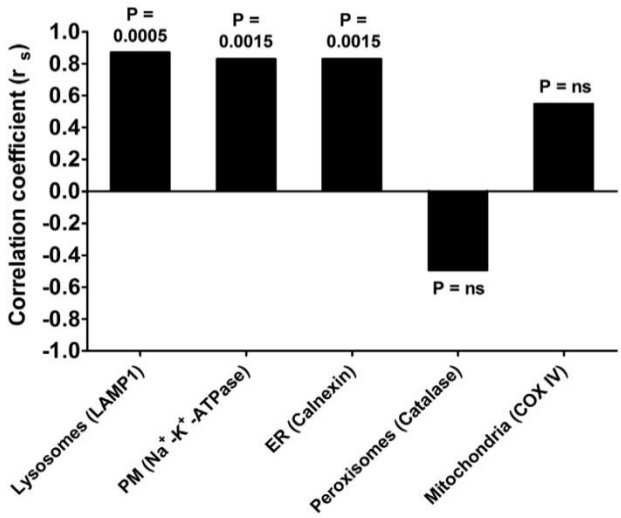

Fig. 7. Spearman's correlation coefficients (r_s) for the correlation between the band intensity of the αTTP expression and the band intensities of the cell organelle markers for the lysosomes (LAMP1), plasma membrane (Na^+-K^+-ATPase), endoplasmic reticulum (calnexin), peroxisomes (catalase) and mitochondria (COX IV) in αTTP-expressing HepG2-TTP cells incubated for 72 h. Correlations are significant at $P < 0.05$. P-values for the correlations are reported above each bar.

leukemia cells, which also had most αT present in the microsomal fractions [15,16]. Only αT, but not γT, αT3, or γT3 concentrations significantly correlated with the mitochondria marker intensity in all cell lines (Fig. 6E), irrespective of αTTP expression, suggesting that αTTP is not involved in mitochondrial targeting. The mitochondrial localization of αT observed here is in agreement with observations in rats fed respectively injected with αT [13,14], in wild type and NPC1 mice fed with αT [17], and PC12 cells [15]. In human T-leukemia cells, on the other hand, both αT3 and αT were present in mitochondria [16]. The lack of significant correlations of γT, αT3, and γT3 concentrations with the mitochondria and of all four congeners with peroxisomes (Fig. 6D and E) agrees with the function of these organelles in the metabolism of vitamin E in general and the preferential metabolism of the non-αT congeners in particular. The initial ω-hydroxylation of the parent vitamin E occurs in the endoplasmic reticulum, this alcohol metabolite is then ω-oxidized in the peroxisomes to yield the 13′-carboxychromanol forms, which are then β-oxidized in the mitochondria to ultimately yield the sidechain-shortened carboxyethyl hydroxychromanol metabolites (reviewed in [3,31]). Hence, the metabolites rather than the parent compounds are expected to localize in these organelles.

The comparison of αTTP-expressing (HepG2-TTP) with the non-expressing HepG2 and HepG2-EV cells revealed only a minor impact of the protein on the intracellular localization of αT, γT, αT3, and γT3 (Fig. 6). The most pronounced and significant difference was observed for the localization of γT in peroxisomes. In the absence of αTTP (HepG2 and HepG2-EV cells), γT was not correlated with the peroxisomal marker, when αTTP was expressed (HepG2-TTP), a significant negative correlation was observed (Fig. 6D), suggesting that the protein prevented the transport of γT to peroxisomes, where it is metabolized. This observation gives further support to our previous findings in these cells that γT metabolism to γ-carboxyethyl hydroxychromanol is reduced in cells with a moderate expression of αTTP and even more strongly in cells with a high expression of αTTP [6].

In order to better understand if αTTP may indeed interact with the organelles involved in vitamin E catabolism, we computed a non-parametric Spearman's correlation test to identify the organelles associated with the expression of αTTP. αTTP expression correlated mainly with the organelle markers for the endoplasmic reticulum, plasma membrane, and lysosomes (Fig. 7), The association with the lysosomes and plasma membrane is in line with the current understanding of the intracellular localization and trafficking of αTTP in hepatocytes (see introduction and literature cited there). The correlation of αTTP with the endoplasmic reticulum, the organelle in which the first and rate-limiting step in vitamin E catabolism takes place [3], is in agreement with our previous findings that αTTP expression reduces the metabolism of γT [6].

To the best of our knowledge, we are the first to directly compare the intracellular localization of the four vitamin E congeners αT, γT, αT3 and γT3. Our observation that all four congeners, irrespective of their methylation pattern and sidechain saturation, are primarily associated with the lysosomal compartment, endoplasmic reticulum, and the plasma membrane (Figs. 6C- 4E), suggests that only small, if any, differences in intracellular trafficking exist between αT, γT, αT3 and γT3 in cultured liver cells. What is more, the expression of αTTP in the liver cells did not bring about any major shifts in the intracellular distribution of the four T and T3, indicating that the protein, despite its preferential binding of αT [9], does not determine the intracellular localization of vitamin E. Because all four congeners were mainly present in lysosomes, endoplasmic reticulum, and the plasma membrane and because αTTP did not significantly affect this localization, there must be other processes, perhaps simple passive diffusion, involved in the trafficking of vitamin E within liver cells. This is further supported by previous observations that vitamin E accumulates mainly in organelles with a high lipid content [15,16].

4. Conclusions

While in the present experiment and in agreement with published literature (see discussion above) γ-configuration of the chromanol ring and unsaturation of the sidechain promoted the uptake of tocochromanols into cultured hepatocytes, it cannot be ruled out that this is caused by the observed instability of tocopherols in cell culture media rather than differences in cellular uptake. The expression of αTTP did not affect the incorporation of T and T3 into HepG2 cells. Based on our data, ring methylation and sidechain saturation of vitamin E also do not appear to be major determinants of its intracellular localization, with the exception of mitochondria, for which a significant correlation was only found for αT. αTTP expression in liver cells did not substantially influence the intracellular distribution of αT, γT, αT3 and γT3.

In summary, our findings suggest that neither the methylation pattern of the chromanol ring or the sidechain saturation of vitamin E congeners, nor the cytosolic αTTP are major factors controlling the intracellular localization of vitamin E in cultured liver cells. This may be indicative of passive processes, such as diffusion, as main driving forces of the distribution of vitamin E forms within the cell.

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Conflict of interest

The authors declare no competing financial interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.07.027.

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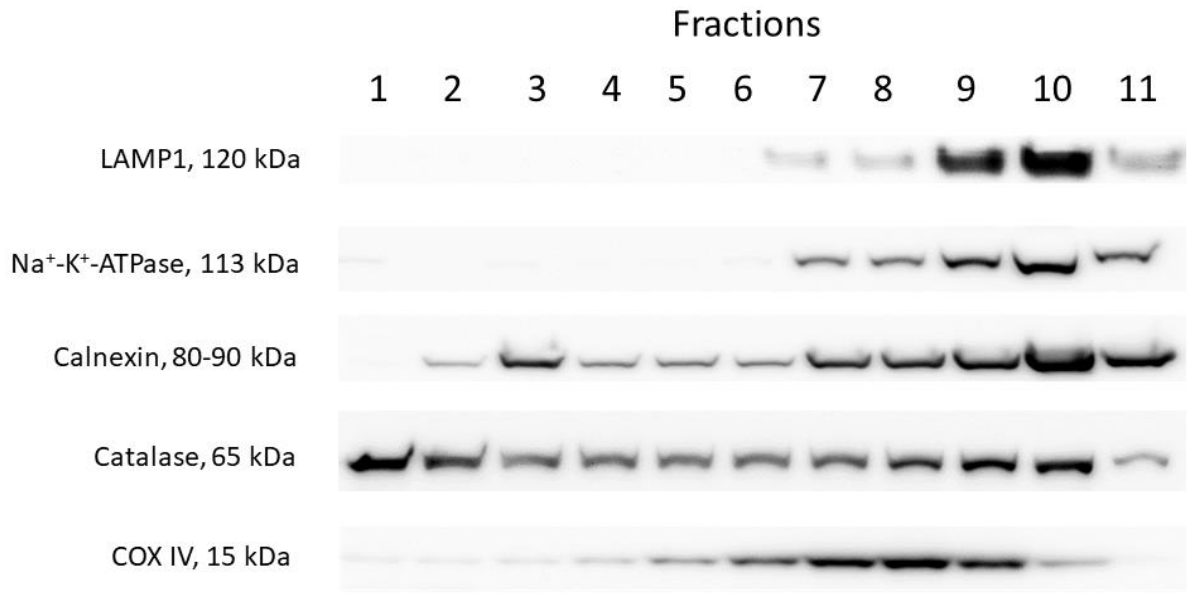

Supplementary figure 1. Representative Western blots of cell organelle markers for lysosomes (LAMP1), plasma membrane (Na^+-K^+-ATPase), endoplasmic reticulum (calnexin), peroxisomes (catalase) and mitochondria (COX IV) for the 11 subcellular fractions obtained from HepG2 cells.

Supplementary table 1. Mean concentrations (± standard error of the mean; n = 3) of α-tocopherol and α-tocotrienol in cell culture medium and cell lysates of HepG2 cells and HepG2 cells transfected with an empty vector (HepG2-EV) or an expression vector carrying the TTP cDNA (HepG2-TTP) at different times after incubation with 50 μmol/L of α-tocopherol or α-tocotrienol.[1]

Time (h)	Concentration in cell culture medium (μmol/L culture medium)						Concentration in cells lysates (μmol/g cell protein)					
	α-tocopherol			α-tocotrienol			α-tocopherol			α-tocotrienol		
	HepG2	HepG2-EV	HepG2-TTP	HepG2	HepG2-EV	HepG2-TTP	HepG2	HepG2-EV	HepG2-TTP	HepG2	HepG2-EV	HepG2-TTP
0.5	8.4 ± 1.3^{b}	2.4 ± 0.3^{a}	4.7 ± 0.7^{a}	1.7 ± 0.1^{a}	3.9 ± 0.5^{a}	3.8 ± 0.8^{a}	1.4 ± 0.6^{a}	0.9 ± 0.2^{a}	0.6 ± 0.2^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
1	9.5 ± 1.2^{b}	2.3 ± 0.4^{ac}	4.8 ± 0.6^{c}	1.4 ± 0.2^{a}	3.9 ± 0.5^{ac}	3.9 ± 0.8^{ac}	1.0 ± 0.3^{a}	1.4 ± 0.4^{a}	0.9 ± 0.3^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
2	4.5 ± 0.3^{b}	2.0 ± 0.3^{ac}	4.3 ± 0.6^{b}	1.7 ± 0.3^{a}	3.8 ± 0.5^{bc}	3.9 ± 0.8^{bc}	1.1 ± 0.4^{a}	2.0 ± 0.6^{a}	1.0 ± 0.3^{a}	0.5 ± 0.2^{a}	1.5 ± 0.5^{a}	0.8 ± 0.2^{a}
4	4.8 ± 0.8^{b}	1.8 ± 0.2^{a}	2.9 ± 0.4^{ab}	1.4 ± 0.2^{a}	3.7 ± 0.3^{ab}	4.3 ± 1.1^{b}	2.9 ± 0.9^{a}	1.7 ± 0.4^{a}	1.5 ± 0.2^{a}	1.1 ± 0.3^{a}	2.8 ± 0.8^{a}	1.7 ± 0.6^{a}
6	3.5 ± 0.4^{a}	2.0 ± 0.3^{a}	3.0 ± 0.5^{a}	1.8 ± 0.3^{a}	3.4 ± 0.6^{a}	3.6 ± 1.0^{a}	5.2 ± 1.0^{b}	1.5 ± 0.4^{a}	1.7 ± 0.2^{a}	3.8 ± 1.2^{ab}	1.7 ± 0.4^{a}	2.8 ± 0.6^{ab}
24	2.6 ± 0.4^{ab}	1.9 ± 0.6^{ab}	2.6 ± 0.3^{ab}	1.3 ± 0.3^{a}	3.0 ± 0.4^{ab}	3.8 ± 0.9^{b}	3.9 ± 0.3^{a}	3.6 ± 1.3^{a}	3.4 ± 0.2^{a}	6.6 ± 2.2^{a}	6.0 ± 1.8^{a}	8.4 ± 2.8^{a}
48	2.4 ± 0.2^{ab}	1.7 ± 0.4^{ab}	1.7 ± 0.3^{ab}	1.4 ± 0.2^{a}	2.6 ± 0.5^{ab}	3.4 ± 0.7^{b}	5.9 ± 0.8^{a}	3.8 ± 0.9^{a}	2.9 ± 0.3^{a}	9.0 ± 2.5^{ab}	20.5 ± 6.2^{b}	6.9 ± 1.8^{a}
72	2.2 ± 0.2^{ab}	1.9 ± 0.5^{ab}	1.5 ± 0.1^{a}	1.3 ± 0.2^{a}	2.6 ± 0.6^{ab}	3.4 ± 0.7^{b}	8.8 ± 1.6^{ab}	6.5 ± 1.7^{ab}	3.9 ± 0.5^{b}	14.4 ± 2.8^{a}	6.9 ± 1.4^{ab}	9.4 ± 3.4^{ab}

[1]Comparison of means were performed separately for concentration of α-tocopherol and α-tocotrienol in cell culture medium and cells lysates, respectively. Differences between cells types and compounds at each given time were calculated with a one-way ANOVA using Tukey's posthoc test. Values within a row (for a given compartment) not sharing a common superscript letter are significantly different at $P<0.05$.

