THE BIOAVAILABILITY, POTENCY AND DISTRIBUTION OF DIETARY PALM TOCOPHEROLS AND TOCOTRIENOLS IN THE TISSUES OF RED HYBRID TILAPIA, *Oreochromis* sp.

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by

WANG YAN

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Dedicated to

My beloved father, Mr. Wang Jing-Zhong, mother, Madam Zhang Zheng-Ying, and wife, Qian Yun-Yun

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LIST OF SYMBOLS

Symbol	Illustration
α	Alpha
β	Beta
γ	Gama
δ	Delta

ABBREVIATIONS

Abbreviation	Full Name
BHT	Butyl hydroxyltoluene
CEL	Carboxyl ester hydrolase
CEHC	Carboxyethyl-hydrochroman
CMC	Carboxylmethyl cellulose
CPKO	Crude palm kernel oil
CPO	Crude palm oil
FAME	Fatty acids methyl esters
GC	Gas chromatography
HDL	High density lipoprotein
HPLC	High pressure liquid chromatography
HSI	Hepatosomatic index
IPI	Intraperitoneal fat index
LPL	Lipoprotein lipase
LDL	Low density lipoprotein
NFE	Nitrogen free extract
NPU	Net protein utilization
PCV	Packed cell volume
PER	Protein efficiency ratio
PFAD	Palm fatty acid distillate
PUFA	Polyunsaturated fatty acid
SGR	Specific growth rate
T	Tocopherol
T3	Tocotrienol
TAP	Tocopherol associated protein
TBP	Tocopherol binding protein
ToAc	Tocopheryl acetate
ToSc	Tocopheryl succinate
TTP	Tocopherol transfer protein
VLDL	Very low density lipoprotein

KETERSEDIAAN, KEMUJARABAN DAN PENABURAN TOKOPHEROL DAN TOKOTRIENOL DARIPADA MINYAK KELAPA SAWIT DALAM TISU TILAPIA HYBRID MERAH, *Oreochromis* sp.

ABSTRAK

Dalam eksperimen yang pertama, satu ujian makanan telah dijalankan selama 8 minggu untuk mengkaji ketersediaan α-tokopherol (α-T) secara biologi dengan lapan diet yang berisonitrogenous dan isoenergik. Empat diet piawai (Diet 1 hingga Diet 4) telah dibekalkan dengan α-tokopheryl asetat (α-ToAc) secara berperingkat bermula pada 0, 25, 50, atau 100 mg/kg. Sementara empat diet lagi (Diet 5 hingga Diet 8) mengandungi α-tokopheryl succinate (α-ToSc) sebanyak 50 mg/kg, tokotrienols-rich fraction (TRF, ekstrak dari kelapa sawit mentah, sebanyak 500 mg/kg), asid lemak sulingan sawit (PFAD, 30 g/kg) dan minyak sawit mentah (CPO, 100 g/kg) sebagai sumber vitamin E dalam diet. Oleh yang demikian jumlah keseluruhan α-T dalam kelapan-lapan diet kajian ini ialah 2.98, 28.9, 54.59, 103.39, 46.03, 27.15, 34.20, atau 25.65 mg/kg, masikg-masikg. Setiap diet ini diberikan kepada tiga kumpulan replikat anak ikan tilapia merah (berat awal: 4.14 ± 0.02 g) selama tempoh lapan minggu. Satu persamaan regresi linear piawai bagi α-T dalam diet (dengan y dalam mg/kg) sebagai fungsi kepekatan α-T dalam hati (% dalam μg/g) telah digunakan untuk mencari nilai α-T dalam ujian ke atas diet. Hasilnya didapati bahawa 7.97, 13.83, 24.18 dan 14.44 mg/kg dengan ketersediaan 17.31%, 50.95% 70.72% dan 56.30% α-T yang terdapat dalam α-ToSc, TRF, PFAD dan CPO, masikg-masikg, yang dibekalkan di dalam diet. Apabila kajian terhadap kepekatan vitamin E dijalankan, didapati kepekatan α-T terdapat dalam otot, hati, tisu adipos dan kulit ikan tilapia lebih rendah bagi ikan yang diberi diet α-ToSc berbanding ikan-ikan yang diberikan diet α-ToAc. Tisu-tisu ikan tilapia mempunyai pelbagai kebolehan dalam mengumpulkan tocotrienols. Kepekatan tertinggi didapati dalam tisu adipos (47-60% dari jumlah diet vitamin E), diikuti hati (27-38%), kulit (16-18%) dan otot (kira-kira 17%). Sementara kepekatan thiobarbituric acid-reactive substances (TBARS) dari katalis Fe^{2+}- dan vitamin C, menyebabkan peroksidasi lipid paling rendah dalam tisu otot dan hati ikan-ikan yang diberikan diet TRF. Tiada perbezaan secara signifikan dari segi peratus pertambahan berat, nisbah

penukaran makanan dan nisbah kecekapan protein bagi ikan-ikan yang diberi pelbagai diet yang berbeza ($P>0.05$).

Sementara itu, dalam eksperimen kedua, satu ujian makanan selama 9 minggu telah dijalankan dengan tujuan untuk menilai kesan pemakanan vitamin E ke atas pertumbesaran ikan, peroksidasi lipid dan taburan tokopherol dan tokotrienols dalam pelbagai tisu ikan tilapia merah. Lima diet yang berisonitrogenous dan isoenegenik dibekalkan dengan 0, 30, 60, 120 dan 240 mg/kg dari keseluruhan vitamin E dari TRF. Kemudian didapati tiada perbezaan secara signifikan dalam peratus pertambahan berat ikan, nisbah penukaran makanan dan nisbah kecekapan protein bagi ikan yang diberi diet yang berbeza ($P>0.05$). Kepekatan α-tokopherol, α-tokotrienols, dan γ-tokotrienols dalam otot, hati, plasma, kulit dan tisu adipos meningkat dengan meningkatnya TRF dalam diet. α-tokopherol menyumbang sebanyak 51.2-94.2% pengumpulan vitamin E dalam kebanyakan tisu. Pemendakan tokotrienols didapati paling tinggi dalam tisu adipos. Sebaliknya kepekatan TBARS dalam otot, hati dan plasma ikan tilapia merah yang diberi diet tanpa penambahan vitamin E menunjukkan signifikan yang lebih tinggi ($P>0.05$) berbanding dengan ikan-ikan yang diberikan diet TRF dari minyak kelapa sawit.

Kesimpulannya, hasil dari kajian ini didapati penambahan tokotrienols kelapa sawit boleh meningkatkan kepekatan tokotrienols dalam kebanyakkan tisu ikan tilapia. Disamping itu, ia dapat memberikan perlindungan yang tinggi kepada tisu-tisu ikan tilapia untuk mengelakkan berlakunya peroksidasi lipid yang seterusnya memanjangkan hayat simpanan bagi produk makanan laut.

THE BIOAVAILABILITY, POTENCY AND DISTRIBUTION OF DIETARY PALM TOCOPHEROLS AND TOCOTRIENOLS IN THE TISSUES OF RED HYBRID TILAPIA, *Oreochromis* sp.

