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Biopotency and bioavailability of palm tocopherols and tocotrienols in aquaculture feeds

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Summary of the report

In Experiment 1, a 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 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

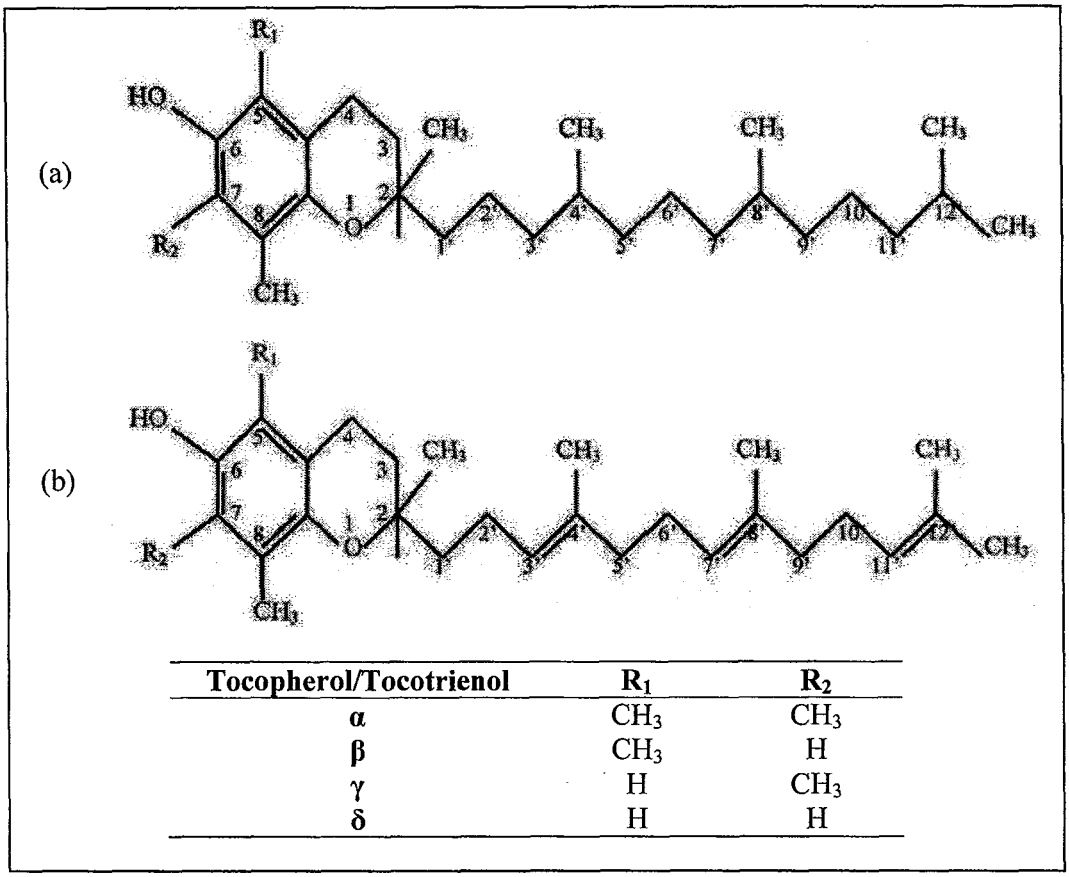
Vitamin E was discovered in 1922 by Evans and Bishop when studying the relationship of nutrition and fertility of rats, considered at the time to be especially important for normal reproduction. Female rats accidentally fed with rancid fat for a long time were observed to have a syndrome featuring a 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



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).