Supplementary table 2. Mean concentrations (± standard error of the mean; n = 3) of γ-tocopherol and γ-tocotrienol in cell culture medium and cell lysates of HepG2 cells and HepG2 cells transfected with an empty vector (HepG2-EV) or an expression vector carrying the TTP cDNA (HepG2-TTP) at different times after incubation with 30 μmol/L of γ-tocopherol or γ-tocotrienol.[1]

Time (h)	Concentration in cell culture medium (μmol/L culture medium)						Concentration in cells lysates (μmol/g cell protein)					
	γ-tocopherol			γ-tocotrienol			γ-tocopherol			γ-tocotrienol		
	HepG2	HepG2-EV	HepG2-TTP	HepG2	HepG2-EV	HepG2-TTP	HepG2	HepG2-EV	HepG2-TTP	HepG2	HepG2-EV	HepG2-TTP
0.5	2.6 ± 0.2^{bc}	2.3 ± 0.3^{bc}	2.0 ± 0.5^{c}	10.3 ± 1.8^{a}	7.3 ± 0.7^{ab}	6.1 ± 1.1^{b}	2.2 ± 2.2^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	3.9 ± 1.3^{a}	3.2 ± 1.9^{a}	1.9 ± 0.4^{a}
1	1.8 ± 0.2^{b}	2.0 ± 0.4^{b}	1.6 ± 0.4^{b}	14.0 ± 3.5^{a}	6.6 ± 0.4^{b}	5.5 ± 0.8^{b}	1.5 ± 1.4^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	12.1 ± 4.1^{a}	3.8 ± 0.4^{b}	2.3 ± 0.4^{b}
2	2.5 ± 0.5^{c}	2.1 ± 0.3^{c}	2.3 ± 0.3^{c}	4.1 ± 1.0^{ac}	7.3 ± 0.5^{b}	5.9 ± 1.0^{ab}	2.6 ± 2.6^{ab}	0.04 ± 0.02^{b}	0.08 ± 0.03^{b}	6.0 ± 2.3^{ab}	7.2 ± 1.0^{a}	5.1 ± 0.8^{ab}
4	2.0 ± 0.1^{b}	1.4 ± 0.5^{b}	2.4 ± 0.1^{b}	6.0 ± 0.4^{a}	7.2 ± 0.4^{a}	5.6 ± 0.8^{a}	1.0 ± 1.0^{b}	0.1 ± 0.1^{b}	0.06 ± 0.04^{b}	25.1 ± 12.3^{a}	9.1 ± 1.0^{ab}	10.4 ± 1.0^{ab}
6	2.0 ± 0.1^{bc}	1.5 ± 0.4^{c}	2.5 ± 0.1^{bc}	10.3 ± 2.4^{a}	7.5 ± 0.9^{a}	6.6 ± 0.8^{ab}	18.0 ± 2.3^{ab}	0.5 ± 0.2^{c}	0.9 ± 0.5^{c}	35.2 ± 9.3^{a}	14.2 ± 1.7^{bc}	15.7 ± 2.0^{bc}
24	1.9 ± 0.1^{b}	1.2 ± 0.3^{b}	2.1 ± 0.1^{b}	8.7 ± 2.2^{a}	5.7 ± 1.5^{b}	5.0 ± 0.5^{ab}	17.3 ± 6.5^{b}	1.4 ± 0.7^{b}	0.9 ± 0.1^{b}	50.9 ± 8.9^{a}	16.4 ± 1.3^{b}	14.2 ± 1.4^{b}
48	1.7 ± 0.1^{b}	1.3 ± 0.3^{b}	2.3 ± 0.1^{b}	8.1 ± 2.3^{a}	3.2 ± 0.7^{b}	3.8 ± 0.5^{b}	12.6 ± 4.8^{b}	2.4 ± 0.4^{b}	2.4 ± 0.8^{b}	35.9 ± 9.1^{a}	13.3 ± 1.2^{b}	18.8 ± 4.9^{ab}
72	1.2 ± 0.2^{a}	1.0 ± 0.3^{a}	1.8 ± 0.4^{a}	4.9 ± 2.6^{a}	2.8 ± 0.4^{a}	2.8 ± 0.4^{a}	8.3 ± 2.3^{b}	6.6 ± 2.2^{b}	2.9 ± 0.7^{b}	39.4 ± 11.2^{a}	19.2 ± 5.9^{ab}	28.4 ± 10.4^{ab}

[1]Comparison of means were performed separately for concentration of γ-tocopherol and γ-tocotrienol in cell culture medium and cells lysates, respectively. Differences between cells types and compounds at each given time were calculated with a one-way ANOVA using Tukey's posthoc test. Values within a row (for a given compartment) not sharing a common superscript letter are significantly different at $P<0.05$.

Supplementary table 3. Percentage of α-tocopherol or α-tocotrienol (Cn) and band intensity (BI) of cell organelle markers separated by density gradient centrifugation for HepG2 cells.

Fraction	Peroxisomes (Catalase)				Mitochondria (COX IV)				Endoplasmic reticulum (Calnexin)				Plasma membrane (Na^+-K^+-ATPase)				Lysosomes (LAMP1)			
	α-T		α-T3		α-T		α-T3		α-T		α-T3		α-T		α-T3		α-T		α-T3	
	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI
1	1.3	25.0	0.0	8.0	1.3	1.0	0.0	0.7	1.3	1.0	0.0	2.0	1.3	4.1	0.0	0.3	1.3	0.9	0.0	0.8
2	1.2	16.7	0.0	11.1	1.2	1.5	0.0	1.6	1.2	7.1	0.0	1.8	1.2	3.0	0.0	0.3	1.2	1.7	0.0	1.3
3	1.5	9.9	0.0	17.9	1.5	1.0	0.0	0.9	1.5	7.8	0.0	1.3	1.5	2.3	0.0	0.3	1.5	1.3	0.0	1.0
4	1.6	7.2	0.9	9.8	1.6	1.0	0.9	1.9	1.6	5.1	0.9	1.4	1.6	1.4	0.9	0.3	1.6	0.6	0.9	1.5
5	1.4	7.0	0.0	7.9	1.4	1.3	0.0	5.5	1.4	4.6	0.0	3.3	1.4	1.2	0.0	0.8	1.4	0.7	0.0	1.7
6	1.8	6.1	1.9	8.6	1.8	4.1	1.9	15.2	1.8	6.4	1.9	11.9	1.8	1.6	1.9	5.4	1.8	0.8	1.9	2.3
7	3.7	7.6	24.3	9.2	3.7	24.5	24.3	43.2	3.7	9.6	24.3	26.2	3.7	4.3	24.3	12.1	3.7	1.6	24.3	14.9
8	22.4	11.0	13.9	11.6	22.4	37.1	13.9	26.6	22.4	17.8	13.9	26.4	22.4	15.7	13.9	27.0	22.4	11.1	13.9	19.0
9	36.7	5.9	25.5	6.9	36.7	18.7	25.5	3.1	36.7	19.2	25.5	15.9	36.7	23.0	25.5	20.5	36.7	37.1	25.5	37.4
10	13.8	3.0	17.3	4.7	13.8	8.2	17.3	0.8	13.8	14.8	17.3	6.2	13.8	22.0	17.3	19.5	13.8	36.4	17.3	16.3
11	14.8	0.6	16.3	4.2	14.8	1.6	16.3	0.6	14.8	6.6	16.3	3.6	14.8	21.4	16.3	13.6	14.8	7.8	16.3	3.9

Data displayed in dark grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is in agreement with the maximum concentration of the compound; and data displayed in light grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is not in agreement with the maximum concentration of the compound.

Supplementary table 4. Percentage of α-tocopherol or α-tocotrienol (Cn) and band intensity (BI) of cell organelle markers separated by density gradient centrifugation for HepG2 cells transfected with an empty vector (HepG2-EV).

Fraction	Peroxisomes (Catalase)				Mitochondria (COX IV)				Endoplasmic reticulum (Calnexin)				Plasma membrane (Na^+-K^+-ATPase)				Lysosomes (LAMP1)			
	α-T		α-T3		α-T		α-T3		α-T		α-T3		α-T		α-T3		α-T		α-T3	
	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI
1	0.1	13.9	0.3	10.1	0.1	1.1	0.3	1.6	0.1	0.3	0.3	0.5	0.1	1.1	0.3	0.4	0.1	0.3	0.3	2.3
2	0.2	12.3	0.0	10.9	0.2	1.1	0.0	3.4	0.2	3.3	0.0	4.5	0.2	1.5	0.0	0.8	0.2	0.7	0.0	2.9
3	1.1	7.4	0.8	8.5	1.1	2.3	0.8	1.0	1.1	8.8	0.8	3.7	1.1	1.6	0.8	1.3	1.1	0.6	0.8	2.7
4	1.6	5.2	0.4	9.3	1.6	1.5	0.4	0.9	1.6	7.3	0.4	3.3	1.6	2.0	0.4	1.5	1.6	0.8	0.4	2.6
5	1.0	6.0	0.5	10.3	1.0	1.2	0.5	1.6	1.0	6.1	0.5	2.9	1.0	2.0	0.5	1.6	1.0	1.7	0.5	2.8
6	1.0	7.3	0.6	11.9	1.0	1.2	0.6	9.6	1.0	5.9	0.6	4.8	1.0	2.0	0.6	3.3	1.0	1.8	0.6	2.7
7	1.2	7.7	13.3	16.1	1.2	2.1	13.3	55.3	1.2	6.2	13.3	17.5	1.2	2.2	13.3	14.7	1.2	1.6	13.3	7.6
8	3.2	9.1	48.1	16.3	3.2	12.0	48.1	20.3	3.2	8.9	48.1	33.2	3.2	6.2	48.1	30.4	3.2	3.9	48.1	31.2
9	25.3	21.2	19.0	3.8	25.3	60.7	19.0	4.5	25.3	19.1	19.0	19.7	25.3	23.9	19.0	22.9	25.3	29.9	19.0	33.0
10	46.1	8.0	5.9	1.5	46.1	15.8	5.9	1.0	46.1	22.8	5.9	7.7	46.1	38.3	5.9	9.9	46.1	45.4	5.9	10.0
11	19.3	2.1	11.1	1.3	19.3	1.0	11.1	0.8	19.3	11.2	11.1	2.3	19.3	19.3	11.1	13.3	19.3	13.3	11.1	2.3

Data displayed in dark grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is in agreement with the maximum concentration of the compound; and data displayed in light grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is not in agreement with the maximum concentration of the compound.

Supplementary table 5. Percentage of α-tocopherol or α-tocotrienol (Cn) and band intensity (BI) of cell organelle markers separated by density gradient centrifugation for HepG2 cells transfected with an expression vector carrying the TTP cDNA (HepG2-TTP).

Fraction	Peroxisomes (Catalase)				Mitochondria (COX IV)				Endoplasmic reticulum (Calnexin)				Plasma membrane (Na^+-K^+-ATPase)				Lysosomes (LAMP1)			
	α-T		α-T3		α-T		α-T3		α-T		α-T3		α-T		α-T3		α-T		α-T3	
	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI
1	0.3	14.5	0.0	10.7	0.3	1.6	0.0	1.1	0.3	0.3	0.0	0.2	0.3	1.3	0.0	0.2	0.3	0.8	0.0	0.7
2	1.2	13.4	0.0	8.6	1.2	3.8	0.0	2.3	1.2	3.8	0.0	3.5	1.2	2.1	0.0	0.4	1.2	0.8	0.0	0.8
3	0.5	10.5	0.0	8.2	0.5	2.7	0.0	1.5	0.5	7.0	0.0	3.6	0.5	2.6	0.0	0.4	0.5	1.3	0.0	0.8
4	1.4	9.5	0.0	7.0	1.4	1.7	0.0	1.3	1.4	6.1	0.0	3.4	1.4	3.0	0.0	0.5	1.4	1.2	0.0	0.8
5	2.1	6.5	0.0	6.8	2.1	1.7	0.0	2.8	2.1	5.6	0.0	3.8	2.1	3.0	0.0	0.5	2.1	1.1	0.0	0.9
6	2.3	7.0	1.2	9.3	2.3	1.9	1.2	6.9	2.3	5.7	1.2	6.5	2.3	3.5	1.2	1.5	2.3	1.1	1.2	1.0
7	4.9	7.8	7.2	17.5	4.9	3.4	7.2	36.6	4.9	7.1	7.2	10.8	4.9	4.9	7.2	8.7	4.9	1.6	7.2	4.3
8	4.8	9.8	18.9	16.3	4.8	10.0	18.9	34.3	4.8	12.4	18.9	21.8	4.8	10.2	18.9	20.1	4.8	4.8	18.9	17.3
9	27.4	10.5	38.9	12.1	27.4	34.9	38.9	9.9	27.4	19.0	38.9	23.8	27.4	16.7	38.9	25.0	27.4	20.0	38.9	36.0
10	30.7	8.4	18.2	2.2	30.7	32.9	18.2	2.1	30.7	22.0	18.2	15.1	30.7	28.6	18.2	21.4	30.7	41.0	18.2	31.2
11	24.4	2.2	15.6	1.3	24.4	5.4	15.6	1.3	24.4	11.1	15.6	7.5	24.4	24.0	15.6	21.3	24.4	26.4	15.6	6.3

Data displayed in dark grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is in agreement with the maximum concentration of the compound; and data displayed in light grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is not in agreement with the maximum concentration of the compound.

Supplementary table 6. Percentage of γ-tocopherol or γ-tocotrienol (Cn) and band intensity (BI) of cell organelle markers separated by density gradient centrifugation for HepG2 cells.

Fraction	Peroxisomes (Catalase)				Mitochondria (COX IV)				Endoplasmic reticulum (Calnexin)				Plasma membrane (Na^+-K^+-ATPase)				Lysosomes (LAMP1)			
	γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3	
	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI
1	0.2	6.9	0.4	12.8	0.2	8.2	0.4	1.9	0.2	0.5	0.4	0.6	0.2	0.8	0.4	0.4	0.2	0.3	0.4	0.6
2	0.7	6.3	1.4	9.3	0.7	7.4	1.4	1.9	0.7	1.6	1.4	5.1	0.7	0.5	1.4	5.4	0.7	0.4	1.4	0.7
3	3.6	4.0	3.9	7.2	3.6	9.6	3.9	2.3	3.6	5.0	3.9	9.0	3.6	1.3	3.9	4.9	3.6	1.3	3.9	0.7
4	4.3	7.4	1.4	6.4	4.3	10.8	1.4	2.1	4.3	12.1	1.4	4.7	4.3	1.9	1.4	2.5	4.3	2.0	1.4	0.5
5	1.2	8.8	2.3	6.4	1.2	5.1	2.3	6.8	1.2	10.8	2.3	4.1	1.2	1.4	2.3	5.8	1.2	1.8	2.3	0.4
6	1.2	8.0	2.8	7.2	1.2	4.4	2.8	23.2	1.2	8.2	2.8	4.3	1.2	1.5	2.8	6.6	1.2	1.9	2.8	0.4
7	1.4	9.0	3.3	9.9	1.4	4.6	3.3	26.7	1.4	10.1	3.3	7.9	1.4	5.1	3.3	12.7	1.4	4.5	3.3	2.0
8	3.7	11.1	5.3	9.8	3.7	8.4	5.3	17.7	3.7	10.0	5.3	8.9	3.7	11.2	5.3	21.4	3.7	9.1	5.3	2.5
9	18.5	20.0	14.4	13.2	18.5	14.4	14.4	11.8	18.5	14.8	14.4	13.7	18.5	21.2	14.4	15.7	18.5	26.0	14.4	23.9
10	41.0	13.9	41.8	12.9	41.0	20.9	41.8	4.0	41.0	14.0	41.8	26.0	41.0	29.2	41.8	17.5	41.0	31.7	41.8	57.1
11	24.0	4.5	23.1	4.9	24.0	6.2	23.1	1.5	24.0	13.0	23.1	15.7	24.0	26.0	23.1	7.3	24.0	21.0	23.1	11.3

Data displayed in dark grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is in agreement with the maximum concentration of the compound; and data displayed in light grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is not in agreement with the maximum concentration of the compound.