ABSTRACT

In Experiment 1, an 8-week feeding trial was carried out to investigate the bioavailability of dietary α-tocopherol (α-T), using eight isonitrogenous and isoenergetic semi-purified diets. The four standard diets (Diets 1 to 4) were supplemented with graded levels of α-tocopheryl acetate (α-ToAc) at 0, 25, 50, and 100 mg/kg diet, respectively. The remaining four test diets (Diets 5 to 8) were supplemented with α-tocopheryl succinate (α-ToSc) at 50 mg/kg, tocotrienols-rich fraction (TRF, extracted from crude palm oil, 500 mg/kg), palm fatty acid distillate (PFAD, 30 g/kg) and crude palm oil (CPO, 100 g/kg), as dietary vitamin E sources. The verified amounts of α-T in eight experimental diets were 2.98, 28.41, 54.59, 103.39, 46.03, 27.15, 34.20 and 25.65 mg/kg, respectively. All the eight diets were fed to triplicate groups of red hybrid tilapia fingerlings (initial mean weight: 4.14 ± 0.02g) for 8 weeks. A standard linear regression equation of available α-T in diet (y in mg/kg) as a function of liver α-T concentration (χ in μg/g) was derived and used to determine the amount of available α-T in the test diets. It was found that 7.97, 13.83, 24.18, and 14.44 mg/kg, corresponding to 17.31%, 50.95%, 70.72% and 56.30%, respectively, of available α-T in α-ToSc, TRF, PFAD and CPO supplemented diets. When exploring vitamin E distribution, we observed that α-T concentrations in muscle, liver, adipose tissue and skin of tilapia were lower in fish fed diets with α-ToSc compared to the α-ToAc. Tilapia tissues varied in their ability to accumulate tocotrienols with the highest concentrations being found in adipose tissues (47-60% of total vitamin E), followed by liver (27-38%), skin (16-18%) and muscle (about 17%). The concentrations of thiobarbituric acid-reactive substances (TBARS) from Fe^{2+}-catalysed vitamin C induced lipid peroxidation in the muscle and liver tissues of fish fed the TRF diet were the lowest. There were no significant differences in fish percent weight gain, feed conversion ratio and protein efficiency ratio among fish given the various test diets (P>0.05).

In Experiment 2, a 9-week feeding trial was conducted to evaluate the effects of dietary vitamin E on fish growth, lipid peroxidation and distribution of tocopherols and tocotrienols in various tissues of red hybrid tilapia. Five semi-purified isonitrogenous and isoenergetic diets were supplemented with 0, 30, 60, 120, and 240 mg/kg, respectively, of total vitamin E derived from TRF. There were no significant differences in fish percent weight gain, feed conversion ratio and protein efficiency ratio among fish given the various test diets ($P>0.05$). Muscle, liver, plasma, skin, and adipose tissue concentrations of α-tocopherol, α-tocotrienol, and γ-tocotrienol increased linearly in response to increasing dietary concentrations originating from the added TRF. α-Tocopherol constituted 51.2–94.2% of the vitamin E composition of various tissues. The deposition of tocotrienols was highest in the adipose tissue. The concentrations of TBARS in muscle, liver and plasma of tilapia fed diets with no added vitamin E were significantly higher ($P<0.05$) than those found in the tissues of fish fed diets supplemented with TRF from palm oil.

Results obtained from the present study indicated that palm tocotrienols supplementation could markedly enhance the tocotrienols concentration in various tilapia tissues and provide higher protection of these tissues against lipid peroxidation, which ultimately would translate to longer shelf-life for seafood products.

Chapter 1. Introduction

1.1 Vitamin E discovery and history

Vitamin E was discovered in 1922 by Evans and Bishop when studying the relationship of nutrition and fertility of rats, considered at the time to be especially important for normal reproduction. Female rats accidentally fed with rancid fat for a long time were observed to have a syndrome featuring a loss of fertility through resorption of the fetus. But later, the symptoms were reversed after supplementing diets with some amounts of fresh lettuce, wheat germ, or dried alfalfa leaves. So Evans and Bishop (1922) concluded that plants contained a specific factor being responsible for the phenomenon observed. Thus it is understandable that the term "Vitamin E" was originally described as a lipid extract from plants, which was essential to maintain fertility. The multiple nature of the vitamin began to emerge in 1936 when Evans et al. isolated and characterized two compounds with vitamin E activity, and designated them as α- and β-tocopherol (T), originating from the Greek letter "tokos" (childbirth) and "phorein" (to bring forth) and the suffix "ol" was added to indicate the phenolic nature. In the following years, two additional tocopherols, γ- and δ- tocopherol (Emerson et al. 1937; Stern et al., 1947) as well as the tocotrienols (T3) (Pennock et al., 1964) were isolated in succession from edible plant oils, such as wheat germ oil, soybean oil and crude palm oil ect. So, today a total of four tocopherols (d-α-, d-β-, d-γ-, and d-δ-) and their corresponding tocotrienols are known to occur in nature. It has been more than 40 years before vitamin E was associated with an antioxidant property in 1966 reported by Epstein et al. The American Food and Nutrition Board in 1968 officially recognized the essential nature of vitamin E (Azzi & Stocker, 2000). Since then, for decades,

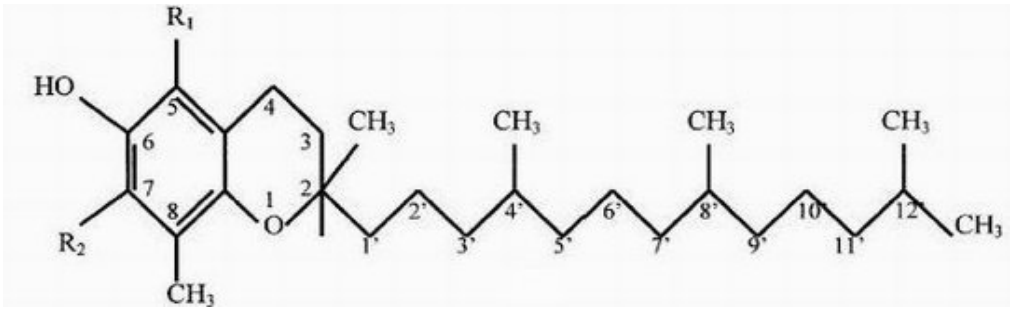
researchers have undertaken numerous research on vitamin E as a radical chain breaking antioxidant in humans and other mammals, as well as in fish.

1.2 Chemistry of tocopherols and tocotrienols

Vitamin E includes two groups of closely structure-related, fat-soluble compounds. Its eight naturally occurring vitamin E isoforms share some resemblance consisting of a common chromanol head and a side chain at the C-2 position. The differences between tocopherols and tocotrienols are determined by their aliphatic tail. Tocopherols have a saturate phytyl chain which has three chiral centers with configuration position at 2, 4' and 8', whereas the tail of tocotrienols is an unsaturated isoprenoid chain with three bonds embedded at 3', 7', and 11' (Figure 1.1). Within one group, the members are designated α, β, γ, and δ relying on the number and the position of the methyl groups attached to the aromatic ring. Chemically synthetic vitamin E, such as tocopheryl or tocotrienyl acetate and tocopheryl succinate, derived from hydrogen atom substituted at C-6 position of phenolic ring on the chromanol head of tocopherol or tocotrienol by acetate or succinate group (Figure 1.2). Nowadays vitamin E has become a generic descriptor for all the molecules that qualitatively exhibit the biological activity of α-tocopherol (NRC, 1993).