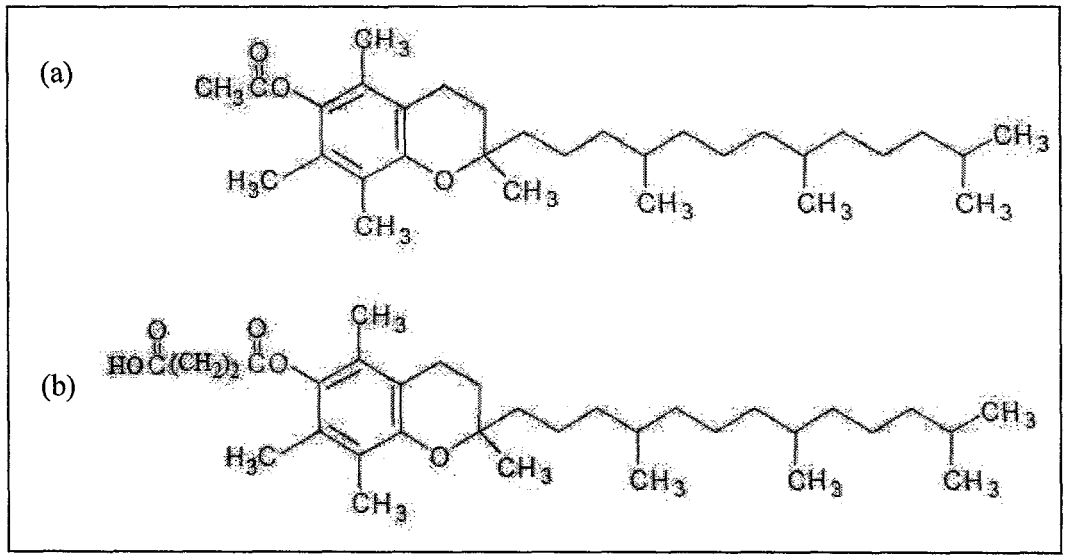


Figure 1.2 Structure of synthetic vitamin E: α-tocopheryl acetate (a) and α-tocopheryl succinate (b).

(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

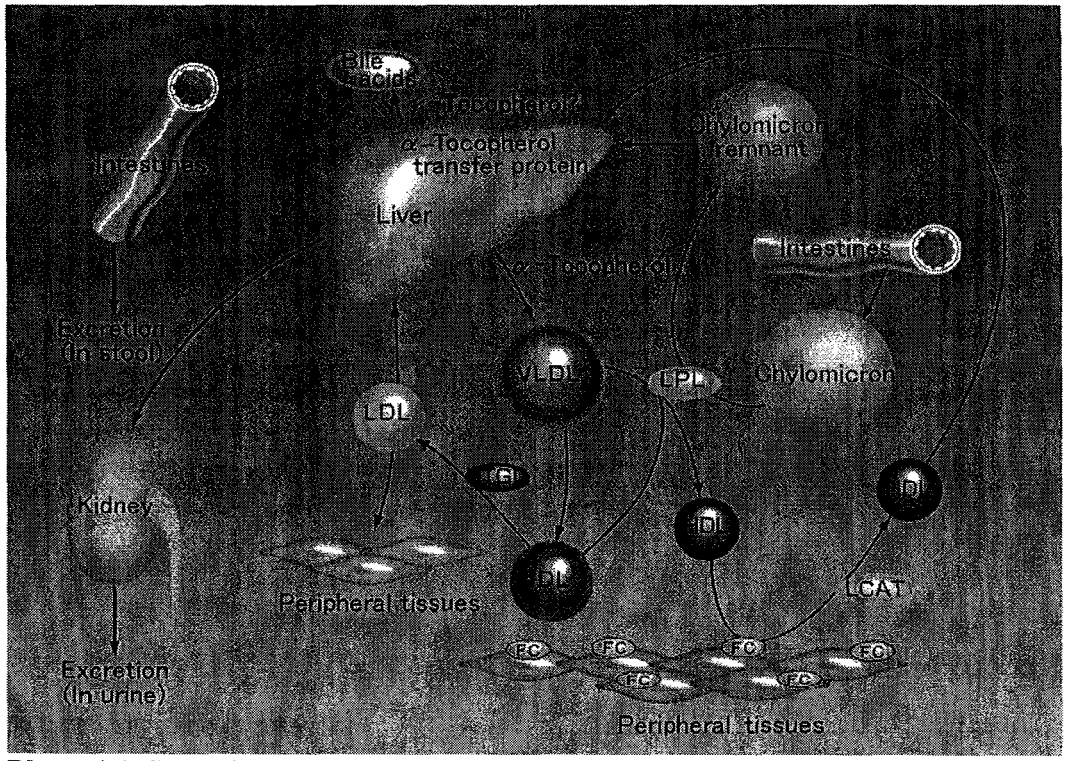


Plate 1.1 Vitamin E absorption and transport in mammalian animal body (source: *http://www.eisai.co.jp/evita_e/kiso4.html*).

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)

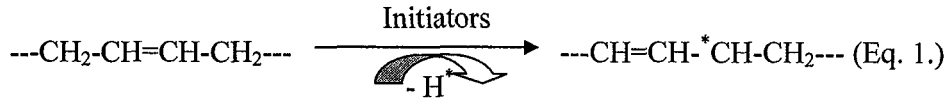
characterized TBP from bovine heart. TBP was only partially sequenced (Blatt, et al., 2001). The exact functions of TAP and TBP remain uncertain.

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

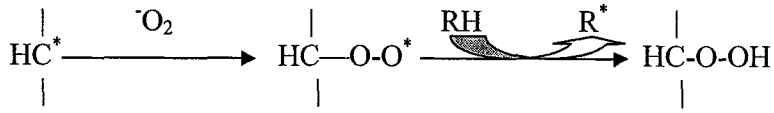
Lipid peroxidation is the introduction of a functional group containing two catenated oxygen atoms, O-O, into unsaturated fatty acids in a free radical reaction (Wheatley, 2000). This progress can generally be divided into three major reactions: initiation, propagation and termination. Polyunsaturated 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.