Supplementary table 7. Percentage of γ-tocopherol or γ-tocotrienol (Cn) and band intensity (BI) of cell organelle markers separated by density gradient centrifugation for HepG2 cells transfected with an empty vector (HepG2-EV).

Fraction	Peroxisomes (Catalase)				Mitochondria (COX IV)				Endoplasmic reticulum (Calnexin)				Plasma membrane (Na^+-K^+-ATPase)				Lysosomes (LAMP1)			
	γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3	
	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI
1	0.6	9.9	0.1	12.3	0.6	9.0	0.1	1.6	0.6	3.1	0.1	3.8	0.6	23.0	0.1	2.2	0.6	3.3	0.1	2.5
2	1.9	10.8	1.9	9.4	1.9	15.2	1.9	2.6	1.9	7.7	1.9	7.1	1.9	5.2	1.9	0.9	1.9	4.7	1.9	2.2
3	3.4	11.2	0.3	6.8	3.4	10.6	0.3	3.4	3.4	8.9	0.3	6.1	3.4	6.1	0.3	1.2	3.4	4.6	0.3	1.1
4	1.6	7.8	0.6	7.4	1.6	8.3	0.6	6.8	1.6	6.9	0.6	5.4	1.6	3.5	0.6	2.8	1.6	2.6	0.6	1.3
5	1.0	9.0	1.5	8.1	1.0	5.6	1.5	4.7	1.0	5.4	1.5	6.3	1.0	3.1	1.5	5.6	1.0	1.5	1.5	3.6
6	1.7	8.6	1.9	8.6	1.7	5.8	1.9	3.0	1.7	4.6	1.9	6.3	1.7	3.4	1.9	9.3	1.7	1.4	1.9	4.9
7	1.8	9.0	10.5	9.2	1.8	7.4	10.5	6.6	1.8	5.9	10.5	9.3	1.8	4.9	10.5	11.9	1.8	1.9	10.5	10.0
8	6.2	11.0	7.9	10.1	6.2	18.0	7.9	30.8	6.2	9.3	7.9	12.4	6.2	20.2	7.9	17.2	6.2	8.5	7.9	13.2
9	36.4	12.0	25.3	17.5	36.4	16.1	25.3	31.4	36.4	16.7	25.3	15.1	36.4	19.3	25.3	19.1	36.4	30.8	25.3	23.6
10	31.5	8.2	26.1	7.4	31.5	3.3	26.1	8.1	31.5	19.0	26.1	13.8	31.5	9.0	26.1	20.7	31.5	28.5	26.1	30.2
11	13.8	2.7	24.0	3.1	13.8	0.8	24.0	1.0	13.8	12.6	24.0	14.4	13.8	2.4	24.0	9.0	13.8	12.1	24.0	7.4

Data displayed in dark grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is in agreement with the maximum concentration of the compound; and data displayed in light grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is not in agreement with the maximum concentration of the compound.

Supplementary table 8. Percentage of γ-tocopherol or γ-tocotrienol (Cn) and band intensity (BI) of cell organelle markers separated by density gradient centrifugation for HepG2 cells transfected with an expression vector carrying the TTP cDNA (HepG2-TTP).

Fraction	Peroxisomes (Catalase)				Mitochondria (COX IV)				Endoplasmic reticulum (Calnexin)				Plasma membrane (Na^+-K^+-ATPase)				Lysosomes (LAMP1)			
	γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3		γ-T		γ-T3	
	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI	Cn	BI
1	0.0	11.6	0.6	19.2	0.0	7.0	0.6	6.9	0.0	1.0	0.6	0.9	0.0	0.6	0.6	1.0	0.0	1.1	0.6	1.2
2	2.7	10.5	2.0	12.1	2.7	4.9	2.0	5.8	2.7	4.0	2.0	6.0	2.7	0.5	2.0	4.9	2.7	1.3	2.0	1.0
3	2.4	11.7	1.3	8.8	2.4	7.4	1.3	4.4	2.4	6.2	1.3	11.0	2.4	0.8	1.3	1.1	2.4	1.6	1.3	1.0
4	1.2	8.9	0.9	7.8	1.2	8.7	0.9	7.1	1.2	7.8	0.9	5.0	1.2	1.0	0.9	0.8	1.2	1.2	0.9	0.6
5	0.8	10.2	0.8	6.5	0.8	10.5	0.8	5.0	0.8	6.2	0.8	4.5	0.8	1.2	0.8	0.6	0.8	1.3	0.8	0.6
6	1.9	9.5	0.6	5.6	1.9	8.3	0.6	8.1	1.9	7.8	0.6	3.6	1.9	2.4	0.6	0.9	1.9	1.5	0.6	0.6
7	4.3	9.4	1.1	5.2	4.3	5.7	1.1	16.0	4.3	12.9	1.1	6.5	4.3	5.1	1.1	2.7	4.3	3.9	1.1	0.7
8	11.1	7.3	3.0	6.6	11.1	16.2	3.0	27.9	11.1	19.2	3.0	7.7	11.1	10.4	3.0	18.1	11.1	9.9	3.0	7.0
9	20.3	8.1	13.8	15.5	20.3	16.1	13.8	12.7	20.3	9.7	13.8	16.3	20.3	23.1	13.8	57.9	20.3	25.4	13.8	40.6
10	40.2	8.0	34.4	22.9	40.2	9.7	34.4	5.2	40.2	17.5	34.4	26.4	40.2	29.8	34.4	11.4	40.2	36.4	34.4	43.4
11	15.1	4.8	41.5	2.2	15.1	5.6	41.5	1.1	15.1	7.8	41.5	12.1	15.1	25.0	41.5	0.7	15.1	16.5	41.5	3.3

Data displayed in dark grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is in agreement with the maximum concentration of the compound; and data displayed in light grey boxes indicated that the maximum band intensity of the organelle marker in the fractions is not in agreement with the maximum concentration of the compound.

Supplementary table 9. Non-parametric Spearman´s rank order correlation between the percentage of α-tocopherol or α-tocotrienol and the percentage of band intensity of cell organelle markers separated by density gradient centrifugation for HepG2 cells and HepG2 cells transfected with an empty vector (HepG2-EV) or an expression vector carrying the TTP cDNA (HepG2-TTP).

Cell type	Correlation parameter	Peroxisomes (Catalase)		Mitochondria (COX IV)		Endoplasmic reticulum (Calnexin)		Plasma membrane (Na^+-K^+-ATPase)		Lysosomes (LAMP1)	
		α-T	α-T3	α-T	α-T3	α-T	α-T3	α-T	α-T3	α-T	α-T3
HepG2	r_s [1]	-0.6000	-0.4513	0.7727	0.2559	0.7182	0.7583	0.7182	0.8328	0.6636	0.8886
	P value [2]	0.0510	0.1635	0.0053	0.4476	0.0128	0.0068	0.0128	0.0015	0.0260	0.0003
HepG2-EV	r_s	-0.08182	-0.009091	0.6273	0.3364	0.9727	0.6818	0.9545	0.9364	0.8182	0.5455
	P value	0.8110	0.9788	0.0388	0.3118	< 0.0001	0.0208	< 0.0001	< 0.0001	0.0021	0.0827
HepG2-TTP	r_s	-0.4727	0.2479	0.7818	0.4958	0.8545	0.9439	0.9636	0.9249	0.8545	0.9439
	P value	0.1420	0.4624	0.0045	0.1209	0.0008	< 0.0001	< 0.0001	< 0.0001	0.0008	< 0.0001

[1] Spearman´s correlation coefficient (r_s) ranges from -1 to +1, with perfect positive correlation at value +1.

[2] Correlations are significant at the 0.05 level (two tailed).

Supplementary table 10. Non-parametric Spearman´s rank order correlation between the percentage of γ-tocopherol or γ-tocotrienol and the percentage of band intensity of cell organelle markers separated by density gradient centrifugation, for HepG2 cells and HepG2 cells transfected with an empty vector (HepG2-EV) or an expression vector carrying the TTP cDNA (HepG2-TTP).

Cell type	Correlation parameter	Peroxisomes (Catalase)		Mitochondria (COX IV)		Endoplasmic reticulum (Calnexin)		Plasma membrane (Na^+-K^+-ATPase)		Lysosomes (LAMP1)	
		γ-T	γ-T3	γ-T	γ-T3	γ-T	γ-T3	γ-T	γ-T3	γ-T	γ-T3
HepG2	r_s [1]	0.3455	0.1727	0.5909	0.2000	0.8636	0.9091	0.8909	0.7909	0.8909	0.7636
	P value [2]	0.2993	0.6147	0.0609	0.5574	0.0012	0.0003	0.0005	0.0055	0.0005	0.0086
HepG2-EV	r_s	0.2091	0.1364	0.1182	0.4455	0.9455	0.9545	0.1909	0.7091	0.8545	0.7909
	P value	0.5393	0.6937	0.7345	0.1730	< 0,0001	< 0.0001	0.5765	0.0182	0.0015	0.0055
HepG2-TTP	r_s	-0.7091	0.05455	0.1545	-0.1818	0.6455	0.9273	0.7909	0.4545	0.9636	0.7455
	P value	0.0182	0.8812	0.6538	0.5950	0.0368	0.0001	0.0055	0.1635	< 0,0001	0.0112

[1] Spearman´s correlation coefficient (r_s) ranges from -1 to +1, with perfect positive correlation at value +1.

[2] Correlations are significant at the 0.05 level (two tailed)

Chapter 4

The bioavailability of α-tocomonoenol in mice, contrary to α-tocopherol, is low but may also be controlled by the hepatic α-tocopherol transfer protein

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The bioavailability of α-tocomonoenol in mice, contrary to α-tocopherol, is low but may also be controlled by the hepatic α-tocopherol transfer protein

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Abstract

Tocomonoenols are structurally related to the vitamin E family of compounds and are distinguished from the saturated tocopherols and threefold unsaturated tocotrienols by their characteristic single double bond at carbon 11' in the sidechain. The hepatic α-tocopherol transfer protein (TTP) has a higher affinity for α-tocopherol (αT) than for all other vitamin E congeners and is required for the maintenance of normal αT concentrations in blood and tissues. Its role in the tissue distribution of α-tocomonoenol (αT1) is currently unknown. We therefore investigated the tissue distribution of αT1 compared to αT in 2-3 months-old female wild-type ($TTP^{+/+}$) and TTP knockout ($TTP^{-/-}$) mice fed a diet with either αT or αT1 for 2 weeks. Concentrations of αT and αT1 were measured in blood, small intestine, liver, lungs, heart, kidneys, spleen, adipose tissue, and brain. αT1 was only found in blood, not in tissues, at a concentration of ca. 40% of that of αT in $TTP^{+/+}$ mice (0.186 ± 0.079 μmol/L). αT concentrations in $TTP^{+/+}$ mice were in the order of adipose tissue > brain > heart > spleen > lungs > kidneys > small intestine > liver, and reached 0.560 ± 0.065 μmol/L in blood. As expected, loss of TTP function in $TTP^{-/-}$ mice resulted in almost complete depletion of αT in all tissues. Interestingly, αT1, contrary to αT, was still present in blood of $TTP^{-/-}$ mice. In conclusion, we found that αT1 is bioavailable and reaches the blood in mice, but does not accumulate in tissues. αT1 may not depend entirely on TTP function for its secretion in the systemic circulation.

Keywords:
α-tocopherol transfer protein knockout mice; α-tocopherol; α-tocomonoenol; adipose tissue; blood, liver; TTP; protein expression; depletion.

1. Introduction

Vitamin E comprises eight structurally related lipid-soluble compounds composed of a chromanol ring attached to a saturated (tocopherols (T)) or threefold unsaturated (tocotrienols (T3)) 16-carbon sidechain; with the prefixes α, β, γ, or δ designating the number and position of methyl groups substituted at the chromanol ring [1].

A novel vitamin E derivative with the structural feature of a single double bond at carbon 11' was reported for the very first time in 1995, named α-tocomonoenol (αT1; Figure 1) [2], and later detected in palm, pumpkin, and sunflower oils [3-5].

From the gastrointestinal tract, all eight vitamin E congeners are absorbed and transported to the liver to a similar extent, but then the liver selectively secretes α-tocopherol (αT) into the bloodstream for distribution in the body, while the non-αT forms are preferentially metabolized via a cytochrome P450-dependent pathway [6]. It has been suggested that the selective retention of αT is the result of an interaction of the catabolic pathway with the hepatic α-tocopherol transfer protein (TTP) [7], a cytosolic protein that preferentially binds αT (100%) over the others congeners, β-tocopherol (38%), γ-tocopherol (9%), δ-tocopherol (2%) and α-tocotrienol (12%) [8].

TTP is expressed primarily in liver, but has been detected in other tissues as rat brain, spleen, lung, and kidney [9]; in rat uterus [10] and eye retina [11], suggesting that its expression in other organs regulates distinct tissue-specific accumulations of the vitamin [12].

Humans with mutations in the *Ttpa* gene encoding TTP develop ataxia with vitamin E deficiency (AVED) and are unable to maintain normal αT plasma concentrations [6, 12].

Previous studies into the tissue distribution of αT alone or in combination with their homologues all-rac-αT, α-tocotrienol, γ-tocopherol and γ-tocotrienol reported a wide distribution of αT in blood and tissues, primarily in the liver, lungs, spleen and brain; the deletion of the *Ttpa* gene in mice (TTP knockout mice) leads to the depletion of αT from all tissues [7, 13-22].