Like other vitamins, the chemical properties of vitamin E also include bioactivity other than antioxidant ability. To assess the bioactivity of vitamin E isoforms, the classical rat foetal gestation-resorption assay is the routine manner to be adopted

(a)



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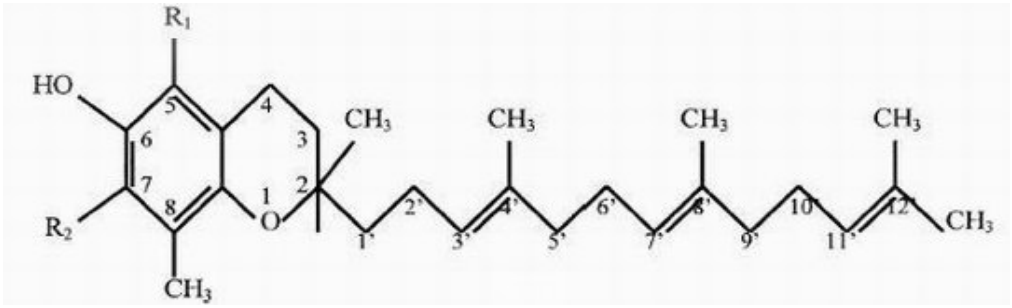
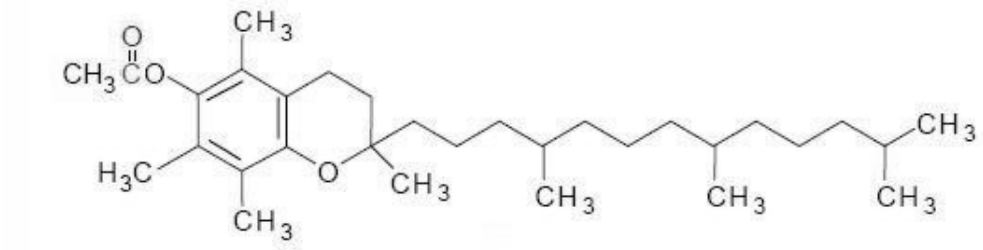


Tocopherol/Tocotrienol	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 1.1 Structure of naturally occurring tocopherols (a) and tocotrienols (b) Munné-Bosch & Alegre (2002).

(a)



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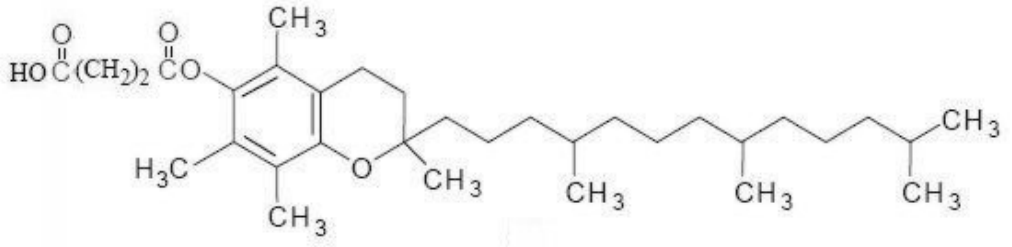


Figure 1.2 Structure of synthetic vitamin E: α-tocopheryl acetate (a) and α-tocopheryl succinate (b).

(Evans & Bishop, 1922; Leth & Søndergaard, 1977; Weimann & Weiser, 1991). Historically, vitamin E activity (one international unit, IU) has been defined as 1 mg of all-rac-α-tocopheryl acetate while RRR-α-tocopherol equalled 1.49 IU. For dietary purposes, vitamin E activity is expressed as the α-tocopherol equivalent (α-TE) which is the activity of 1 mg RRR-α-tocopherol (Papas, 1999). So based on this, each of the natural vitamin E isoforms, according to the amount of vitamin E necessary to prevent foetal resorption in pregnant and vitamin E-deficient rats, is assigned a biopotency factor indicated as below: α-T, 1.0; β-T, 0.5; γ-T, 0.1; δ-T, 0.03; α-T3, 0.3; β-T3, 0.05; γ -T3, 0.01 (Sheppard and Pennington, 1993; Drotleff and Ternuies, 1999). The factor for δ-T3 is presently unknown. Meanwhile, antioxidant activities among various vitamin E isoforms seemed uncertainly correlated with their biological activities. Though it is generally agreed that the relative antioxidant activity of tocopherols *in vivo* is in the order of α>β>γ>δ (Burton and Ingold, 1986; Burton & Traber, 1990; Dillard et al., 1983), there is a wide spread confusion concerning their relative potency *in vitro* (Burton & Ingold, 1981). Cooney et al. (1993) and Kamat et al. (1995 & 1997) reported that γ-tocopherol and tocotrienols had more potent antioxidant capabilities than α-tocopherol, respectively.

1.3 Vitamin E availability in nature

In nature, only plants can synthesize vitamin E compounds. Therefore, vitamin E is a very important and essential dietary nutrient for humans and animals to maintain normal physiological functions of their various tissues (Kamal-Eldin and Appelqvist, 1996). Tocopherols are present in oil seeds, fruits roots, tubers, cotyledons, hypocotyls,

stems, leaves, and flowers (e.g., sepals and petals) of higher plants (Munné-Bosch & Alegre, 2002). Most of them predominate in the α-tocopherol form, except seeds, which show either α- or γ-isoform, together with minor quantities of other tocopherols or tocotrienols. Tocopherols were also found in some photosynthetic bacteria fungi, algae (Munné-Bosch & Alegre, 2002). In contrast, the tocotrienols are rather concentrated in cereal grains (i.e. oat, barley, and rye) and certain vegetable oils (i.e. palm oil and rice bran oil) (Theriault et al., 1999).

Table 1.1 shows the vitamin E contents (mg/kg) determined in selected vegetable oils and animal fats. According to this table, it is apparent that either α- or γ-T is the most abundant vitamin E isoform in most of vegetable oils, such as sunflower oil , canola oil, safflower oil, soybean oil, corn oil, and peanut oil. Only negligible amounts of tocotrienols were present in canola oil and soybean oil. However, α-T is almost the exclusive vitamin E isoform present in animal-origin oils or fats, except for lard, containing γ-T and α-T3. Compared to these oils or fats mentioned-above, palm oil is unique because it contains more sufficient tocotrienols than tocopherols, with the former accounting for about 80%. The vitamin E compositions of crude palm kernel oil (CPKO), crude palm oil (CPO), and palm fatty acid distillate (PFAD) to be used as dietary lipid sources in the present two studies were analysed and determined by HPLC in our Fish Nutrition Lab, and the results are summarized in Table 3.1.

Table 1.1 Tocopherols and tocotrienols contents of selected oils and fats (mg/kg)[1].

Fats and oils	Tocopherols				Tocotrienols			
	α	β	γ	δ	α	β	γ	δ
Vegetable oil								
Sunflower	487.0	-	51.0	8.0	-	-	-	-
Canola	210.0	1.0	42.0	0.4	0.4	-	-	-
Safflower	342.0		71.0					
Soybean	75.0	15.0	797.0	266.0	2.0	0.1		0.3
Corn	112.0	50.0	602.0	18.9	-	-	-	-
Peanut	130.0		214.0	21.0				
Palm oil	279.0	-	61.0	-	274.0	-	398.0	69.0
Fish oil								
Cod liver	220.0	-	-	-	-	-	-	-
Herring	92.0	-	-	-	-	-	-	-
Menhaden	75.0	-	-	-	-	-	-	-
Animal Fat								
Lard	12.0	-	7.0	-	7.0	-	-	-
Tallow	27.0	-	-	-		-	-	-

[1]Sheppard and Pennington (1993).

1.4 Vitamin E absorption and transport

The pathway of vitamin E absorption and its transport in the animal body is well illustrated in Plate 1.1. Vitamin E, due to its hydrophobicity, requires special transport mechanisms in the aqueous environment of the plasma, body fluids and cells. After being ingested, dietary vitamin E isoforms are absorbed in the gut by passive diffusion together with other non polar lipids, such as triglycerides and cholesterol (Bjørneboe et al., 1990; Kayden & Traber 1993; Ricciarelli, et al., 2001). Bile, produced by the liver, emulsifies the tocopherols incorporating them into micelles along with other fat-soluble compounds, thereby facilitating absorption. It is generally believed that esters of α-

tocopheryl acetate and α-tocopheryl succinate, are hydrolysed in the gut by pancreatic esterases before being absorbed as free α-tocopherol in humans and mammals (Bjørneboe et al., 1990; Papas, 1999) and may be also in fish (Hung et al., 1982). Normal biliary and pancreatic functions are therefore necessary for absorption of vitamin E.