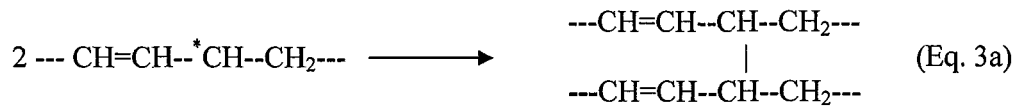
(Eq. 1.)

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).

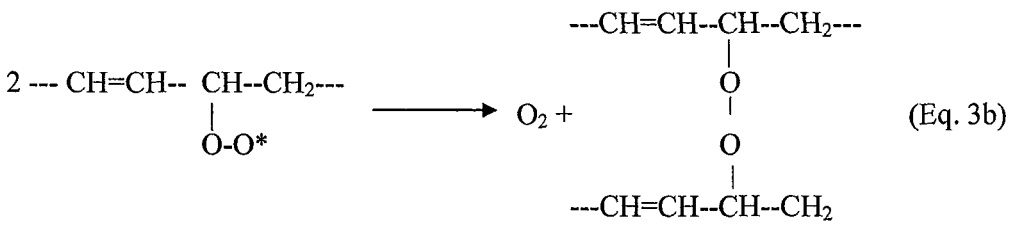


(Eq. 2.)

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

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

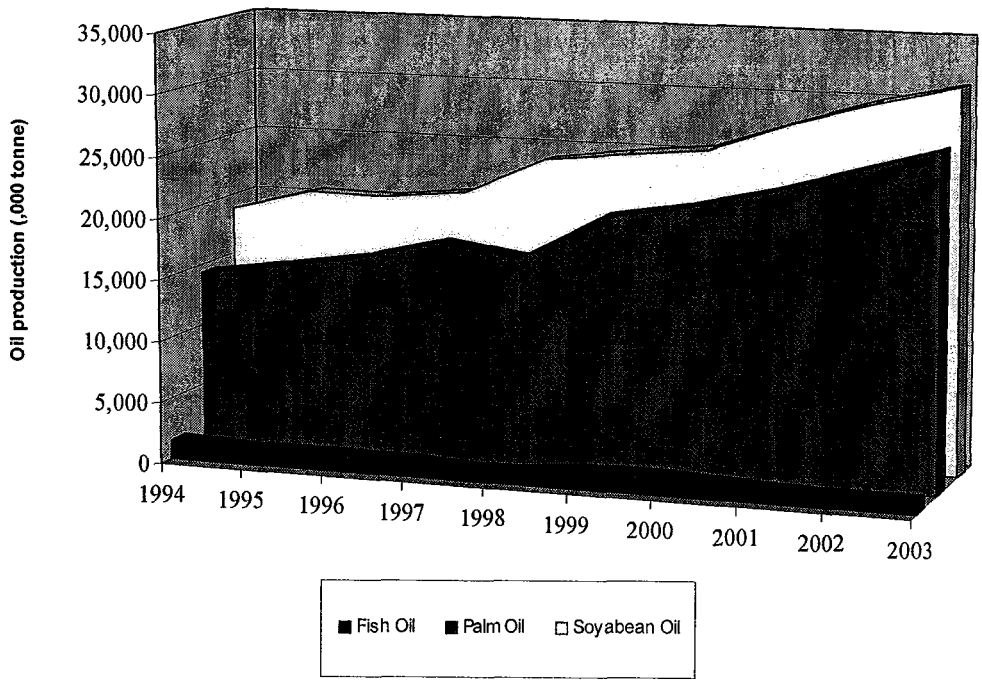


Figure 1.4 Production of fish oil, crude palm oil, soybean oil in the world from 1994 to 2003 (Source: Oil World Weekly, 1998-2003).

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.