The uptake of dietary αT1 and its tissue concentration have hitherto only been studied in a single trial in mice, in which αT1 was detected in the liver and brain [23].

A deeper understanding of the tissue distribution and accumulation of αT1 and their regulation, potentially by TTP, is required to elucidate its potential to exert the biological activity of vitamin E in mammals. We therefore investigated the tissue distribution of αT1 and αT in wild-type and TTP knockout mice ($TTP^{-/-}$) administered identical dietary doses for two weeks.

2. Materials and Methods

2.1. Test compounds and diets

RRR-α-tocopherol (αT, ≥95%, CAS number 59-02-9, cat#KP5101) was from DSM (Grenzach, Germany) and R-α-tocomonoenol (αT1; ≥99.5% pure, extracted from vitamin E capsules as previously described [24]) was a kind gift from Prof. Dr. Walter Vetter (Institute of Food Chemistry, University of Hohenheim, Germany). αT and αT1 (165 mg each) were by separately dissolved in 1 mL of ethanol and mixed thoroughly in 275 g of vitamin E-stripped corn oil (Dyets, Bethlehem, PA, USA). The fortified oils were used in the preparation of semisynthetic standard rodent diet (vitamin E free standard diet, C1000; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) containing 5% oil and 30 mg/kg of αT respectively αT1.

2.2. Animal experiment

All animal procedures were carried out in accordance with the FELASA guidelines for the care and use of laboratory animals and approved by the regional presidium Stuttgart (Baden-Württemberg, Germany; trial no. V 342/17 BC). Forty-four female mice (2-3 months old, 21.2 ± 0.6 g) from our colony at the University of Hohenheim were fed for two weeks the αT-containing diet before twenty-two C57BL/6 wild-type mice ($TTP^{+/+}$) and twenty-two homozygous TTP knockout mice ($TTP^{-/-}$, genotype confirmed by PCR) were randomized into four groups of 11 animals and animals from each genotype were fed the vitamin E free standard diet (modified C1000; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) fortified with either 30 mg/kg of αT or αT1. Concentrations of αT and αT1 in the diets were confirmed by HPLC and were ≤ 1 mg/kg of αT or αT1 (see Supplementary Figure 1). Mice were housed in groups of maximum 4 per cage in a controlled environment (22 ± 2 °C, 55 ± 10% humidity, 12 h light/dark cycle) and had free access to feed and water. After 2 weeks, animals were fasted for 12 h, anaesthetized with CO_2, and killed by cervical dislocation. Blood was collected into heparinized tubes, and tissues (small intestine, liver, lungs, heart, kidneys, spleen, adipose tissue and brain) were excised and snap-frozen in liquid nitrogen. All samples were immediately stored at -80 °C until further analysis.

2.3. HPLC analysis

All chemicals used were of highest purity and purchased from Sigma-Aldrich (Taufkirchen, Germany), JT Baker (Phillipsburg, United States) or Merck (Darmstadt, Germany). Methanol was HPLC gradient grade and water was deionized and filtered (Millipore, Billerica, MA, USA). αT and αT1 were extracted from tissues (200 mg) and whole blood (100 µL) and saponified as previously described [25]. Prior to HPLC analysis, extracts were re-suspended in 100 µL methanol/water (85:15, v/v) and transferred to amber HPLC vials. Twenty microliter of the extract was injected into a Jasco HPLC (system controller LC-Net II/ADC, two pumps X-LCTM 3185PU, mixing unit X-LCTM 3180MX, degasser X-LCTM 3080DG, autoinjector X-LCTM 3159AS, column oven X-LCTM 3067CO, fluorescence detector FP-2020 Plus; Jasco, Germany). Test compounds were separated on a Phenomenex KinetexTM PFP column (2.6 µm particle size, 150 x 4.6 mm) maintained at 40 °C, using methanol/water (85:15, v/v) at a flow rate of 1.7 mL/min, for a total run of 15 minutes. The fluorescence detector was operated at excitation/emission wavelengths of 296/325 nm, peaks were

recorded and integrated using Chrompass software (version 1.9. 302.1124, Jasco), and quantified against external standard curves using the authentic compounds.

2.4. GC/MS analysis

To confirm the presence or absence of αT and αT1, crude extracts of liver samples were redisolved in 500 µL n-hexane, treated by column chromatography for purification and analyzed by gas chromatography with mass spectrometry (GC/MS) as previously described [24].

2.5. Western blot analysis for TTP expression

Liver protein homogenates were prepared in radioimmunoprecipitation assay buffer (Tris, 50 mM; NaCl, 150 mM; sodium dodecyl sulfate (SDS), 0.1%; sodium deoxycholate, 0.5%; Triton X100, 1%; EDTA, 20 mM (pH 7.2); dithiothreitol, 1 mM; protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany)) and stored at -80 °C until further analyses. The amount of protein in the supernatant was determined via Bradford assay [26] and 40 µg of protein per lane was separated by 10% SDS gel electrophoresis and transferred to polyvinylidenefluoride (PVDF) membranes, blocked for 1 h at room temperature in blocking buffer (5 % bovine serum albumin (BSA; Sigma-Aldrich, Taufkirchen, Germany) in tris-buffered saline Tween-20 (TBST: 0.8 % (w/v) NaCl, 0.24 % (w/v) Tris-HCl (pH 7.6), 0.05 % (v/v) Tween 20 in H2Odd; Sigma-Aldrich, Taufkirchen, Germany)) and incubated with the primary antibodies (TTP (1:1000, ab155323); β-actin (1:1000, #4967, Cell Signaling Technology, Danvers, USA)).

The primary antibodies were diluted in 5% BSA in TBST and incubated overnight at 4 °C. Membranes were washed three times with TBST and incubated for 1 h at room temperature with the secondary antibody (goat anti-rabbit peroxidase conjugated (1:10000, cat#401353, Calbiochem/Merck Millipore, Darmstadt, Germany)). Membranes were washed three times with TBST and bands visualized using AceGlowTM Essential chemiluminescence solutions A and B (Peqlab Biotechnologie GmbH, Erlangen, Germany) and 20X LumiGLO® Reagent and Peroxide Solutions (Cell Signaling Technology, Cambridge, UK), and intensities were recorded on a Fusion FX imaging system and band intensities were quantified using the FusionCapt Advance software (Vilber Lourmat, Eberhardzell, Germany). Expression of the protein α-TTP was tested using the housekeeping protein β-actin as loading control.

2.6. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Differences between group means were calculated by One-way Analysis of Variance with a Tukey's multiple comparison test or by unpaired t-test with Welch's correction (heterogeneity of variances). Reported values represent means ± standard error of the mean (SEM). Differences were considered significant at $P<0.05$.

3. Results and discussion

To investigate the tissue distribution of αT1 and its regulation by TTP, we fed 2-3 months-old wild type ($TTP^{+/+}$) and TTP knockout mice ($TTP^{-/-}$, Figure 2) diets containing 30 mg/kg αT1 or αT for two weeks. No significant differences in body weight gain, final body weight, and absolute and relative (referred to body weight) organ weights were observed between the experimental groups (data not shown), indicating a normal, healthy and comparable performance of the mice during the experiment.

To the best of our knowledge is the first time that the presence of αT1 is reported in the blood of $TTP^{+/+}$ and $TTP^{-/-}$ mice. In a previous mouse trial, αT1 was found in the liver and brain, but in agreement with our findings, not in the lung and spleen [23].

αT was present in the blood and all examined tissues, with the exception of the liver, of $TTP^{+/+}$ mice fed αT or αT1 (Figure 3). Interestingly, αT concentrations in the blood of $TTP^{+/+}$ mice fed αT1 were numerically higher, but not significantly different, than in the $TTP^{+/+}$ mice fed αT (Figure 3H). αT was also present in αT1-fed mice, probably because all animals received αT-containing diets until the beginning of the trial, and the short duration of the trial did not result in a depletion of αT in the tissues. Another potential explanation, which could also partly explain this and thus deserves further investigation, is the conversion of αT1 to αT by the saturation of the side-chain. Such a side-chain saturation would be in line with the observed concentrations of αT in the blood and tissues of $TTP^{+/+}$ mice fed αT1. Earlier investigations reported that the metabolism of tocotrienols involves enzymes, probably 2,4-dienoyl-coenzyme A-reductase, that catalyze the saturation of the side-chain, similar to those involved in the β-oxidation of unsaturated fatty acids [27].

αT concentrations in αT-fed $TTP^{+/+}$ mice were in the order of adipose tissue > brain > heart > spleen > lungs > kidneys > small intestine > liver, and reached 0.560 ± 0.065 μmol/L in blood (Figure 3). αT1 was only found in blood, not in tissues, at a concentration of ca. 40% of that of αT in $TTP^{+/+}$ mice (0.186 ± 0.079 μmol/L; Figure 4). Relatively high αT concentrations in adipose tissue was reported before [13, 16, 28]; and was explained by the comparably low turnover of adipocytes, which slowly accumulate and release αT [16, 29]. Adipose tissue was also reported as the main storage for α- and γ- tocotrienols and MDT [14, 16, 18, 28, 30].

As expected, deletion of the *Ttpa* gene in mice resulted in almost complete depletion of αT from tissues and blood (Figure 3), highlighting its importance for the maintenance of an adequate αT status, as previously reported [7, 13, 22, 31]. In agreement with our understanding of the role of TTP in mediating the selective secretion of αT from the liver into the bloodstream [6], αT concentrations in the small intestine were not under the control of TTP and therefore similar in $TTP^{+/+}$ and $TTP^{-/-}$ mice.

Interestingly, αT1 was numerically reduced in the blood of $TTP^{-/-}$ mice, but contrary to αT, it was still present in the blood (Figure 4). Earlier observations reported a TTP-independent distribution of α-tocotrienol into the bloodstream [15]. Nevertheless, our results cannot rule out that TTP is involved in the distribution of αT1. The observed reduction in αT1 concentrations in the blood of $TTP^{-/-}$ mice suggest that αT1 may not require TTP activity to the same extent as αT for the secretion into the blood (Figure 3H), but there may still be a TTP-influence on αT1 bioavailability.

Overall, αT concentrations in tissues were ca. 10-fold lower than the values reported in tissues of mice and rats fed diets containing 30-100 mg αT per kg diet [7, 13, 16, 18, 28]. These low

αT concentrations in tissues are more similar to values reported in mice and rats after feeding of αT-deficient or αT-free diets [16, 28, 32].

The unexpected depletion of αT and αT1 in the livers of our mice was confirmed by GC/MS analyses (see Supplementary Figure 2, data interpretation based on previous publication [24]). It was previously reported that αT is secreted from the liver at a higher rate, thus possibly depleting the liver of αT, when the intake of the vitamin is inadequate [29, 33]. A complete reduction of αT in the liver was reported before in $TTP^{-/-}$ mice fed a vitamin E-depleted diet from the age of 3 to 18 months [31], for male Wistar rats fed a diet containing < 0.4 µg/g αT for 6 weeks followed by an αT-free diet for 7 days [28]; and an almost complete depletion of αT in the liver was observed in male Fisher 344 rats after 6 months feeding with an αT-deficient diet [34], as well as in male Wistar rats fed a vitamin E-free diet from 6 until 10 weeks old [16].

The low αT concentrations in tissues and the depletion of αT and αT1 in the livers made us questioning about the integrity of the vitamin E in the experimental diets. It is possible that the vitamin E added to vitamin E-free corn oil to prepare our experimental diets was rapidly oxidized and the final concentrations in the diets might thus have been significantly lower than planned. We therefore retrospectively measured the concentrations of αT and αT1 in the experimental diets, which were supposed to be 30 mg/kg, but indeed were ≤ 1 mg/kg diet (Supplementary Figure 1).

Even though we cannot currently rule out alternative explanations, it is possible that the low intake of αT in our animals led to a redistribution of αT from the liver to peripheral tissues, resulting in the observed depletion of vitamin E from this organ and the lower values in the other tissues.

4. Conclusion

In conclusion, we report, for the first time, the absorption of αT1 into the blood of mice. Contrary to αT, αT1 was still found in the blood of mice not expressing TTP, but at lower concentrations than in wild-type mice. These results suggest that αT1 secretion from the liver may not depend to the same extent as αT on the function of TTP, but may still be partly controlled by it.

Conflict of interest

The authors declare no competing financial interest.

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List of abbreviations

αT, α-tocopherol; αT1, α-tocomonoenol; MDT, marine-derived tocopherol; $TTP^{+/+}$, wild-type mice; $TTP^{-/-}$ homozygous TTP knockout mice; TTP, α-tocopherol transfer protein.

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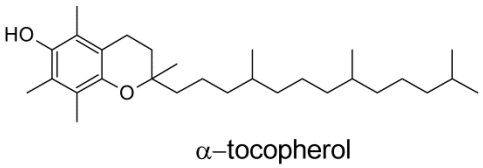
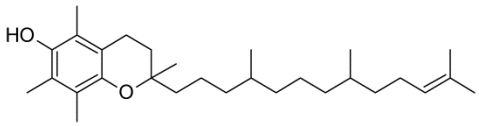


α–tocopherol



α–tocomonoenol

Figure 1. Chemical structures of α-tocopherol and α-tocomonoenol.

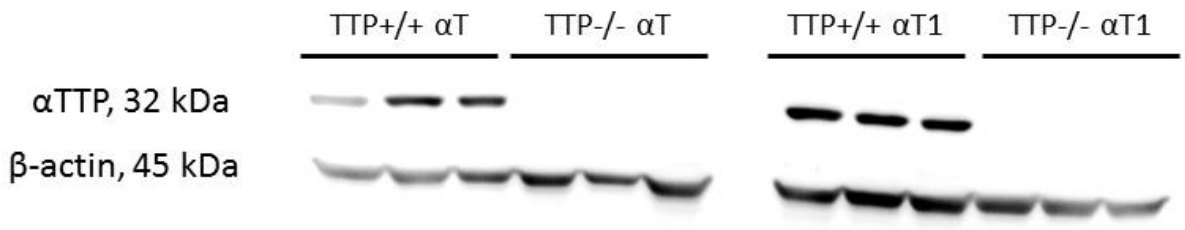


Figure 2. Representative Western blots of hepatic α-tocopherol transfer protein expression in $TTP^{+/+}$ and $TTP^{-/-}$ mice (n=11) used in the feeding trial.