After vitamin E is absorbed from the small intestine, tocopherols, together with triglycerides, phospholipids, cholesterol and apoliproteins, are re-assembled to chylomicrons by the *Golgi* body of the mucosa cells (Brigelius-Flohé & Traber, 1999; Azzi & Stocher, 2001). Vitamin E is then mainly carried to the liver with the chylomicrons which is later catabolized rapidly by lipoprotein lipase (LPL) to form the remnants. This process is similar for all forms of vitamin E tested (Brigelius-Flohé & Traber, 1999). Tocopherols in the chylomicron remnants are secreted by the liver into very low density lipoproteins (VLDL) which would be partially converted by LPL to low density lipoproteins (LDL) which holds the largest part of plasma tocopherols and appears to exchange them readily with high-density lipoproteins, HDL (Kayden & Traber, 1993; Papas, 1999). Wallaert and Babin (1994) reported that the metabolism of LDL in fish is similar to that in mammals when employing trout as the experimental model. Cohn (1992) reported that peripheral tissue in rabbits can acquire tocopherols from LDL by receptor mediated endocytosis as well as by exchange. Hung et al. (1982) observed that LDL carried most of the radioactivity 32 hours after oral administration of radioactively labeled α-tocopherol and α-tocopheryl acetate to young rainbow trout (*Salmo gairdneri*). Lie et al. (1994) found highest relative levels of α-tocopherol in HDL and LDL of the serum of Atlantic salmon (*Salmo salar*) undergoing vitellogenesis. This is probably mirroring a transfer α-tocopherol from the muscle to the developing

gonads, which is consistent with the proposed roles of HDL and LDL in tocopherol transfer (Kayden & Traber, 1993), although the accurate mechanism with regard to tissue uptake of tocopherols is somewhat not well understood as yet.

Absorption of tocotrienols appears to be similar to tocopherols. But their transport and tissue uptake, however, appears quite different from α-tocopherol. According to some studies performed on humans and rats, it is believed that tocotrienols disappear from the plasma with chylomicron clearance and are preferentially deposited with substantial amounts into the skin (Pearson & Barnes, 1970; Ikeda et al., 2000) and in conjunction with triglycerides into the adipose tissue (Hayes et al., 1993; Ikeda et., 2001 & 2003). However, so far, there is little information on deposition of tocotrienols into fish tissues (Runge et al. 1992; Ng et al., 2004).

1.5 Tocopherol regulatory proteins

α-tocopherol transfer protein (α-TTP), tocopherol associated protein (TAP), and tocopherol binding protein (TBP) are made up of tocopherol regulatory proteins which specifically bind tocopherols and determine tissue tocopherol levels in human, rats and bovine (reviewed by Blatt et al., 2001). α-TTP, a 30-35-kDa protein, was first found in rats' liver (Murphy & Mavis, 1981), later detected in rat brain, spleen, lung and kidney (Hosomi, et al, 1998) and in also mice uteri (Jishage et al, 2001). It has also been successfully purified from rat liver cytosol (Sato et al., 1991; Yoshida et al., 1992). Hosomi et al. (1997) reported relative affinities of purified hepatic α-TTP for α-T among vitamin E analogs for transfer between liposomes and membranes *in vitro*, with

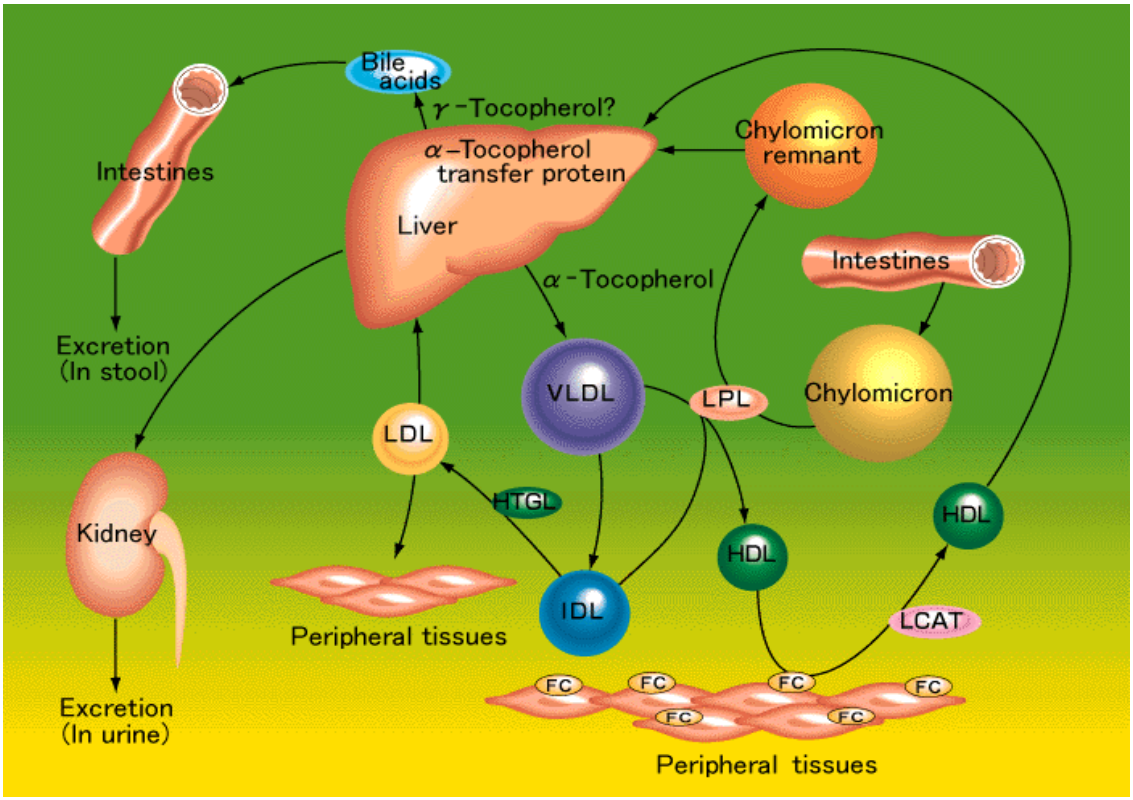


Plate 1.1 Vitamin E absorption and transport in mammalian animal body (source: *http://www.eisai.co.jp/evita_e/kiso4.html*).

100% for RRR- α-T, 38% for β-T, 9% for γ-T and 12% for α-T3. Apparently, α-TTP discriminates between vitamin E analogs upon the basis of the number of methyl groups on the chromanol ring. It was thought that hepatic α-TTP plays important roles in facilitating preferential secretion of α-T from the liver into the blood (Traber, 1988) causing VLDLs to be enriched with α-T (Cohn et al., 1988).

TAP, a 46-kDa protein, has been found in human tissues (Zimmer et al., 2000) and after being purified and sequenced, also demonstrated its homology to α-TTP (Shibata et al., 2001). Human TAP levels were highest in adult liver, and other tissues levels, when expressed as percentages of liver TAP, were not more than 40% (Zimmer et al., 2000). Dutta-Roy et al. (1993) first found TBP, a 14.2-kDa cytosolic protein, also manifested the ability to bind α-T in rat liver and heart. Gordon et al. later (1995)

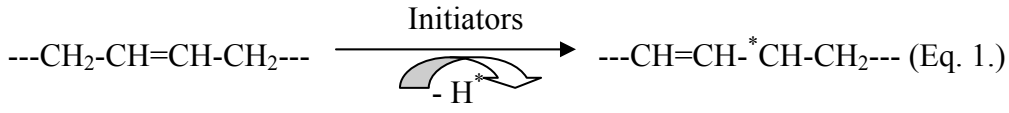
characterized TBP from bovine heart. TBP was only partially sequenced (Blatt, et al., 2001). The exact functions of TAP and TBP remain uncertain.