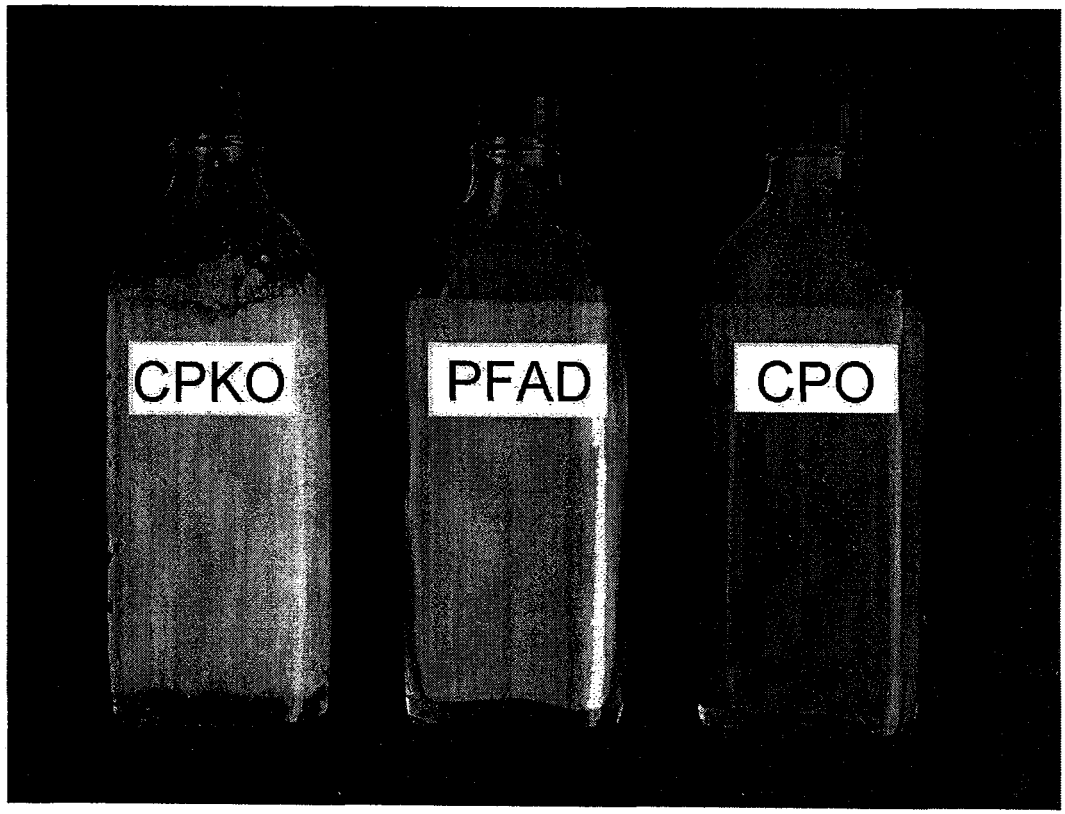


Plate 2.1 Crude palm kernel oil (CPKO), Palm fatty acid distillate (PFAD), Crude palm oil (CPO).

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.

Table 2.3 Composition (g/100 g dry diet) of the eight semi-purified experimental diets used in Experiment 1.

Ingredient [1]	Diets							
	1	2	3	4	5	6	7	8
	ToAc-0	ToAc-25	ToAc-50	ToAc-100	ToSc	TRF	PFAD	CPO
Casein (vitamin-free)	32.47	32.47	32.47	32.47	32.47	32.47	32.47	32.47
Gelatin	6	6	6	6	6	6	6	6
CPKO	10	10	10	10	10	10	7	–
Vitamin E source added [2]	–	0.05	0.10	0.20	0.005	0.05	3.00	10.00
White Dextrin	27.11	27.11	27.11	27.11	27.11	27.11	27.11	27.11
Vitamin premix [3]	3	3	3	3	3	3	3	3
Mineral premix [4]	4	4	4	4	4	4	4	4
Dicalcium Phosphate	1	1	1	1	1	1	1	1
CMC	2	2	2	2	2	2	2	2
α -Cellulose	14.42	14.37	14.32	14.22	14.41	14.37	14.42	14.42

[1]All purified feed ingredients were purchased from Sigma-Aldrich (MO, USA) except for the α-cellulose from Liang Traco (Penang, Malaysia) and elements of mineral premix from Fluka (Basel, Switzerland).

[2]α-ToAc, α-ToSc, TRF, PFAD and CPO were contributed as Diet 1- Diet 4, Diet 5, Diet 6, Diet 7 and Diet 8' s vitamin E source, respectively.

[3,4]See Section 2.1.2

In Experiment 2, dietary protein and energetic requirements were the same as those of Experiment 1, 35% of protein and 14.64 kJ of energy per g diet. CPKO and TRF were used as sole dietary lipid and vitamin E source, respectively, for all of the five experimental diets. The dietary lipid level was fixed at 10%. Diet 1 to Diet 5 were added total vitamin E amounts at 0, 30, 60, 120, 240 mg/kg, respectively, which corresponded to dietary TRF added at levels of 0, 138.89, 277.78, 555.56, and 1111.11 mg/kg. Table 2.4 shows composition of the five experimental diets (g/100g dry diet) used in Experiment 2.

Table 2.4 Composition (g/100g dry diet) of the five semi-purified experimental diets used in Experiment 2.

Ingredient[1]	Diets				
	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
V- free Casein	32.12	32.12	32.12	32.12	32.12
Gelatin	6.00	6.00	6.00	6.00	6.00
CPKO	10.00	10.00	10.00	10.00	10.00
Dextrin	27.56	27.56	27.56	27.56	27.56
Vitamin mix[2]	3.00	3.00	3.00	3.00	3.00
Mineral mix[3]	4.00	4.00	4.00	4.00	4.00
TRF[4]	0.00	0.14	0.28	0.56	1.11
Dicalcium phosphate	1.00	1.00	1.00	1.00	1.00
CMC	2.00	2.00	2.00	2.00	2.00
α-cellulose	14.32	14.18	14.04	13.76	13.21

[1,2,3]See footnote of Table 2.3

[4]Tocotrienol-rich fraction, contains (g/kg): d-α-T, 46.00; d-α-T3, 48.00; d-γ-T3, 94.00; d-δ-T3, 28.00.