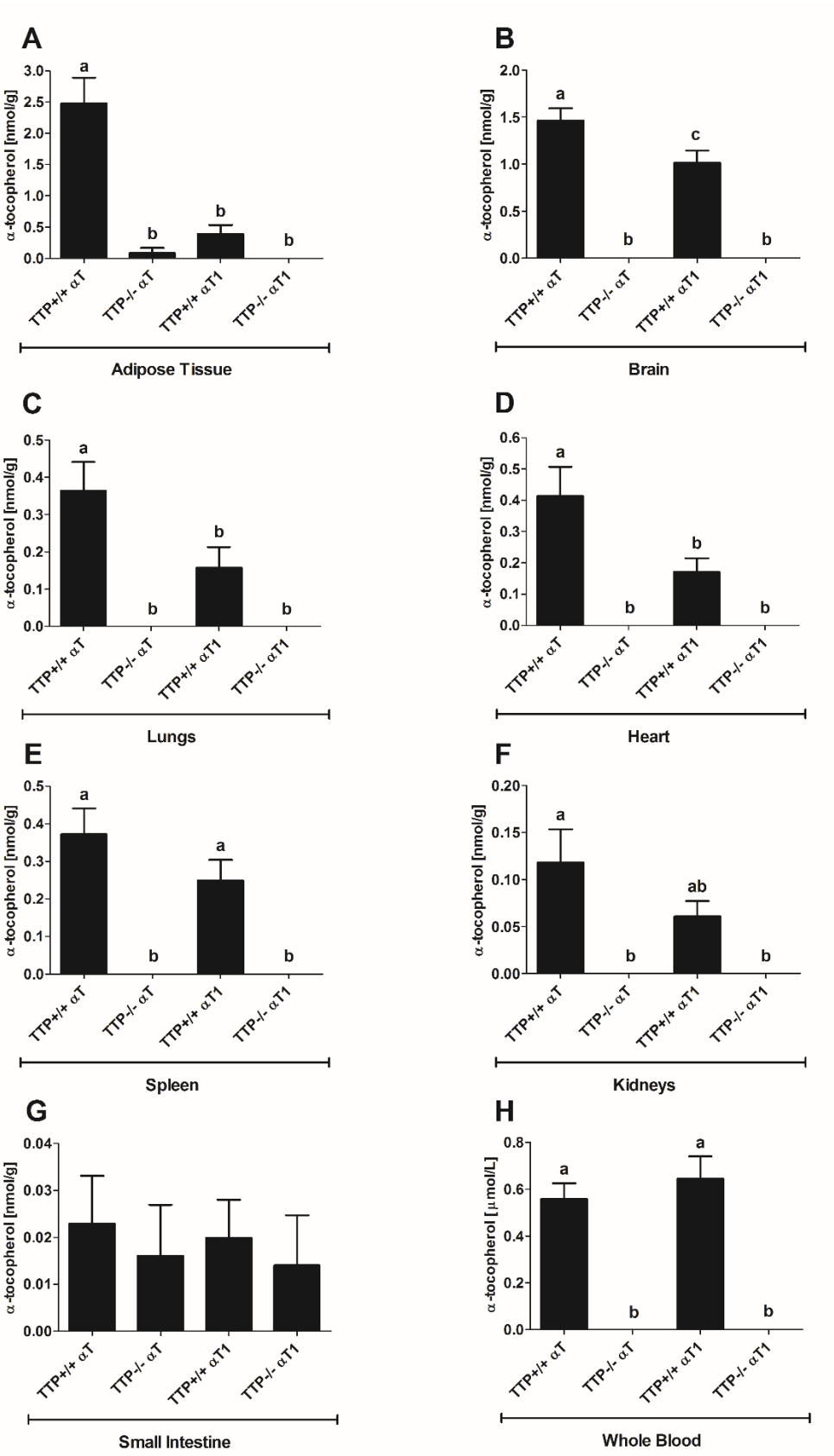


Figure 3. Mean tissue concentrations (error bars represent standard error of the mean; n = 11) of αT in $TTP^{+/+}$ and $TTP^{-/-}$ mice fed a standard diet with either αT or αT1 for 2 weeks. Bars not sharing a common letter are significantly different at $P<0.05$.

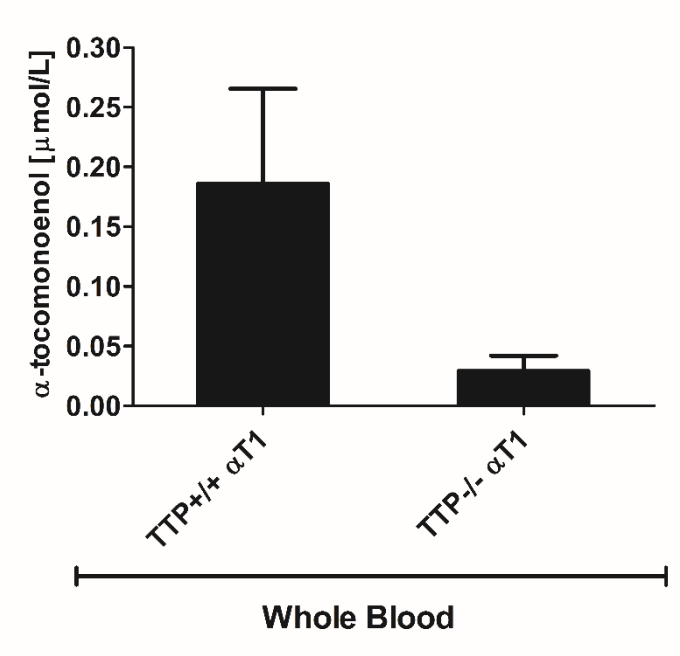
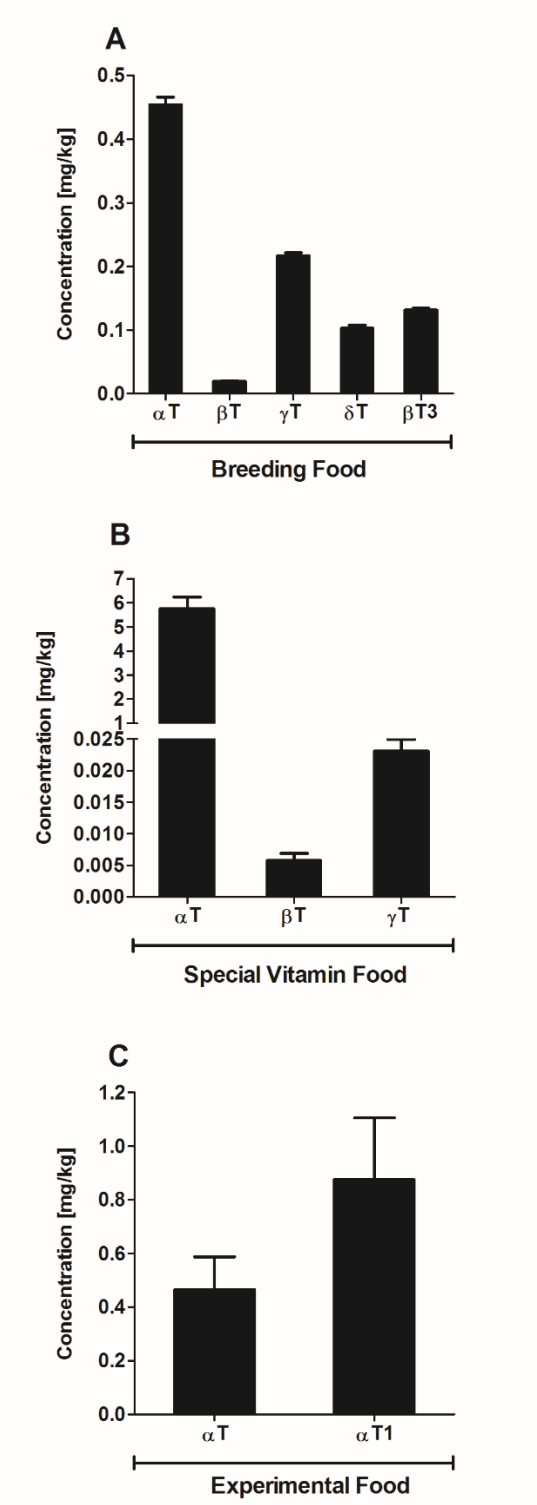
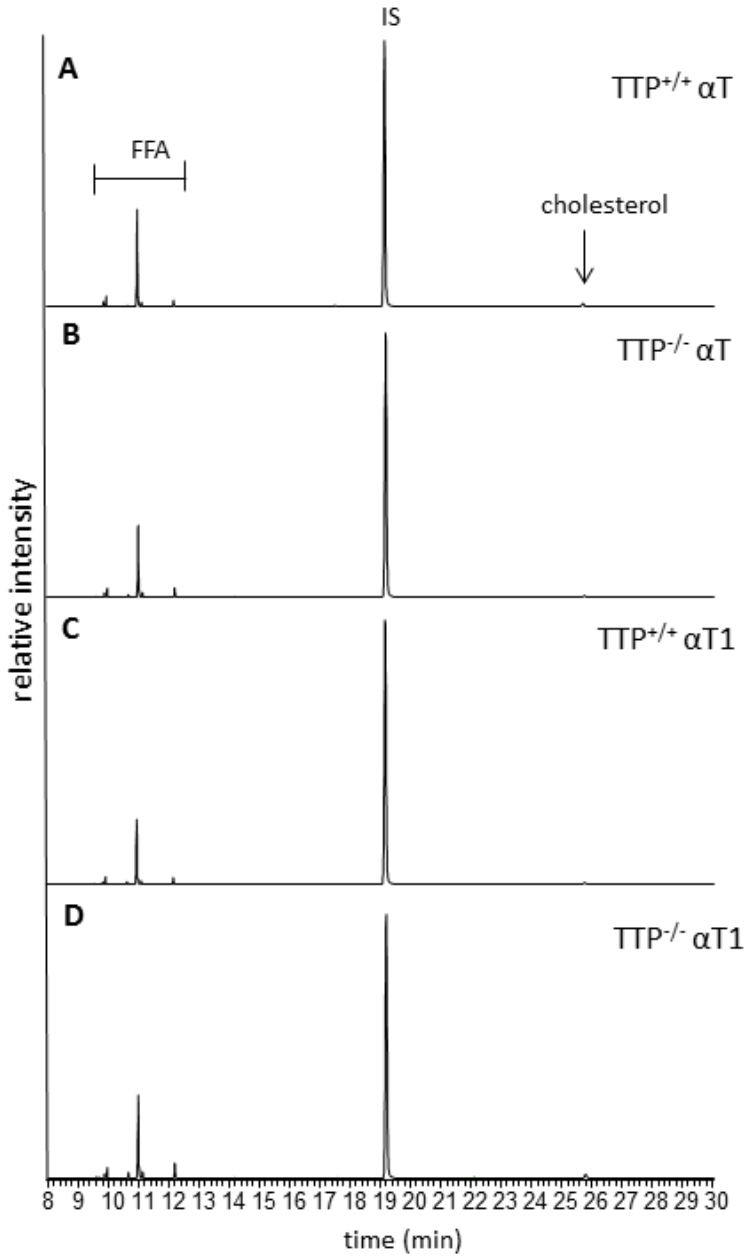


Figure 4. Mean whole blood concentration (error bars represent standard error of the mean; n = 11) of αT1 in $TTP^{+/+}$ and $TTP^{-/-}$ mice fed a standard diet with either αT or αT1 for 2 weeks.



Supplementary Figure 1. Mean concentrations (error bars represent standard error of the mean; n = 3) of vitamin E in the breeding (A), special vitamin (B) and experimental (C) foods fed the $TTP^{+/+}$ and $TTP^{-/-}$ mice during their life. The breeding food is produced by ssniff Spezialdiaeten GmbH (Soest, Germany) and the special vitamin and experimental diet are produced by Altromin Spezialfutter GmbH & Co (KG, Lage, Germany).



Supplementary Figure 2. Representative GC/MS chromatograms (full scan mode) of the analyzed liver extracts of TTP+/+ and TTP-/- mice fed a standard diet with either αT (A and B, respectively) or αT1 (C and D, respectively) for 2 weeks. Peaks from 10-12 minutes belong to free fatty acids (FFA), peak at 19 minute is the internal standard (IS) 5-alpha-cholestane and the slight peak at 26 minute corresponds to cholesterol. αT and αT1 were reported to appear at 24 and 25 minutes, respectively, and their absences were noted in all experimental groups.

Chapter 5

Concluding discussion

The present thesis aimed at increasing our knowledge of the non-αT congeners of vitamin E with respect to their occurrence in food, their intracellular localization upon uptake into liver cells, and their tissue distribution in mammals. Because the intracellular localization and tissue distribution of the main vitamin E congener αT is under the control of the hepatic TTP, the role of this protein in the intracellular and intra-organismic trafficking of the non- αT congeners was a second focus of the current investigations.

The **first aim** of the thesis, namely to determine the vitamin E profiles of Costa Rican palm oils, was the basis for the subsequent aims and experiments. The Ph. D. project started with an inventory of the vitamin E profile and content in oils of Costa Rican Palm fruits. To this end, six varieties and two extraction procedures were studied. The used varieties belong to the *Elaeis* species with industrial importance in the palm oil market of Costa Rica and were selected in cooperation with ASD Costa Rica Breeding Division. In total, three *Elaeis Guineensis*, two *Elaeis Oleifera* and one interspecific hybrid varieties were selected for detailed analyses.

The two oil extraction techniques selected, chemical and mechanical extraction, are the two most common and commercially relevant extraction procedures. Chemical extraction mainly uses hexane as a solvent, which extracts the majority of lipids and thus has a higher oil yield than mechanical extractions. A disadvantage is the co-extraction of unwanted artifacts, such as waxes, which compromises the oil quality. The required subsequent purification process to ensure a complete removal of solvent residues in the oil is time- consuming, costly and environmentally unfriendly. Mechanical extraction is therefore the preferred procedure in the palm oil industry, even though the oil yield is lower than chemical extraction, the procedure is cleaner, cheaper, and less time-consuming [57, 58].