To date, there is still no reported work on fish α-TTP, TAP or TBP. One, however, can speculate that tocopherol regulatory proteins, or at least α-TTP should be present, according to the fact that α-T is the predominant vitamin E *in vivo*, regardless of the experimental fish being assigned to individual non-α-T or a mixture of non-α-Ts (Sigurgisladottir et al., 1994; Hamre and Lie, 1997; Hamre et al., 1998; Parazo et al., 1998; Ng et al., 2004).

1.6 Lipid peroxidation

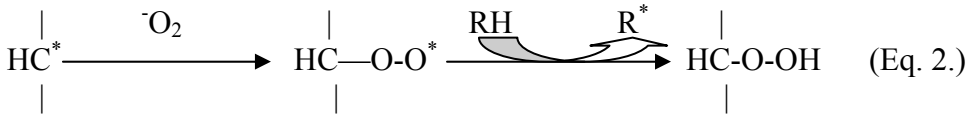
Lipid peroxidation is the introduction of a functional group containing two catenated oxygen atoms, O-O, into unsaturated fatty acids in a free radical reaction (Wheatley, 2000). This progress can generally be divided into three major reactions: initiation, propagation and termination. Polyunsaturated fatty acids susceptible to free radical attack are initiated by the formation of a carbon-centered radical by the abstraction of a hydrogen atom at one of the double bonds of the lipid (Eq 1.) (Wheatley, 2000). The rate-limiting reactions can be catalyzed by heat, light, trace metal transition ions, some enzymes and so on. *In vivo*, some amounts of free radicals originating from oxygen are generated through normal metabolism, by electron transport, phagocytotic activity and by certain enzymes (oxigenases, cytochrome P_{450}) (Rice-Evans and Burdon, 1993). Low levels of radical may be necessary for regulation of cell growth and development (Rice-Evans and Burdon, 1993). Oxidative challenge is encompassed when the formation of radicals exceeds the capacity of the antioxidant

defence. Such a situation may develop after tissue injury or inflammation, as a result of exposure to a wide variety of external factors, including pollutants, oxidative drugs, heavy metals, heat, UV, or deficiency of antioxidant nutrients (e.g., vitamin E). Lipid peroxidation is also one of major causes of quality deterioration during the storage of fats, oils or other lipid-rich foods.



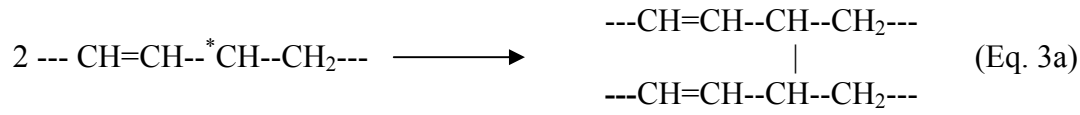
(Eq. 1.)

The active radicals generated in the initial progress undergo bond rearrangements from methylene-interrupted 1,4-pentadiene structures into conjugated 1,3-pentadiene systems and also react with available atmospheric triplet oxygen at a very high rate to produce lipid peroxide radicals, which can abstract more hydrogen, thus propagating the chain (Eq 2.) (Wheatley, 2000). The resultant lipid hydroperoxides then decompose to form alkoxyl (R-O*-) and peroxyl (R-O-O*-) which can participate in chain propagation reations, or degrade to give rise to a great variety of carbonyl secondary oxidation products, such as alkane, alkenes, aldehydes, ketones, alcohol, esters and acids (Fenaille, 2001).

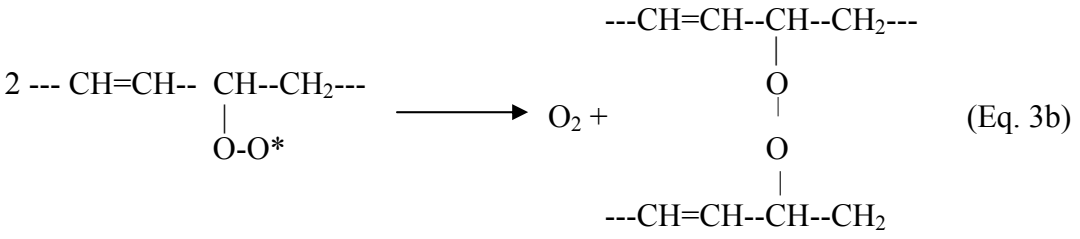


(Eq. 2.)

The chain reaction is terminated by reactions between radicals producing dimers and higher polymers which are not active again to initiate a new chain reaction (Eq. 3a, 3b) (Kamal-Eldin, 1996; Wheatley, 2000).



(Eq. 3a)



(Eq. 3b)

1.7 Antioxidant function of vitamin E

It is assumed that α-T is positioned with the phytyl chain buried in the hydrophobic inner part of the membrane, while the chromanol ring, which carries the reactive and polar OH- group, resides at or near the membrane surface (Kagan et al., 1993). α-T competes with PUFA in donating a hydrogen atom to the lipid peroxyl radical (Figure 1.3), thereby breaking the chain of reactions involved in lipid peroxidation. The discovery of higher antioxidant potency of α-T3 over α-T in recent years was hypothesized to be due to the combined effects of three factors (Serbinova, 1991): (1). α-T3 was mentioned to have a higher recycling efficiency from its chromanoxyl radicals than α-T; (2). α-T3 is significantly less associated in clusters and more

uniformly distributed in membrane bilayers than α-T; (3). α-T3 has a strong disordering effect on membrane lipids which makes interaction of the chromanols with lipid radicals more efficient.

In nonpolar homogenous solutions, lipid peroxyl radicals react approximately 10^5 times faster with α-T than with PUFA (Table 1.2). The tocopheroxyl radical (Figure1.3) is resonance stabilized, and reacts slowly with PUFA (Table 1.2). The relative attack rates of peroxyl radicals on α-tocopherol to PUFA decline dramatically in more polar solutions and in lipid dispersions, but apparently not enough to reverse the reaction directions (Ingold et al., 1993). From measurement with red blood cell ghosts and linoleate miscelles, Buettner (1993) calculated that one molecule of α-T can protect approximately 1000 molecules of PUFA against oxidation, which is in accordance with ratios of α-T to PUFA found in animal tissues (Poukka et al., 1974).

Table 1.2 Rates of selected reactions in lipid oxidation, between lipids and α-T (Ingold et al., 1993).

Reaction	**Rate Constant** ($M^{-1} \cdot s^{-1}$)
PUFA-OO^* + PUFA-H = PUFA-OOH + $PUFA^*$	60
PUFA-OO^* + α-T = PUFA-OOH + α -T^*	3×10^6
A -T^* + PUFA-H = α-T + $PUFA^*$	≈ 0.01