In both two experiments, vitamin-free casein and gelatin were used as dietary protein sources for all experimental diets, with the provided levels set at 29% and 6%, respectively. The energy content provided by other than protein and lipid in all diets was contributed by white dextrin. Dextrin was chosen since several fish species (reviewed by Wilson, 1994) have been shown to be able to utilize dextrin as effective as lipid as an energy source within certain carbohydrate to lipid ratios. Mineral premix, vitamin E-free vitamin premix, CMC, $CaHPO_4$, were incorporated into the experimental diets with the levels fixed at 4%, 3%, 2%, and 1%, respectively. The formulations of mineral and vitamin E-free vitamin E premixes were described in Section **2.1.2**. α-cellulose served as the non-nutritive filler (NRC 1993).

2.3 Diet preparation

According to dietary formulations shown in Tables 2.4 and 2.5, the amounts of all ingredients, except oils, vitamin premix, and vitamin E, of each diet desired for pelleting, were weighed in advance into individual plastic containers, each of which corresponded to one diet. These dry ingredients were mixed homogenously in a Hobart mixer, after being added the desired amount of vitamin premix and vitamin E source (α-ToAc, α-ToSc, or TRF) for those diets not using endogenous vitamin E contents of dietary lipids as the sole vitamin E source. Before the semi-solid oils (CPKO, PFAD, or CPO) were added to the Hobart mixer, they had been melted at a temperature of 55°C in advance. The oils were thoroughly mixed with the ingredient mixture and distilled water was added to form a dough containing around 30-40% of water. The wet dough was cold-pelleted through a 3-mm die of a meat mincer to form spaghetti-

like strands, which were fans dried in a wooden shelf cabinet for around 5 hours at room temperature in subdued light. The strands were then broken down into 2 to 5 mm pellets and stored in air-tight freezer bags at -20 °C until used.

2.4 Verification of energy source in experimental diets

The energy content was calculated by using standard physiological fuel values of 4 kcal/g carbohydrate and protein, and 9 kcal/g of lipid (Lee & Putnam, 1973) in both Experiment 1 and Experiment 2:

- Metabolizable m energy (kJ)
  = [Total dietary energy donated by protein (4 kcal/g)
  + lipid (9 kcal/g) + carbohydrate (4 kcal/g) in diet] × 4.184 kJ*

  * Conversion factor: 1 kcal = 4.184 kJ.

- Protein energy (%)
  = [Energy donated by protein (kJ) / metabolizable energy (kJ)]/100

- Protein :Energy ratio (mg/kJ)
  = Dietary protein (mg) / metabolizable energy (kJ)

2.5 Fish management and samples collection

In both Experiment 1 and Experiment 2, red hybrid tilapia, *Oreochromis* sp. was used as the biological model to conduct the vitamin E nutrition studies.

2.5.1 Fish management

Red hybrid tilapia fingerlings of 2.5-inch size were purchased from a local hatchery. Upon arrival, they were stocked into a 1000-l round fiber-glass tank supplied with fresh flow-through water and continuous aeration to maintain water quality at our Fish Nutrition Lab. The fish were fed a commercially available floating fingerling diet (Dindings Feeds, Malaysia) twice daily for 2 weeks before they were used for the feeding trial. Then after being fasted for 24 hours, all fish were fed with the control diet (no exogenous vitamin E) for 1 week. The feeding time was the same as the time scheme to be applied during the actual feeding trials.

Plate 2.2 shows the Aquaria setup used in the present two feeding trials. At the start of the feeding experiments, groups of 12 red hybrid tilapia fingerlings (mean weight, 4.14 and 6.00 g for Experiment 1 and Experiment 2, respectively) were weighed and stocked into each aquarium (L × W × H= 70cm × 30cm × 46cm), which were provided with a 14:10 diurnal light : dark cycle of fluorescent lighting controlled by a timer. Each aquarium was supplied with continuous aeration. The static system was employed in both Experiment 1 and Experiment 2 (Ng et al., 2000). Eighty percent of the water in each aquarium was changed daily at 18:00h, 1 hour after the second daily ration allowance was fed to the experimental fish. If necessary, an appropriate volume of sodium thiosulfate (Na_2SO_3) solution was added to each aquarium to neutralize chlorine derived from fresh city tap water.