The obtained results **(Chapter 2)** demonstrated that in all varieties and irrespective of the extraction procedure, α- and γ-tocotrienols were the most abundant congeners in Costa Rican palm oils. Oils from five of the six palm varieties also contained α-tocomonoenol, one of the more recently discovered vitamin E derivatives bearing a single double bond at carbon 11' **(Chapter 2)**.

Once the two most abundant tocotrienols (αT3 and γT3) were identified during the characterization of the oils, they were selected for further studies with respect to their cellular uptake and intracellular distribution in cultured liver cells. This **second aim** of the thesis addressed, for the first time, the intracellular trafficking of these compounds and compared them to their respective tocopherol counterparts (αT and γT). In order to elucidate whether or not the TTP is involved in the intracellular trafficking of these vitamin E congeners, a human hepatoma cell line (HepG2) expressing TTP was used for the experiments and compared to the parent HepG2 clone not expressing TTP. An optimized method of subcellular

fractionation with self-generated gradients of Iodixanol, an iodinated density gradient media capable of organelles isolations of 80-90% yield and high purity [59], was used and reproducibility of the separation was monitored with the density of the Iodixanol **(Chapter 3)**.

αT1, which was detected in the Costa Rican Palm oils, was also reported in other fruits and oils (see introduction). There is, however, a lack of studies regarding its behavior *in vivo*. The **third aim** of the thesis was therefore to characterize the tissue distribution of αT1 in mice in comparison to αT and to investigate the influence of TTP **(Chapter 4)**. To this end, a rodent model with mice deficient in TTP ($TTP^{-/-}$) and TTP-expressing wild type ($TTP^{+/+}$) mice was used. An animal model was required to study tissue distribution, but also because the metabolic enzymes in the liver (CYP, enzymes of β-oxidation) and TTP in combination with the enzyme systems in other organs, such as the small intestine and kidney, control the flow of vitamin E in the body and its excretion [60].

The main results of the present thesis are summarized below:

Characterization of vitamin E profile in oils of Costa Rican Palm fruits extracted by chemical and mechanical extraction

- All eight vitamin E congeners, α-, β-, γ- and δ-tocopherols and -tocotrienols, and α-tocomonoenol were detected in the six varieties of palm oils extracted (*E. Guineensis* Tanzania, *E. Guineensis* Deli Dami, *E. Guineensis* Deli x Nigeria, *E. Oleifera* Quepos, *E. Oleifera* Manaos and Hybrid OxG).
- The concentrations of the vitamin E congeners in the oils were in the order of γT3 > αT3 > δT3 > αT ≥ βT3 > γT ≈ βT ≈ δT among the studied varieties, irrespective of the genotype and extraction procedure.
- Total vitamin E content of the palm oils is almost entirely made up of tocotrienols (>91%), and the genotype Quepos had by far the highest total vitamin E content, which consisted of 99.8% tocotrienols.
- Total vitamin E content ranged from 163-525 μg/g for the *Elaeis Guineensis*, 254-892 μg/g for the *Elaeis Oleifera* and 222-471 μg/g for the Hybrid OxG.
- Hexane extraction yielded up to 2.5-fold higher total vitamin E compared to screw press extraction (hexane extraction, 333-892 μg/g; screw press extraction, 163-624 μg/g); and the differences in the vitamin E extraction efficiencies were due to vitamin E losses in the byproducts (residual peel, residual press cake, centrifugation byproduct).
- The αT1 contents of the analyzed oils were, irrespective of the extraction method, in the order *E. Guineensis* Tanzania (3.0-2.73 μg/g) > Hybrid OxG (0.59-1.39 μg/g) = *E. Guineensis* Deli x Nigeria (0.677-0.85 μg/g) > *E. Oleifera* Quepos (0.20-0.42 μg/g) = Manaos (0.18-0.48 μg/g) > *E Guineensis* Deli Dami (n.d.-0.078 μg/g).
- In standard reversed-phase HPLC analyses, αT1 eluted between βT and γT and partly overlapped with the latter. A co-elution of the αT1 with γT was observed for the pumpkin seed oil, which is characterized by its high content of γT (63.5%). This co-

elution may thus, particularly when αT1 is present at appreciable concentrations, lead to an overestimation of the γT content of foods.

As detailed in **Chapter 2**, the concentrations and relative abundances of the tocotrienols and tocopherols in the palm oils analyzed are in agreement with previous studies, even though the values are subject to large variability. Tocotrienols are by far the most abundant tocochromanols in the palm oils. Vitamin E congeners identified in the palm oils are the same, irrespective of the genotype and extraction procedure, and although the order of abundance of the congeners follows the same path for all genotypes, their total concentration and as consequence the total vitamin E content varies. The fact that total vitamin E was overall in the order of *Elaeis Oleifera* ≥ Hybrid OxG ≥ *Elaeis Guineensis* supports the genetic background inherent to the varieties. *Elaeis Oleifera* has a higher content of vitamin E than the *Elaeis Guineensis*, and through the hybridization, the breeders aimed at achieving a variety with a vitamin E content between the *Elaeis Guineensis* and *Elaeis Oleifera* [61].

The observation that the differences in the vitamin E yields between extractions are caused by losses with the extraction byproducts, established the importance of the maximum utilization of the fruit parts during processing. As mentioned in **Chapter 2**, reported concentrations of vitamin E retained in the palm fruit fiber are 2-fold higher than the concentrations in the crude oil. Chemical extraction can be improved by extracting the peel together with the mesocarp and mechanical extraction can be enhanced by changing to hydraulic press and adding water during the pressing process, enhancing the leaching of the oil out of the press cake [58]. The design of the screw press allows the entrapment of fruit debris in the equipment, reducing the oil released.

The obtained results of this first part of the project support the importance of palm oil as one of the few good sources of tocotrienols. Tocotrienols have many health beneficial activities (e.g. antioxidant, hypocholesterolemic agent) and may have a role in the prevention of degenerative diseases, including cardiovascular and age-related diseases and cancer [12]. As palm oil tree is the sixth major crop cultivated in Costa Rica [62], its promotion among agricultural and food industries may contribute to ensuring food security in Costa Rica.

The role of the α-tocopherol transfer protein in the cellular uptake and intracellular distribution of α- and γ-tocotrienols

- Tocotrienols were taken up by the cells faster than the tocopherols and the γ-congeners more than the α-congeners, irrespective of the expression of TTP, initially suggesting that the methylation of the chromanol ring and the sidechain saturation are important factors on the cellular uptake.
- A steady uptake into the cells over time was observed until 24 h, afterwards the concentrations slightly declined, suggesting the start of the metabolic degradation of the congeners.
- A significant instability of the congeners in the order of αT > αT3 > γT3 > γT was observed in the cell culture medium upon addition and further declines were observed after 30 minutes. This questioned if the observed trend in the cellular absorption of the

congeners is due to differences in the cellular uptake and suggested that the true reason may be differences in the stability of the vitamin E congeners in the medium.

- Intracellular distribution indicated that, irrespective of TTP-expression in liver cells, the four congeners correlated mainly with the markers for the endoplasmic reticulum, plasma membrane, and lysosomes, but not with those for the peroxisomes, and only αT correlated with mitochondria.
- The only influence of the TTP on the intracellular distribution was observed for the localization of the γT in peroxisomes, which negatively correlated with the organelle in the presence of the protein, suggesting a prevention of the transport of γT to peroxisomes, and therefore to its metabolism.
- TTP expression correlated mainly with the organelle markers for the endoplasmic reticulum, plasma membrane and lysosomes, which is in agreement with the understanding of its trafficking in hepatocytes and its role in preventing vitamin E metabolism.

The results obtained in this cell culture experiment revealed that the structural differences (chromanol ring methylation pattern or sidechain saturation) between αT, αT3, γT3 and γT are not important factors behind the cellular uptake and intracellular distribution, since no significant differences were observed between congeners. The results further demonstrate that the expression of the TTP does not regulate the intracellular trafficking (uptake and distribution) of the congeners in the cultured liver cells.

Even though in the early stage of the cellular uptake experiments, the T3 and γ-congeners were apparently taken up faster, the fact that the faster increase of the T3 in cells were not accompanied by a faster decrease in T3 concentrations in culture medium, gave the idea that the differences in uptake might be due to a stability problem of the T over T3 rather than a better uptake of T3.

As discussed in the **Chapter 3**, an increased uptake of the T3 over T was reported before in other cell culture models, and was attributed to the higher intermembrane mobility of the T3. However, the instability of the studied congeners directly upon addition and afterwards in the culture medium does not allow stating an effect of the chemical structure in the cellular uptake. Possible explanations behind the lack of stability of the congeners are summarized in **Chapter 3**. In future experiments, it would be important to improve the stability of the compounds in the culture medium before conducting the cultured experiments, for example by using different solvents to dissolve the compounds and/or adding antioxidants to the medium (e.g. vitamin C and butylated hydroxytoluene).

The lack of influence of TTP on cellular uptake of the congeners was not unexpected. It had been reported before that other proteins, the low-density lipoprotein receptor-related protein (LRP) or the scavenger receptor class B type I (SR-BI) mediate the uptake of vitamin E into hepatocytes [63, 64].

The observed intracellular distribution of the congeners and of TTP among the organelles is in line with the understanding of the intracellular trafficking of αT. When αT enters the hepatocyte, it is transported to the late endosome (e.g. lysosomes), where TTP translocates

from the cytosol to mediate αT transport from the late endosomes to the plasma membrane. Then the protein interacts with phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane, which causes a conformational change that releases αT into the plasma membrane. Once released, αT exits the plasma membrane through an ABC-type transporter. TTP translocates again to αT-containing organelles to repeat the cycle [20, 43].

The positive correlations of the intracellular congener concentrations with the endoplasmic reticulum and lack of correlation with the peroxisomes and mitochondria (except for αT) agree with the function of these organelles in vitamin E metabolism. These are the sites where the first, second, and third steps in the metabolism of vitamin E occur (see **Chapter 3**). Congeners are present in the endoplasmic reticulum at the beginning of the degradation, but afterwards the metabolites rather than the parent compounds are expected to localize in the peroxisomes and mitochondria.

The positive correlation of TTP with the endoplasmic reticulum and the negative correlation of γT with the peroxisomes in the presence of TTP, suggest a role of the protein in the metabolism of the congeners, in particular γT. As reported before, in cultured cells TTP reduced the metabolism of γT, suggesting that binding of the non-αT congeners to TTP partially protects their side-chain from ω-hydroxylation (first degradation reaction) [44].

The results obtained during this part of the project gave novel insights into the intracellular sites where the non-αT congeners reside and thus provides novel opportunities for the investigation of potential biological activities of these compounds in these organelles. The finding that TTP lacks a significant role in the uptake and intracellular distribution of the vitamin E congeners supports previous findings that stablished TTP is involved in the secretion of mainly αT from the liver cells. As described in **Chapter 3**, simple passive diffusion or organelle-lipid content can be driving forces behind the distribution of the congeners within the cell.

The role of the α-tocopherol transfer protein in the tissue and blood distribution of α-tocomonoenol

- αT1 was only found in blood, not in tissues, in wild-type ($TTP^{+/+}$) mice at a concentration of 0.186 ± 0.079 μmol/L, approximately 40% of the concentration of αT in the blood of the same type of mice.
- Accumulation of αT in wild-type ($TTP^{+/+}$) mice was in the order of adipose tissue > brain > heart > spleen > lungs > kidneys > small intestine > liver (from 2.48 ± 0.41 nmol/g in adipose tissue to undetectable in liver) and reached 0.560 ± 0.065 μmol/L in blood.
- Deletion of the TTP in knockout ($TTP^{-/-}$) mice resulted in almost complete depletion of αT in tissues and blood, with the exception of the small intestine, whereas no significant differences in αT concentrations were observed between the $TTP^{+/+}$ and $TTP^{-/-}$ mice.
- αT1 concentrations in blood of $TTP^{-/-}$ mice did not significantly differ from $TTP^{+/+}$ mice, but were numerically lower (ca. 25% of the concentration in $TTP^{+/+}$ mice).

Differences in the tissue and blood distribution of the αT1 compared to αT were observed. αT1 is bioavailable and reaches the blood in mice, but does not accumulate in tissues. Contrary to αT, αT1 was still detectable in the blood of $TTP^{-/-}$, suggesting that TTP may not, or only to a limited extent, be required for the secretion of αT1 into the systemic circulation.

The third part of the project gave, for the first time, insights into the overall tissue distribution of αT1, and how it can be influenced by the presence of TTP. Just one study was performed earlier on the accumulation of αT1 in liver, brain, spleen and lung of wild-type mice fed 2 weeks with a vitamin E extract from tuna oil, containing αT1, MDT, αT, βT, γT and δT. This study reported the presence of αT1 in liver and brain, not in spleen and lung [65]. However, the study has some loopholes. The exact αT1 dose administered is not clearly stated; instead the authors only gave the relative distribution of the different congeners in the feed. Therefore, the results of the tissue accumulation are limited and difficult to compare. The reported absolute concentration of the αT1 in the mouse tissues remains unclear. The novelty of our present research was the use of ≥ 99.5% pure αT1 instead of a vitamin E mixture, which ruled out possible influences of the co-ingestion of the other congeners on the bioavailability of αT1. We furthermore gave a more comprehensive overview of the tissue distribution, by analyzing a larger number of tissues.

$TTP^{-/-}$ mice are a valuable model to investigate the role of the protein on vitamin E trafficking and were successfully employed, as can be seen from the expected and observed depletion of αT from tissues and blood in the $TTP^{-/-}$ mice. The fact that TTP did not control the concentrations of αT in the small intestine is in agreement with its lack of involvement in the uptake of vitamin E into cells (see also **Chapter 3**) and its role in the liver, namely facilitating the secretion of αT into the blood (see also the discussion in **Chapter 4**). Regarding the importance of TTP for regulating αT1 concentrations, the current study is inconclusive. The fact that αT1, but not the TTP-ligand with the highest affinity, αT, was detectable in the blood of $TTP^{-/-}$ mice, suggests that TTP might not be required or importantly involved in its secretion. On the other hand, the blood concentrations of αT1 in $TTP^{-/-}$ mice were only ca. 25% of those measured in wildtype mice, which could be seen as evidence for a role of TTP in the secretion.