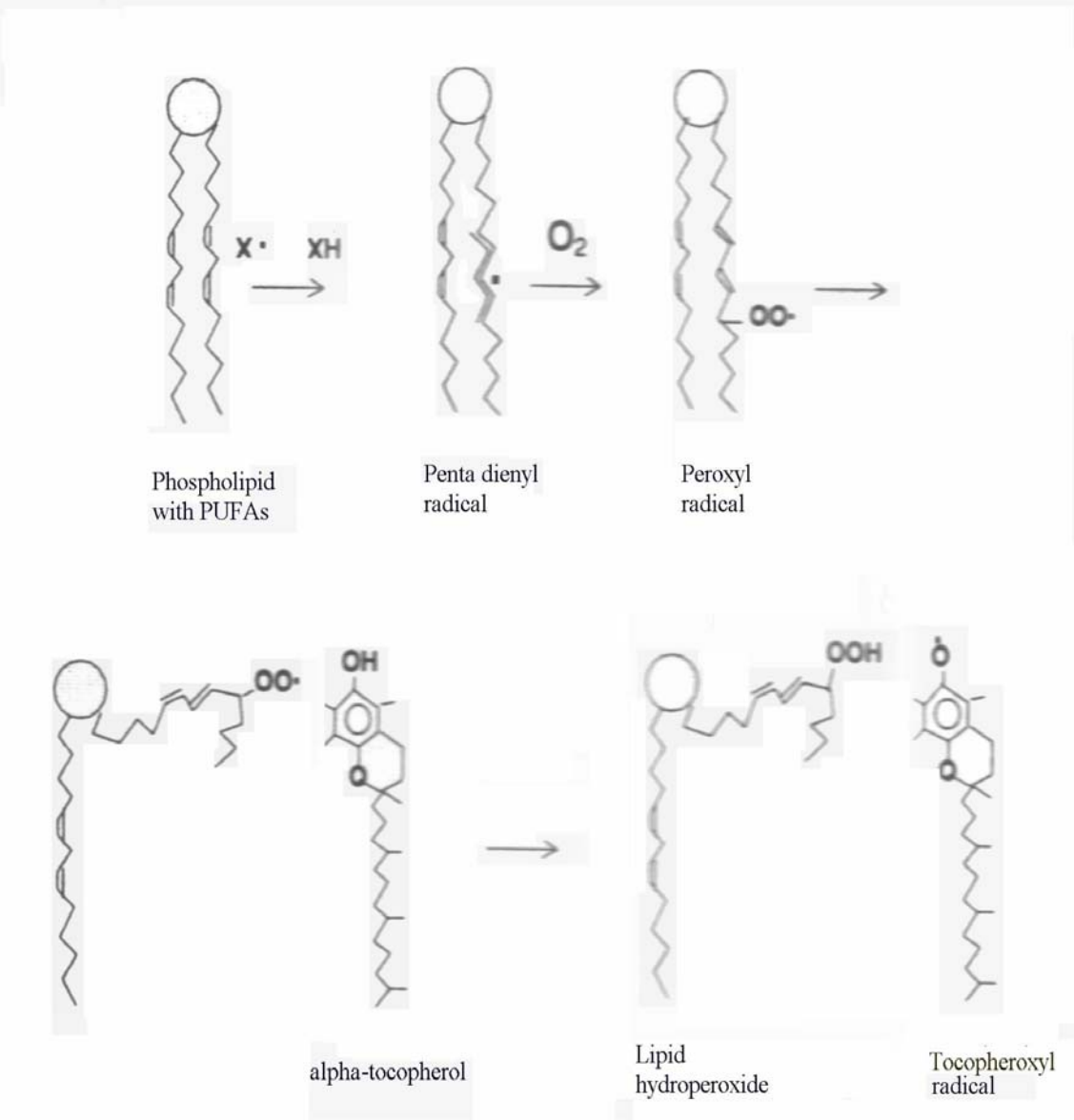


Figure 1.3 Proposed mechanism for the reaction of α-tocopherol with oxidizing lipids. The peroxyl radical group formed during lipid oxidation is polar and floats to the surface of the membrane where it can react with α-T, rendering a lipid hydroperoxyl and the tocopheroxyl radical (Buettner 1993).

1.8 Vitamin E in fish nutrition

Vitamin E, similar to other vitamins, is a low-molecular weight compound which is essential to life, but it can not as a rule be synthesized (or are synthesized only in

insufficient quantities) by the higher animals, and hence to avoid serious metabolic disorders and maintain the animal's normal life process, vitamin E must be supplied as part of their diet (Steffens, 1989).

In fish, the dietary requirements for vitamin E have been established for chinoook salmon (Woodall et al.1964), common carp (Watanabe et al., 1970ab), rainbow trout (Cowey et al., 1981; Watanabe, 1981), channel catfish (Murai & Andrew, 1974, Lovell 1984; Wilson et al., 1984), yellowtail (Shimeno, 1991), Atlantic salmon (Hamre & Lie, 1995), African catfish (Baker & Davies, 1996a), Korea rockish fish (Bai & Lee, 1998), hybrid striped bass (Kocabas & Gatlin, 1999), and tilapia (Satoh, 1987; Roem et al, 1989; Shiau & Shiau, 2001). Based on either regression analysis of weight gain, feed efficiency data, or biochemical analysis (e.g. blood hemolysis, ascorbic acid- induced lipid peroxidation of liver microsomes), it is determined that dietary requirements of fish for vitamin E ranged from 25 - 119 mg/kg (NRC, 1993). The amount of vitamin E requirement may differ from one fish species to another, or even within the same species when different experimental circumstances are applied (e.g. different dietary lipid sources used or different degrees of unsaturation of lipid sources), its vitamin E requirement will also be affected and hence be different..

Although the fat-soluble vitamin E is difficult to deplete from tissues and requires elaborate manipulations to cause deficiency symptoms to occur in experimental animals (Traber, 1999), deficiency signs of vitamin E in a wide range of fishes were still visually known. These signs were similar and included poor growth, poor food conversion, muscular dystrophy involving atrophy and necrosis of white muscle fibers, exophthalmia, ascites, edema of heart, muscle, and other tissues due to increased

capillary permeability allowing exudates to escape and accumulate, which were often green in color as a result of hemoglobin breakdown; anemia and impaired erythropoiesis; depigmentation; and ceroid pigment in the spleen and liver (NRC, 1993; Halver, 2002). The incidence and severity of these deficiencies has been shown to be enhanced when diets deficient in both vitamin E and selenium were fed to Atlantic salmon (Poston et al., 1976), rainbow trout (Bell et al., 1985; Bell & Cowey, 1985), and channel catfish (Gatlin et al., 1986). These observations demonstrated a significant interaction between selenium and vitamin E in the nutrition of fish (NRC, 1993). However, some scholars found no beneficial effect of vitamin E which was administrated alone or in combination with selenium as a prophylaxis for Hitra disease in Atlantic salmon (Salte et al. 1988), and for channel catfish (Wise, 1993a). To prevent those visible deficiency signs depicted above, vitamin E alone, or together with another nutrient -- vitamin C, plays an important role in maintaining proper antioxidant defence system in fish (Montero et al., 1999 & 2001; Mourente et al. 2000; Tocher et al., 2002) and immune responses (Blazer and Wolke, 1984; Hardie et al., 1990; Ortuño et al., 2000; Wise et al., 1993b; Sealey & Gatlin, 2002).

Aside from roles of vitamin E in fish health, it also functions on oxidative stability of fish fillet. Elevated dietary levels of vitamin E can result in its significant deposition in the fish muscle, which in turn can effectively prolong the storage duration or shelf-life of frozen fish fillet (Boggio et al., 1985; Frigg, et al., 1990; Scaife et al., 2000; Ruff et al., 2002a, b). Vitamin E was also reported to enhance the deposition of carotenoids into the fish muscle, thus beneficial to fish flesh quality and texture (Pozo et al, 1988; Sigurgisladottir et al., 1994; Bjerkeng et al., 1999).

The worldwide production of fish oil has staggered for a long time, and in recent years has once again tended to decline (Figure1.5). To maintain a continuous and sustainable aquaculture development, it is urgent and also necessary to find a substitute for fish oil which should not compromise fish health and product quality. Gratifying, extensive studies on the replacement of fish oil by various oils have been performed for the past decades. Among them, some work using palm oil or blend with other oils as dietary lipid and energy source produced encouraging results (Legendre et al., 1995; Al-Owafeir & Belal, 1996; Shiranee & Natarajan; 1996; Ng et al., 2001, 2002 & 2003, Bell et al, 2002). However, it has to be pointed out that in these studies, no any explorative work was done using one of minor components of palm oil, vitamin E, as dietary vitamin E source.