In both of the feeding trials, each test diet (8 and 5 diets for Experiment 1 and Experiment 2, respectively) was fed to randomly assigned triplicate groups of fish. The

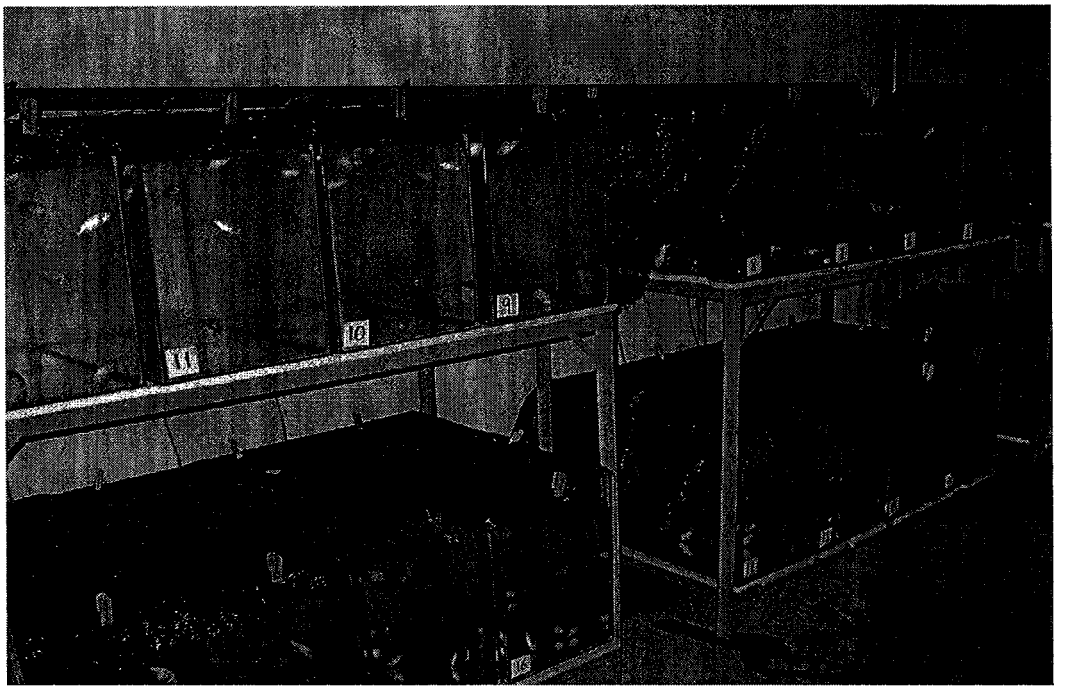


Plate 2.2 Aquaria setup used in the two feeding trials.

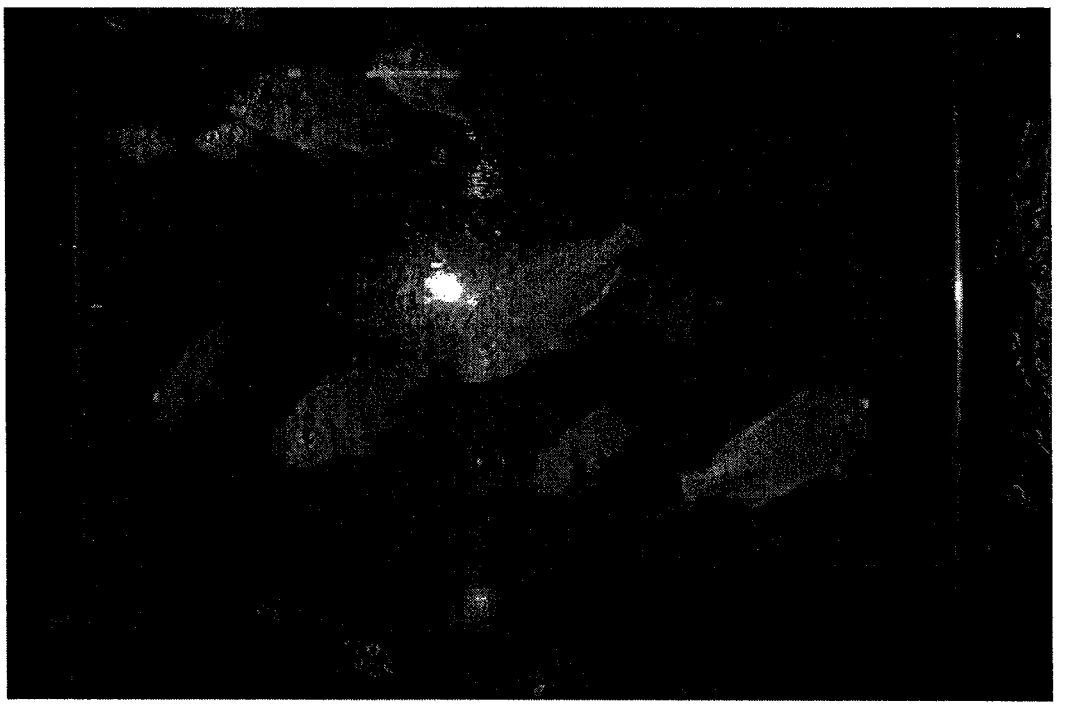


Plate 2.3 A close-up of tilapia reared in the aquarium.

fish were fed close to apparent satiation at a rate of 3.5% of their wet body weight per day. This daily feeding ration was divided into two equal feedings at 0900 and 1700h. Fish were batch weighed by aquarium once every week, and the daily feeding ration was adjusted accordingly. The feeding duration for Experiment 1 and Experiment 2 were 8 and 9 weeks, respectively. Plate 2.3 showed a close-up of red hybrid tilapia reared in an aquarium at the Fish Nutrition Laboratory in Universiti Sains Malaysia.