One of the biggest surprises observed during this experiment was the lack of αT in the liver of the mice, which was confirmed by GC-MS analysis. Therefore, we analyzed the concentrations of αT in the different diets fed to the mice during their life (the standard maintenance diet used in the colony, the high-αT diet used for breeding of $TTP^{-/-}$ mice, and the experimental diets) and found vitamin E values below the expected ones and in the range from marginal to deficient αT consumption (see discussion on **Chapter 4**). This undesired loss of αT in the foods during the experiment might explain the observed results in the liver of both mice genotypes. As explained in **Chapter 4**, a similar depletion of αT from the liver was observed before when diets deficient in αT were fed to rodents. The lack of dietary vitamin E might induce its acquisition from liver stores and secretion into the blood to satisfy αT requirements of the extrahepatic tissues.

We also observed a degradation of αT1 in the experimental diets, which might have contributed to the lack of accumulation of αT1 in the tissues. However, the observed presence

of αT1 in blood, despite the low content of the diet, is a promising result, as the bioavailability of αT1 is a requirement for further studies into any potential biological activities and health benefits.

Accomplishments and future research opportunities

Overall, the present thesis accomplished the aim of increasing our knowledge regarding food sources for the non-αT congeners αT3, γT3 and αT1 and their processing, intracellular trafficking, and in vivo tissue distribution in mice.

A first report on the vitamin E profile of oils from Costa Rican palm fruits and of αT1 concentrations in these varieties was achieved. Of particular interest is that the non-αT congeners represent the majority of vitamin E in this oil (see **Chapter 1**). A broader screening of potential food sources of non-αT congeners, especially αT1, should focus on lipid-rich foods not yet characterized, but consumed or underutilized, i.e. seed oils, algae, green leafy vegetables, and marine products.

Besides, the project described for the first time the cellular uptake and intracellular distribution of αT3 and γT3 in hepatic cells, and the tissue and blood distribution of αT1 in mice. The use of cultured cells and animal models capable of an expression, or not, of the TTP added value to the research, since the potential role of this protein in the trafficking of the non-αT congeners was addressed for the first time.

The research of the intracellular distribution of αT, γT αT3 and γT3 in hepatic cells can be complemented with the further analyses of the metabolites in the different organelles. Metabolites are expected in the endoplasmic reticulum, peroxisomes and mitochondria. However, their concentrations in the organelles and to which extent they are controlled by TTP are unknown.

For αT1, there are plenty of future research opportunities due to the novelty of the congener and scarceness of related scientific publications. Experiments on the cellular uptake and intracellular distribution in hepatic cells, comparable to the one performed for αT, γT αT3 and γT3, are necessary to understand its intracellular trafficking and whether or not the TTP is required for its secretion from the cells. Another interesting contribution on the research towards αT1 would be the measurement of the affinity of TTP for αT1, because previous investigations observed an influence of TTP affinities for the vitamin E congeners on their distribution in extrahepatic tissues and metabolism [39]

In continuation of the results obtained in the mouse trial (**Chapter 4**), further studies investigating the dose-response of the absorption of αT1 in $TTP^{-/-}$ mice might be required to clarify if TTP is involved in the secretion of αT1 into the bloodstream. Furthermore, a trial feeding mice with αT1-depleted, –standard, or –supplemented diets would be important to conclusively study the tissue distribution and potential dose-responses on the absorption of αT1. A trial employing different dietary doses also allows the measure of indicators of vitamin E activity (e.g. HO-1 and CD36 protein expression), with which effects were previously observed using diets with αT [54, 55].

To conclude, studies on the metabolism of αT1 are highly warranted. It was pointed out before that the unsaturated side-chain of the tocotrienols is metabolized by enzymes involved in the metabolism of unsaturated fatty acids. Three enzymes, the 2,4-dienoyl-coenzyme A reductase, 3,2-enoyl-coenzime A isomerase and enoyl-coenzime A hydratase, work together in saturating the double bonds one by one, starting from the end of the side-chain [22]. If the side-chain of the αT1 can be saturated similar to that of the tocotrienols, this could lead to its conversion into αT or into the long-chain metabolites of αT. αT and its long-chain metabolites are known for their biological activities and health benefits (see **Chapter 1**), and a potential conversion of the αT1 gives an interesting twist on the essentiality of this congener.

In general, significantly more is known about αT compared to any of the non-αT congeners. Based on the contributions of the present thesis to the scientific progress, new opportunities for future research on the biological activities and potential health-benefits of the non-αT congeners have arisen.

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Contributions to publications

M.Sc. Andrea Irías-Mata collected the palm fruits and developed the methodologies for oil extraction in Chapter 2; designed and performed part of the cell culture experiments with HepG2 cells in Chapter 3; designed and collaborated on the ethical application and animal experiment development in Chapter 4; participated on the extraction and analysis by HPLC of vitamin E in oils, cells and tissues on Chapters 2, 3 and 4, respectively; performed the statistical analysis for data presented and discussed in Chapter 2, 3 and 4; and wrote the first draft of all manuscripts in Chapters 2, 3 and 4.

Prof. Dr. Jan Frank actively participated on the design of the experiments and supervised all aspects of the results presented in Chapter 2, 3 and 4; and co-wrote and edited all manuscripts in Chapters 2, 3 and 4.

BTA. Nadine Sus established and validated the vitamin E HPLC method used, and actively supported in the analysis by HPLC in Chapters 2, 3 and 4; collaborated in the design and performed of the cell culture experiments with HepG2 cells in Chapter 3; and actively participated in the animal experiment development in Chapter 4.

Dr. Wolfgang Stuetz actively supported in the analysis by HPLC of vitamin E in Chapter 2; and contributed in the development of the mechanical methodology for oil extraction in Chapter 2.

Prof. Dr. Walter Vetter and **Dr. Simon Hammann** established, validated and conducted the analysis by GC-MS of vitamin E in Chapter 2; and provided their methodology and results on this matter for Chapter 2. As well, **Prof. Vetter** with **Dipl.-LM-Chem. Marco Müller** developed the analysis by GC-MS and provided the respective results for Chapter 4.

Dr. Maren Podszun established and validated the intracellular distribution method used as part of the cell culture experiments with HepG2 cells in Chapter 3.

M.Sc. Katrin Gralla and **Lic. Aracelly Cordero-Solano** participated on the extraction and analysis by HPLC of vitamin E of palm oil fruits in Chapter 2; and besides **Miss Cordero-Solano** contributed in the development of the methodologies for oil extraction in Chapter 2.

M.Sc. Denise Woerner participated on the development of the intracellular distribution method used as part of the cell culture experiments with HepG2 cells in Chapter 3; and conducted part of the intracellular distribution experiments in Chapter 3.

M.Sc. Daniela Stock and **M.Sc. Sandra Flory** performed part of the cell culture experiments with HepG2 cells in Chapter 3, and conducted the extraction and analysis by HPLC of vitamin E in Chapter 3; and besides **Miss Flory** contributed in the statistical analysis for the data presented in Chapter 3.

Maria-Lena Hug performed the vitamin E analysis and protein expression experiments in the mice livers, executed the genotype analyses of the mice, and collaborated in the animal experiment development in Chapter 4.

Other activities during the doctoral studies

Poster presentations or contributions in Scientific Congress during the doctoral studies

Andrea Irías-Mata, Wolfgang Stuetz, Nadine Sus & Jan Frank. **2017**. Tocotrienols, Tocopherols and Tocomonoenols in Palm Oils of Costa Rican Palm Fruits (*Elaeis sp*): A Comparison Between Six Varieties and Two Oil Extraction Methods. Third International Congress Hidden Hunger. Food Security Center, University of Hohenheim. Stuttgart, Germany.

Andrea Irías-Mata, Nadine Sus, Daniela Stock & Jan Frank. **2017**. Role of α-tocopherol transfer protein (αTTP) on the cellular uptake and intracellular localization of α-tocopherol (αT) and α-tocotrienol (αT3) in cultured liver cells. IUNS 21st ICN International Congress of Nutrition. Buenos Aires, Argentina.

Sandra Flory, **Andrea Irías-Mata** & Jan Frank. **2018**. The cellular uptake and subcellular localization of γ-tocotrienol and γ-tocopherol in cultured hepatocytes as a function of α-tocopherol transfer protein expression. 55. Scientific Congress of the German Nutrition Society. Stuttgart, Germany.

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Tocotrienols, Tocopherols and Tocomonoenols in Palm Oils of Costa Rican Palm Fruits (*Elaeis sp*): A Comparison Between Six Varieties and Two Oil Extraction Methods

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Introduction

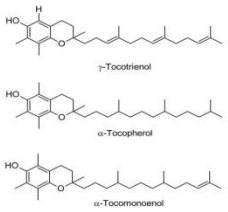
Palm oil is extracted from the ripened mesocarp of the fruits of the palm oil tree (family *Arecaceae*, genus *Elaeis*) and is a rich source for tocotrienols, which make up around 80% of its total vitamin E content, and a source for tocomonoenols.

In Costa Rica, two *Elaeis* species have industrial importance, the *Elaeis Guineensis* from Central and West Africa, and *Elaeis Oleifera* from Central and South America; and lately interspecific hybrid varieties with enhanced nutritional quality have been developed.

The two most common palm oil extraction procedures involve either mechanical pressing with a screw or hydraulic press or the extraction with organic solvents. Yet, it remained unknown how these two different extraction processes differ regarding their vitamin E yield.

Methodology

Fully ripened fruit mesocarp of three *Elaeis Guineensis*, two *Elaeis Oleifera* and one Hybrid OxG genotypes were extracted using mechanical screw press and chemical extraction with hexane; and vitamin E profiles were elucidated and quantified by HPLC-FLD. GC-MS was used for the identification of alpha-tocomonoenol.



Results

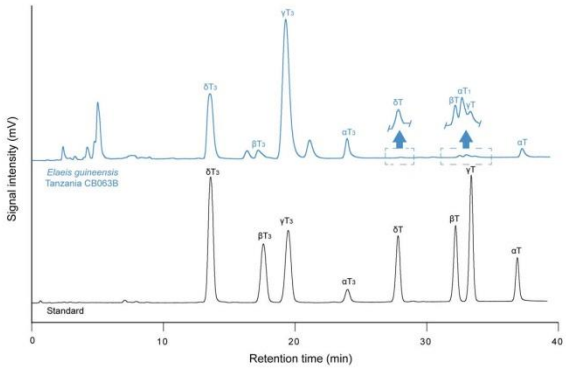


Figure 1. Representative HPLC-FLD chromatograms of tocopherols and tocotrienols in a calibration standard (black) and a palm oil of the genotype *Elaeis Guineensis* obtained by screw press extraction (blue). The identity of α-tocomonoenol (αT1) was confirmed by GC-MS

Tocotrienols were the most abundant (91,9-99,8%); with **γ-Tocotrienol** as the major compound in all genotypes. **α-Tocopherol** was the most abundant tocopherol, while other tocopherols (β-, γ-, δ-T) and **α-tocomonoenol** were detected at minor concentrations.

Significant differences in vitamin E profiles between genotypes were observed and the variety Quepos had the by far highest content of total vitamin E (892 µg/g oil; 99.8% tocotrienols, 0.2% tocopherols and α-tocomonoenol); and the genotype Tanzania had the highest content of α-tocomonoenol (1.7%). Chemical extraction with hexane afforded up to 2.5-fold higher vitamin E yields than screw press extraction; which may be related with the lower oil yield obtained by screw pressing.

Table 1. Mean concentrations (± standard deviation; n=6; all in µg/g) of tocotrienols, tocopherols and α-tocomonoenol in palm oils from Elaeis sp genotypes.[1]

Compound	Concentration in palm oil [µg/g]					
	Tanzania CB063B	Deli Dami CB087F	Deli x Nigeria CB082C	Quepos CB9204	Manaos CB1201	Hybrid OxG CB127B
Screw Press Extraction						
αT1[2]	2.7 ± 0.2^{a}	0.078 ± 0.079^{b}	0.677 ± 0.099^{c}	0.20 ± 0.12^{b}	0.18 ± 0.25^{b}	0.59 ± 0.11^{c}
Total T	9.5 ± 0.5^{a}	13.0 ± 2.0^{b}	10.3 ± 1.3^{a}	1.1 ± 0.2^{c}	0.090 ± 0.079^{c}	5.5 ± 1.2^{d}
Total T3	151.1 ± 9.2^{a}	150.5 ± 19.2^{a}	199.0 ± 28.5^{ab}	**622.2 ± 43.2^{c}**	253.7 ± 28.1^{d}	215.9 ± 33.2^{bd}
Total VE	163.3 ± 9.7^{a}	163.6 ± 20.6^{a}	210.0 ± 29.7^{ab}	**623.5 ± 43.5^{c}**	253.9 ± 28.2^{b}	222.0 ± 34.5^{b}
Chemical Extraction with Hexane						
αT1[2]	3.0 ± 2.6^{a}	nd[3]	0.85 ± 0.68^{b}	0.42 ± 0.22^{b}	0.48 ± 0.33^{b}	1.4 ± 0.6^{ab}
Total T	12.8 ± 8.0^{a}	17.4 ± 4.7^{a}	20.3 ± 3.1^{a}	1.7 ± 1.1^{b}	1.8 ± 0.8^{b}	4.4 ± 1.3^{b}
Total T3	354.0 ± 77.6^{a}	315.2 ± 116.2^{a}	504.2 ± 16.9^{a}	**890.3 ± 219.6^{b}**	524.6 ± 180.8^{a}	466.1 ± 138.4^{a}
Total VE	369.8 ± 83.0^{a}	332.6 ± 119.6^{a}	525.3 ± 18.8^{a}	**892.4 ± 220.4^{b}**	526.9 ± 181.1^{a}	471.9 ± 139.1^{a}

[1] Comparison of means using Tukey's multiple comparison test; values within a row not sharing a common superscript letter are significantly different at $p < 0.05$.
[2] Expressed as α-tocopherol equivalents.
[3] nd=not detected.

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Reference
144/1850

Title: Role of α-tocopherol transfer protein (αTTP) on the cellular uptake and intracellular localization of α-tocopherol (αT) and α-tocotrienol (αT3) in cultured liver cells

Authors: Andrea-Irías Mata*, Nadine Sus, Daniela Stock, Jan Frank

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Background and Objectives:

The intracellular trafficking and localization of αT is poorly understood and its preferential retention has been attributed to its high affinity to the hepatic cytosolic protein αTTP. The role of αTTP in the absorption and intracellular distribution of αT and other vitamin E forms, such as αT3, was therefore studied.