Ng et al. (1998) had developed a bioassay for available niacin from dietary ingredients using liver NAD levels as the response measure. There is currently no information available on the bioavailabity of vitamin E from dietary ingredient sources in fish diets. So a similar bioassay procedure may also be adopted to evaluate the bioavailability of vitamin E from vitamin E-rich oils.

To date, almost all work on fish vitamin E nutrition dealt with either adding α-tocopherol or α-tocopheryl acetate in fish diets since α-tocopherol has the highest biological activity. From the published information of some scientific journals, there were only limited reports on other vitamin E isoforms, e.g. γ- and δ-T deposition in salmonids (Watanabe et al., 1981; Sigurgisladottir et al., 1994; Hamre and Lie, 1997; Hamre et al., 1998; Parazo et al., 1998). Most recently, Ng et al. (2004) reported α-, γ- and δ- tocotrienols deposition in African catfish, using one of the by-products of

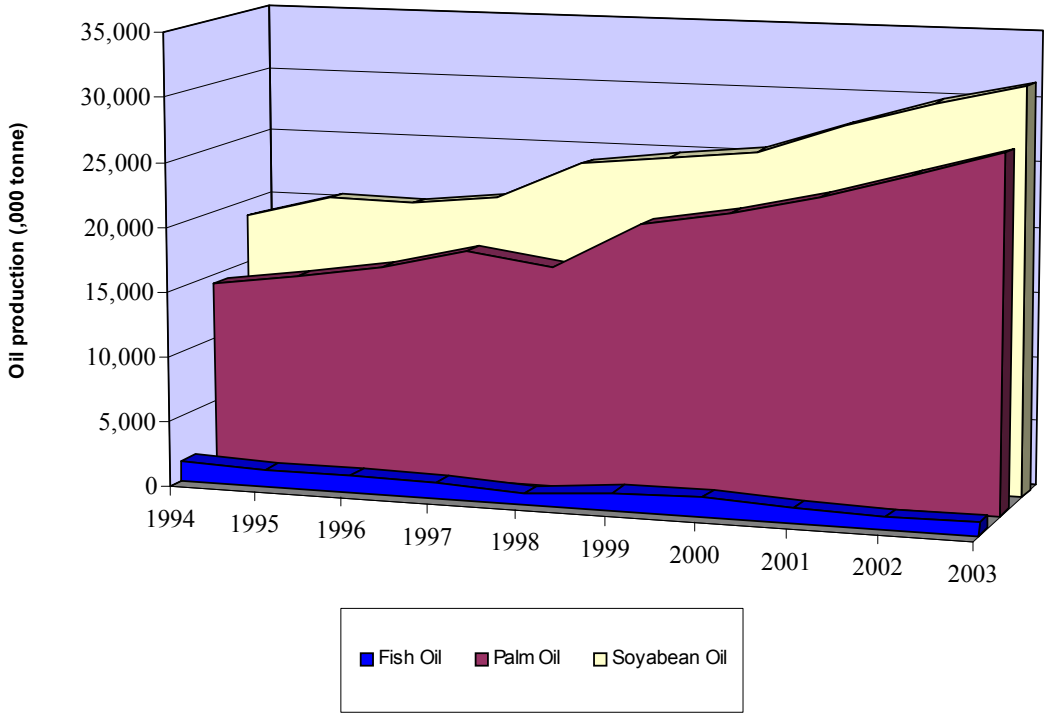


Figure 1.4 Production of fish oil, crude palm oil, soybean oil in the world from 1994 to 2003 (Source: Oil World Weekly, 1998-2003).

palm oil industries, namely palm fatty acid distillate (PFAD), which is abundant in tocotrienols, to partially or fully replace dietary fish oil. As to α-tocopheryl succinate, Jensen et al. (1999) reported that it was not a good vitamin E source for broilers, while to date no study has been conducted on this vitamin E source for fish. Tocotrienol-rich fraction (TRF) extracted from crude palm oil has been used in several *in vivo* or *in vitro* studies in rats on the antioxidant capability and distribution of tocotrienols (Kamat et al., 1995 & 1997; Ikeda et al., 2000 & 2001). No similar studies in fish have been done so far. Thus there is a necessity to conduct such studies to evaluate the bioavailability of vitamin E and distribution of palm tocotrienols in fish.

1.9 Aims of the study

1. To determine the bioavailability of different vitamin E sources for red hybrid tilapia (Experiment 1.).

2. To evaluate the oxidative stability of tilapia tissue when fed different vitamin E sources (Experiment 1) or increasing dietary levels of palm vitamin E (Experiment 2).

3. To estimate the potency of total vitamin E from palm oil-based products (such as CPO, PFAD and TRF) (Experiment 1) when deposited in tilapia tissues .

4. To determine the distribution of tocotrienols in different tissues of tilapia (Experiment 1 & 2).

The results from these two studies will better help us understand the nutritive value and metabolism of tocopherols and tocotrienols from palm oil-based products and their possible use as novel dietary vitamin E sources in fish feeds.

Chapter 2. Materials and Methods

2.1 Dietary ingredients

Dietary ingredients used in the study which consisted of Experiment 1 and Experiment 2, if bearing no origins or no specific explanations in the following context, were all purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.1.1 Dietary lipid and vitamin E sources

Because the two experiments were undertaken to explore some effects of vitamin E from different sources on fish, it was important and also necessary to use dietary ingredients containing only trace or no endogenous vitamin E content at all. Of all the non-vitamin E dietary ingredients to be used in the study, we can easily employ vitamin E-free constituents to formulate experimental diets, except for dietary lipid sources. Vitamin E is extensively distributed in a wide variety of vegetable oils and animal fats. Among those oils which have low levels of vitamin E content is crude palm kernel oil (CPKO). From two previous works done in our fish nutrition lab, Ng et al. (2001 & 2003) reported that dietary inclusion of 10% CPKO did not have any negative effects on growth performance of tilapia and African catfish, respectively. Therefore, CPKO was used as dietary lipid in the present study. In Experiment 1, *all-rac*-α-tocopheryl acetate (α-ToAc), *all-rac*-tocopheryl succinate (α-ToSc), and tocotrienol-rich fraction (TRF) were used as dietary vitamin E sources in Diets 2-4, Diet 5, and Diet 6, respectively. Synthetic *all-rac*-α-tocopheryl acetate is currently used in commercial

fish feeds as the dietary vitamin E source and was intended for comparison purposes. Palm fatty acid distillates (PFAD) or crude palm oil (CPO), serving as parts or the whole of added dietary lipid, as well as dietary vitamin E source, were used in Diet 7 and Diet 8, respectively. In Experiment 2, only CPKO and TRF were used as dietary lipid, and vitamin E source, respectively. Plate 2.1 shows CPKO, PFAD, CPO stored at room temperature. These three oils were obtained from Keck Seng (M) Sdn. Bhd (Johor, Malaysia). Their analysed tocopherols and tocotrienols concentrations by our lab were summarized in Table 3.1.