2.5.2 Samplings

At the inception of feeding trials of both the present two experiments, 5 fish were weighed, sacrificed, de-scaled and also de-skined. In Experiment 1, only fish muscle were excised, pooled, wrapped in parafilm and kept under -20 °C for subsequent vitamin E and fatty acid analysis to determine their respective baseline levels and original constituents. In Experiment 2, besides fish muscle, skin and liver were also sampled and kept for subsequent vitamin E analysis.

2.5.2.1 Blood collection and hematological analysis

At the termination of each feeding trial, all fish were weighed, and sacrificed by a sharp-blow on the head. Blood were collected into capillary tubes by severing the caudal peduncle of all the fish each aquarium for measurement of hematocrit (packed cell volume, PCV). Hematocrit was determined by the micro-centrifugation method through calculating the percentage occupied by the length of packed red blood cells

over the total length of blood sample in the capillary tube. A heparinized vacutainer tube was used to pool blood of fish from the same tank for hemolysis analysis.

The hemolysis analysis method developed by Draper and Csallany (1969) was adopted. Briefly, three drops of blood were added to a tube containing 3 ml saline-phosphate buffer (pH 7.4). The tube was centrifuged for 10 minutes at 500 × g. The supernate was decanted and the red blood cells were resuspended in 4 ml of phosphate buffer. 1 ml of cell suspension was transferred to two tubes either containing 4 ml buffer or 4 ml distilled water. The tubes were incubated in a water bath 37°C for 4 hours (in Experiment 1) or at 24°C for 15 hours (in Experiment 2). The optical absorbance of the supernate was read at 415 nm against a buffer blank. Percent hemolysis was computed from absorbance in buffer and absorbance in distilled water.

2.5.2.2 Fish tissue sampling

After blood collection, fish were further paunched to take out all of innards, de-scaled and de-skined. Fish liver and intraperitoneal fat were separated and weighed for the calculation of hepatosomatic index (HSI) and intraperitoneal fat index (IPI), respectively. Fish liver (without gall bladder) samples from half (3-6) of the randomly sampled fish, depending on the fish mortality of the tank, were pooled, wrapped in parafilm and kept under -20°C until subsequent vitamin E and thiobarbituric acid reactive substance (TBARS) analysis. Fish intraperitoneal fat and skin from all sampled fish were pooled, wrapped in parafilm and kept under -20 °C for subsequent vitamin E analysis. For fish muscle, three packages of samples were collected and kept

under the same condition as mentioned above. Each package was made up of pooled muscle dissected from 2 - 4 fish (randomly assigned again after fish were removed of their viscera), depending on the fish left from the tank being sampled. These fish muscle samples were later used for vitamin E, TBARS and fatty acid analysis.

In Experiment 2, samples taken for vitamin E and TBARS analysis were sampled using the procedure similar to those done in Experiment 1, with the only exception that 3-4 fish were kept for the whole body proximate analysis.

2.6 Evaluation of fish growth performance and feed efficiency

Fish growth performance was assessed by measuring percent weight gain (%), daily weight gain, and specific growth ratio (SGR). Feed efficiency was evaluated by feed conversion ratio (FCR), feed efficiency ratio (FER), and protein efficiency ratio (PER). The above parameters were calculated as follows:

- Weight gain (%)

$$= 100 \times \frac{[\text{Mean final fish weight (g)} - \text{Mean initial fish weight (g)}]}{\text{Mean initial fish weight (g)}}$$

- Specific growth rate (SGR) (%/day)

$$= 100 \times \frac{[\text{Ln(final fish weight, g)} - \text{Ln(initial fish weight, g)}]}{\text{Feeding Duration (Days)}}$$

- Daily weight gain (g/day)

$$= \frac{[\text{Mean final fish weight (g)} - \text{Mean initial fish weight (g)}]}{\text{Feeding Duration (Days)}}$$

- Feed Conversion Ratio (FCR)

  $$= \frac{\text{Total dry feed fed (g)}}{\text{Total wet weight gain (g)}}$$

- Feed Efficiency Ratio (FER)

  $$= \frac{\text{Total wet weight gain (g)}}{\text{Total dry feed fed (g)}}$$

- Protein Efficiency Ratio (PER)

  $$= \frac{\text{Total wet weight gain (g)}}{\text{Total protein intake (g)}}$$

2.7 Evaluation of somatic indices

Evaluation of somatic indices, hepatosomatic index (HSI) and intraperitoneal fat index (IPI), for tilapia in both Experiment 1 and Experiment 2, were calculated as a percentage of organ or tissue to the whole-body weight according to Peres and Oliva-Teles (1999):