Methods:

Time dependent absorption of 50 µM αT and αT3 for up to 72 h was investigated in HepG2 cells with and without αTTP expression. Intracellular distribution after 24 h or 6 h incubation was studied using a density gradient centrifugation. Eleven fractions of increasing density were collected and cell organelle markers determined by Western blotting, and αT and αT3 contents measured by HPLC.

Results:

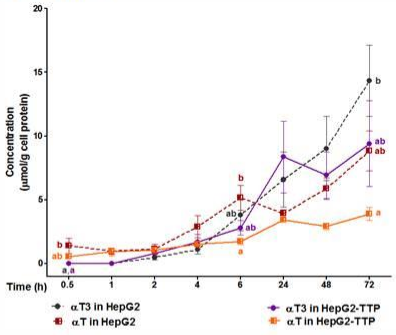

Figure 1. Concentration (mean with SEM, n=3) over time of αT and αT3 in HepG2 and HepG2 with αTTP cells. For each time, vertical points not sharing a common letter are significantly different at $p < 0.05$ (one-way ANOVA + Tukey's). A continuos uptake was observed, with a higher uptake for αT3. αTTP did not reveal a significant enhance in uptake in cells.

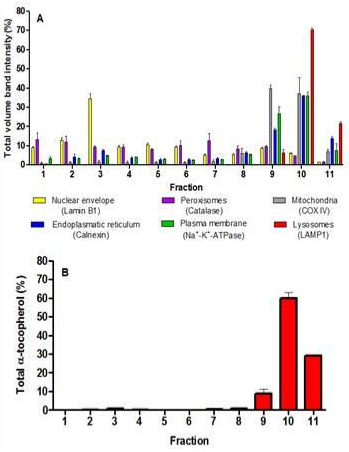

Figure 2. Volume of protein band intensities of subcellular compartments (A) and concentration (mean with SEM, n=2) of αT (B) in HepG2 cells after 6 h incubation. A stronger correlation was observed uniquely between % lysosomes and % αT (linear regression, r^2=0.9701).

Table 1. Non-parametric Spearman's correlation coefficient (r_s) between % αT3 and αT, and the volume of protein band intensity of subcellular compartments in HepG2 and HepG2 with αTTP cells after 24 h incubation. r_s ranges from -1 to +1, with positive correlation at +1; correlations are significant at *p < 0.05.

Cell type	Nuclear Envelope (Lamin B1)		Peroxisomes (Catalase)		Mitochondria (COX IV)		Endoplasmatic reticulum (Calnexin)		Plasma membrane (Na+-K+-ATPase)		Lysosomes (LAMP1)	
	α-T3	α-T	α-T3	α-T	α-T3	α-T	α-T3	α-T	α-T3	α-T	α-T3	α-T
HepG2	-0.4048	-0.5909	-0.4513	-0.6000	0.2559	0.7727*	0.7583*	0.7182*	0.8328*	0.7182*	0.8886*	0.6636*
HepG2-TTP	-0.3337	-0.4818	0.2479	-0.4727	0.4958	0.7818*	0.9439*	0.8545*	0.9249*	0.9636*	0.9439*	0.8545*

Intracellular distribution showed for αT a correlation with mitochondria, endoplasmatic reticulum, plasma membrane and lysosomes (table 1), being stronger with lysosomes at 6 h (figure 2); and for αT3 a correlation with the same compartments except mitochondria was observed (table 1).

Conclusions:

αTTP is not essential for the uptake of the compounds in hepatocytes; its site of action is at a later step. Intracellular distribution in these organelles might be related with their presumed higher levels of oxidative stress; or with the location of αTTP in the hepatocytes, that might be in the lysosomes.

Keywords: α -tocopherol. α -tocotrienol. α-tocopherol transfer protein. Intracellular distribution. Cultured liver cells.

Conflict of Interest: The authors declare no competing financial or personal conflict of interest.

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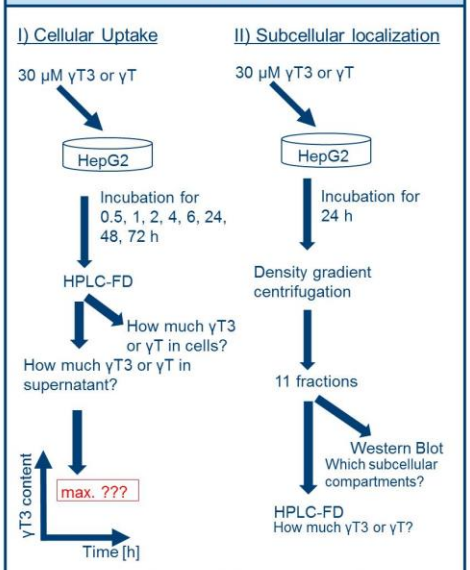
The cellular uptake and subcellular localization of γ-tocotrienol and γ-tocopherol in cultured hepatocytes as a function of α-tocopherol transfer protein expression

Sandra Flory*, **Andrea Irías-Mata**, **Jan Frank**

Background and Objectives

Vitamin E research traditionally focused on α-tocopherol, the predominant vitamin E form in human tissues. Less is known about γ-tocotrienol (γT3) and γ-tocopherol (γT) although they are the predominant forms in edible oils. The mechanisms behind the differences in the body's retention of the different vitamin E forms is not known in detail.
We therefore studied the role of α-tocopherol transfer protein (α-TTP) on cellular uptake and subcellular localization of γT3 and γT in hepatic cell lines.

Methods



→ Compared between HepG2 cells, α-TTP-transfected HepG2 cells and empty vector control HepG2 cells

Results

α-TTP expression did not affect the uptake of γT3 or γT (Figure 1). γT3 and γT correlate with markers for the endoplasmic reticulum, lysosomes and the plasma membrane (Figures 2 and 3) and less with mitochondria, the nuclear envelope and peroxisomes. In α-TTP-expressing cells, γT and γT3 were less localized in the plasma membrane. The correlation of the lysosomes with γT was stronger and with γT3 unchanged in α-TTP-expressing cells. Less γT, but not γT3 localized in the endoplasmic reticulum in α-TTP-expressing cells (Figure 3).

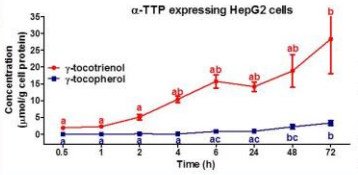


Figure 1. Concentrations of γT3 and γT in cell lysates of cells incubated for up to 72 h.
Mean γT3 and γT concentrations ± SEM of cells incubated for up to 72 h with 30 µmol/L of the compounds. Comparison of means between times for each individual compound (γT3 or γT) were performed by one-way ANOVA with Tukey's posthoc test and points within a line not sharing a common letter are significantly different at $p < 0.05$.

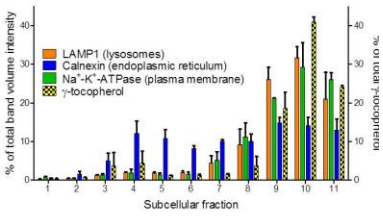


Figure 2. Volume of protein band intensities of LAMP1, calnexin and Na^+-K^+-ATPase and concentration of γT (Mean ± SEM) in the 11 subcellular fractions of HepG2 cells.

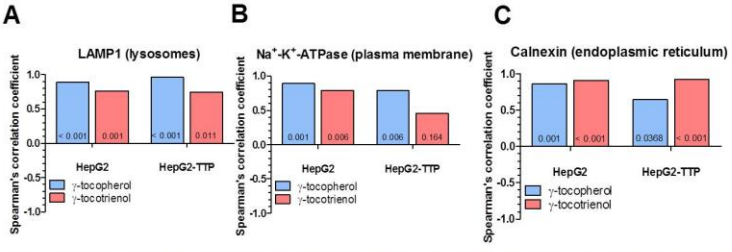


Figure 3. Non-parametric Spearman's rank order correlation coefficients between the percentage of γT3 or γT and the percentage of protein band intensity of LAMP1 (A), Na^+-K^+-ATPase (B) and calnexin (C).
Coefficients are shown for HepG2 cells and α-TTP expressing HepG2 cells (HepG2-TTP). In each column, the p-value is given. Correlations are significant at $p < 0.05$ (two-tailed).

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Conclusion

Our data suggest that α-TTP does not affect the cellular uptake of γT and γT3, but directs both compounds towards lysosomes and reduces their transport to the plasma membrane, especially for γT3. α-TTP furthermore appears to reduce the transport of γT to the endoplasmic reticulum, where the first step of degradation occurs, and thereby delays its metabolism.

Student contributions to the doctoral dissertation and other projects

Supervised students

2016. Katrin Gralla, M.Sc.

Master student in Molecular Nutrition Science. University of Hohenheim.

Thesis: Uptake and antioxidative capacities of palm and pumpkin seed oil tocotrienols and tocopherols in HepG2 cells.

Co-supervisor: BTA. Nadine Sus.

2016. Aracelly Cordero Solano, Lic.

Bachelor exchange student from the School of Food Technology. University of Costa Rica.

Project: Optimization of the screw press extraction conditions for the oil extraction of palm fruits, and their influence on the vitamin E analysis.

2017. Sandra Flory, M.Sc.

Master student in Molecular Nutrition Science. University of Hohenheim.

Thesis: Influence of α-tocopherol transfer protein on the subcellular localization of γ-tocopherol and γ-tocotrienol in HepG2 cells.

2018. Maria-Lena Hug.

Master student in Molecular Nutrition Science. University of Hohenheim.

Thesis: The impact of the α-tocopherol transfer protein on the expression of HO-1 and CD36 in the liver of α-tocomonoenol and α-tocopherol fed mice.

2018. Alina Rüdiger and Anne-Kathrin Huber.

Bachelor students in Nutrition Science. Humboldt Reloaded Programme. University of Hohenheim.

Project: Comparison of vitamin E profiles of different manufactured oils. (Vergleich der Vitamin E-Profile verschieden hergestellter Öle).

Co-supervisor: M.Sc. Sandra Flory.

2018. Beyzanur Serin, Isa Entenmann and Isabel Zurblihn

Bachelor students in Nutrition Science. Humboldt Reloaded Programme. University of Hohenheim.

Project: Vitamin E and fatty acid content in vegetable oils and fats: correlation with color and density? (Vitamin E- und Fettsäuregehalte in pflanzlichen Ölen und Fetten: Korrelation mit Farbe und Dichte?).

Co-supervisor: M.Sc. Sandra Flory.

Others students supervised by co-workers

2015. Denise Woerner, M.Sc.

Master student in Molecular Nutrition Science. University of Hohenheim.

Thesis: Optimization of a density gradient centrifugation method to investigate the subcellular localization of RRR-alpha-tocopherol depending on the expression of alpha-tocopherol transfer protein in HepG2 cells.

Supervisor: Dr. Maren Podszun.

2016. Daniela Stock, M. Sc.

Master student in Molecular Nutrition Science. University of Hohenheim.

Thesis: Intracellular distribution of RRR-alpha-tocopherol in cultured liver cells as a function of alpha-tocopherol transfer protein expression and time.

Supervisor: BTA. Nadine Sus.

Teaching activities

2016-2018. Lecture: Biomarkers of Aging. Module Aging and Age-Related Diseases. Chair: Prof. Dr. Jan Frank. Institute of Biological Chemistry and Nutrition. University of Hohenheim.

2016. Lecture: Role of vitamin E in neurodegenerative diseases. Module Biofunctionality and Safety of Food. Chair: Prof. Dr. Jan Frank. Institute of Biological Chemistry and Nutrition. University of Hohenheim.

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Curriculum vitae

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Nationality: Costa Rican

Education

2019. Dissertation for obtaining the degree of Dr. rer. nat. (Ph.D.) in Nutritional Sciences. Institute of Biological Chemistry and Nutrition. Division of Food Biofunctionality. University of Hohenheim. Stuttgart, Germany. Supervisor: Prof. Dr. Jan Frank.

2012. Master in Science (M.Sc) in Chemistry. University of Costa Rica. San José, Costa Rica

2009. Bachelor in Science (B.Sc) in Chemistry. University of Costa Rica. San José, Costa Rica.

Professional experience

2015-2019. Doctoral student. Institute of Biological Chemistry and Nutrition. Division Food Biofunctionality. University of Hohenheim. Stuttgart, Germany.

06.2014-08.2014. Research intern. Institute of Food Science and Biotechnology. Division Plant foodstuff Technology. University of Hohenheim. Stuttgart, Germany.

2012-2015. Lecturer and Researcher. School of Chemistry and School of Food Technology. University of Costa Rica. San José, Costa Rica.

Awards

2015-2019. Scholarship from the German Academic Exchange Service (DAAD) for the PhD Program "Global Food Security". Food Security Center. University of Hohenheim. Stuttgart, Germany.

Publications

Andrea Irías-Mata, Nadine Sus, Sandra Flory, Daniela Stock, Denise Woerner, Maren Podszun, Jan Frank. **2018**. α-Tocopherol transfer protein does not regulate the cellular uptake and intracellular distribution of α- and γ-tocopherols and -tocotrienols in cultured liver cells. *Redox Biology*. 19, 28-36.

Andrea Irías-Mata, Victor M. Jiménez, Christof Björn Steingass, Ralf M. Schweiggert, Reinhold Carle, Patricia Esquivel. **2018**. Carotenoids and xanthophyll esters of yellow and red nance fruits (Byrsonima crassifolia (L.) Kunth) from Costa Rica. *Food Research International*. 111, 708-714.

Andrea Irías-Mata, Wolfgang Stuetz, Nadine Sus, Simon Hammann, Katrin Gralla, Aracelly Cordero-Solano, Walter Vetter & Jan Frank. **2017**. Tocopherols, Tocomonoenols, and Tocotrienols in Oils of Costa Rican Palm Fruits: A Comparison between Six Varieties and Chemical versus Mechanical Extraction. *Journal of Agricultural and Food Chemistry*. 65, 7476-7482.

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Andrea Irías-Mata & Giselle Lutz. **2014**. Composición química de la biomasa residual de la planta de piña variedad MD2 proveniente de Guácimo, Limón. *Ciencia y Tecnología*. 30 (2): 27-34. (in Spanish).

Andrea P. Irías-Mata & Giselle Lutz. **2013**. Pineapple-stover derived furan compounds as gasoline oxygenate additive. *Research Journal of the Costa Rican Distance Education University*. 5 (2): 279-282.