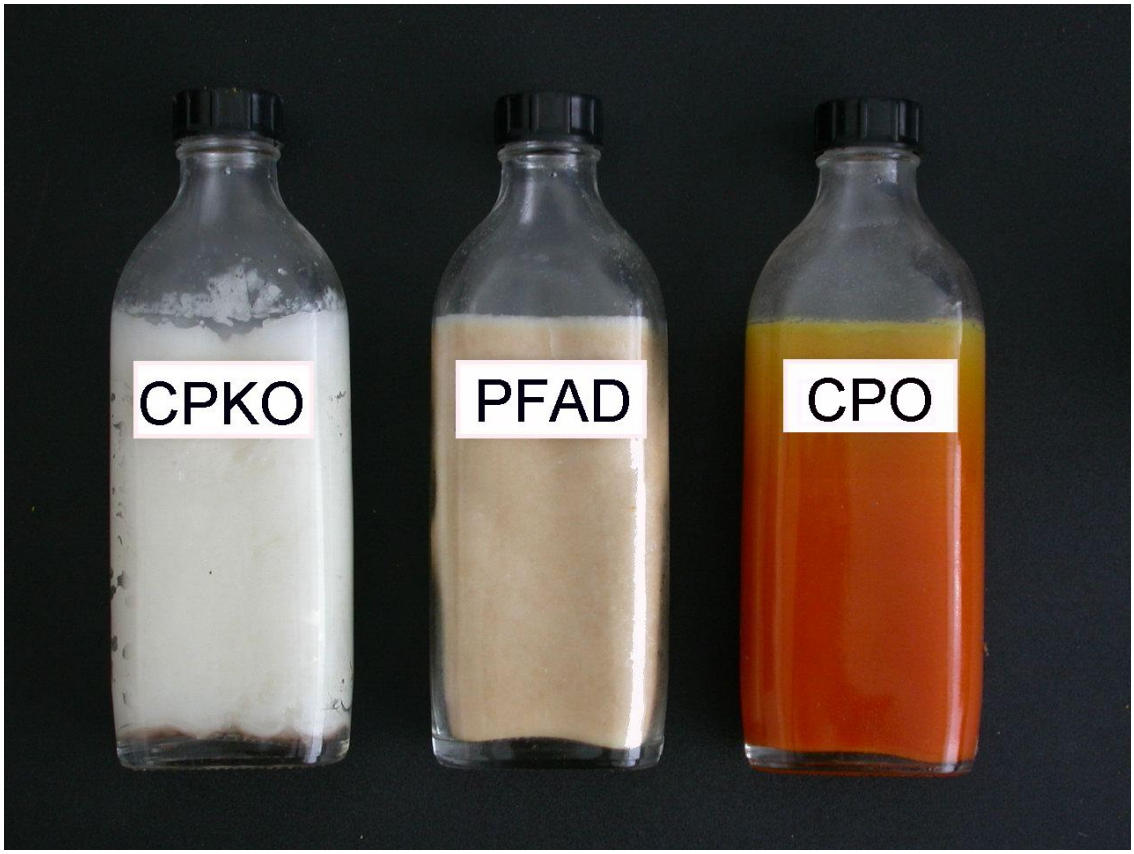


Plate 2.1 Crude palm kernel oil (CPKO), Palm fatty acid distillate (PFAD), Crude palm oil (CPO).

2.1.2 Dietary protein, vitamin and mineral premixes, and other ingredients

Except for vitamin E and the lipid sources, the remaining constituents of experimental diets consisted of vitamin-free casein, gelatin, vitamin E-free vitamin premix, mineral premix, dicalcium phosphate ($CaHPO_4$), carboxylmethyl cellulose (CMC) and α-cellulose (Liang Traco, Malaysia). Table 2.1 and 2.2 shows results of proximate analysis on some of the dietary ingredients for Experimental 1 and Experiment 2, respectively.

Vitamin E-free vitamin premix was prepared by mixing individual purified vitaminers. Their respective amount (g vitaminer/kg premix) was added as follows: ascorbic acid, 45; inositol, 5; choline bitartrate, 136.06; niacin, 4.5; riboflavin, 1; pyridoxine·HCl, 1; thiamin·HCl 0.92; d-calcium panthothenate, 3; retinyl acetate, 0.6; cholecalciferol, 0.083; menadione 1.67; d-biotin, 0.02; folic acid, 0.09; vitamin B12, 0.00135; cellulose, 801.056.

Table 2.1 Protein concentration of the feed ingredients (dry weight basis) in Experiment 1[1]

Ingredients	Dry matter	Moisture	Protein	Lipid
Casein V-free	93.89 ± 0.02	6.11 ± 0.02	89.30 ± 0.24	TR[2]
Gelatin	87.87 ± 0.04	12.13 ± 0.04	107.87 ± 0.22[3]	TR
Dextrin	92.73 ± 0.11	7.27 ± 0.11	ND[4]	ND
CMC[5]	88.53 ± 0.03	11.47 ± 0.03	ND	ND
α-Cellulose	94.71 ± 0.38	5.29 ± 0.38	ND	ND

[1]Values are mean ± s.d. of three replicates of samples analyzed.
[2]TR = trace
[3]Treat as 100% when conducting diet formulations
[4]ND = not detectable
[5]CMC = carboxylmethyl cellulose

Table 2.2 Protein concentration of the feed ingredients (dry weight basis) in Experiment 2[1]

Ingredients	Dry matter	Moisture	Protein	Lipid
Casein V-free	93.22 ± 0.63	6.78 ± 0.63	90.28 ± 0.05	TR[2]
Gelatin	88.75 ± 0.26	11.25 ± 0.26	107.87 ± 0.22[3]	TR
Dextrin	92.49 ± 0.26	7.51 ± 0.26	ND[4]	ND
CMC[5]	85.86 ± 0.64	14.14 ± 0.64	ND	ND
α-Cellulose	96.18 ± 0.40	3.82 ± 0.40	ND	ND

[1,2,3,4,5]See footnote of Table 2.1

Mineral premix was obtained through mixing individual A.R. grade salts, all of which were purchased from Fluka Chemical Co., Basel, Switzerland. The formulation was as follows (g/kg): Calcium Phosphate Monobasic, 135.490; Calcium L-Lactate Hydrate, 327.000; Ferric Citrate, 29.700; Magnesium Sulphate·$7H_2O$, 132.000; Potassium Phosphate Dibasic, 239.800; Sodium Phosphate Monobasic·H_2O, 87.200; Sodium Chloride, 43.500; Potassium Iodide, 0.150; Cuprous Chloride, 0.200; Manganous Sulfate·H_2O, 0.800; Cobalt Chloride·$6H_2O$, 1.000; Zinc Sulfate·$7H_2O$, 3.000; Sodium Selenite, 0.011.

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2.2 Composition and formulation of the experimental diets

In Experiment 1, eight isonitrogenous and isoenergetic semi-purified experimental diets were formulated. Dietary protein and energy levels were set at 35% and 14.64 $kJ \cdot g^{-1}$ diet, respectively. Diet 1 - Diet 6 , Diet 7 and Diet 8 were added either 10% CPKO, 7% CPKO + 3% PFAD, or 10% CPO, respectively. Four standard diets (Diet1 - Diet 4) were added α-ToAc at the level of 0, 25, 50 and 100 mg/kg, respectively, at

the expense of α-cellulose. Dietary vitamin E levels of Diet 5 - Diet 8 added were set at around α-tocopherol equivalents (α-TE) of Diet 3 (50 mg/kg α-ToAc), which was the level believed to meet the minimum requirement of tilapia for vitamin E (Shiau and Shiau, 2001). The following biopotency factors were adopted: *all-rac*-α-ToAc, 0.67; *all-rac*-α-ToSc, 0.60; d-α-T, 1.0, d-β-T, 0.5; d-γ-T, 0.1; d-δ-T 0.03; d-α-T3, 0.3; d-γ-T3, 0.01 (Sheppard and Pennington, 1993; Papas, 1999; Drotleff and Ternes, 1999); d-δ-T3 is unknown, treated as 0. So, 50 mg/kg α-ToSc, 500 mg/kg 20% TRF , 3% PFAD and 10% CPO were added to Diet 5 - Diet 8, respectively, serving as dietary vitamin E contributors. Table 2.3 shows compositions of eight experimental diets (g/100g dry diet) used in Experiment 1.