- Hepatosomatic Index (HSI)

  $$= 100 \times \frac{\text{Liver weight (g)}}{\text{Fish body weight (g)}}$$

- Intraperitoneal Fat Index (IPI)

  $$= 100 \times \frac{\text{Intraperitoneal fat weight (g)}}{\text{Fish body weight (g)}}$$

2.8 Proximate analysis

In both Experiment 1 and Experiment 2, proximate analysis were performed on purified dietary ingredients and also pelleted experimental diets. Vitamin-free casein

and the diets were analysed for moisture, crude protein, crude lipid, fiber and nitrogen-free extract (NFE). NFE was not determined directly, but by the amount remaining after subtracting the total amounts of other proximate constituents from 100%, that is %NFE = 100- (%Ash + % Protein + %Lipid + % Fiber) (Ng et al., 2000). Gelatin, dextrin, CMC and α-cellulose, were analyzed for their moisture content. The procedures to perform these analyses were done according to AOAC standard methods (Association of Official Analytic Chemists, 1997).

2.9 Determination of tocopherol and tocotrienol concentrations

Vitamin E was analysed by high performance liquid chromatography (HPLC) on oils, pelleted diets and fish tissues obtained from the present two studies.

2.9.1 Chemicals and solvents

Butylated hydroxyltoluene (BHT), sodium chloride (ACS-graded, NaOH), potassium hydroxide (ACS-graded, KOH), diethyl ether, absolute ethanol (>99.6%) were purchased from Fisher Scientific Ltd. All HPLC-grade solvents, such as hexane, tetrahydrofuran (THF), ethyl acetate were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.9.2 HPLC equipments and vitamin E standards

The HPLC system consisted of a 5μ Kromasil silica column (250 x 4.6 mm; Metaphase, KL, Malaysia) equipped with a guard column (Upchurch Scientific, Oak Harbor, WA), a Rheodyne 7125 sample injector fitted with a 50μl sample loop, a Jasco PU-980 pump and a Jasco FP-1520 Flourescence Detector (Jasco, Tokyo) with the excitation and emission wavelength set at 296 nm and 330 nm, respectively. The chromatograms were recorded by a Hitachi D-2500 chromato-integrator (Hitachi, Tokyo) at a chart speed of 2.5 mm/min. The isocratic mobile phase used was hexane/tetrahydrofuran (100:4, v/v) at a flow rate of 2 ml/min.

Tocotrienol peaks were identified and quantified with the help of an in-house reference material extracted from palm oil (Tocomin® 50%: Carotech, Malaysia) which was initially calibrated using a tocotrienol standard kit purchased from Merck (Darmstadt, Germany). Tocopherol standards were purchased from Sigma-Aldrich (MO, USA).

2.9.3 Samples preparation

All the following operations should be performed in the subdued lighting conditions. All the solutions or solvents were kept on ice until use.

For the determination of vitamin E concentrations in CPKO, PFAD, and CPO, the cold saponification procedure recommended for vegetable oils as described by

Pocklington and Dieffenbacher (1988) with slight modifications were used to prepare oil samples. Weigh accurately about 2 g of the melt CPKO, PFAD or CPO into a conical flask, to which 8 ml of absolute ethanol, 100mg of BHT and 4 ml of 60% KOH were added. The flask was saponified for 15 minutes by being stirred on a stirrer in room temperature after being flushed with N_2 and covered tightly with the parafilm. After saponification, 50 ml of distilled water was added to the flask and the whole saponification medium was transferred to a 250 ml separating funnel. 50 ml of diethyl ether was used to rinse the conical flask and the solvent was then transferred to the funnel. The separating funnel was shaken vigorously for one minute and then left to allow the separation of two layers to occur. The lower aqueous layer was drawn off and extracted further two times with 50 ml of diethyl ether. The ether extracts to which vitamin E was extracted were combined and washed using distilled water until the solution got neutral. The diethyl ether was then transferred to the rotary evaporator flask for the removal of the diethyl ether under a temperature of <40°C. The liquid residue within the flask was dissolved in hexane and transferred to a 50 ml volumetric flask and made up to the volume. The resultant hexane stock solutions of PFAD and CPO were made a necessary dilution (e.g. 1:25) before 100 µl of aliquots were injected into the HPLC. For the CPKO stock solution, it is unnecessary to dilute.

Samples of diets, fish tissues and plasma were prepared for HPLC analyses using a modified method of Ikeda et al. (2003). Each experimental diet was finely ground, weighed (0.2 g) and transferred into a centrifuge tube, to which 0.2 ml of 2% NaCl, 2 ml ethanol containing 0.2% Butylated hydroxytoluene (BHT) and 0.4 ml of 60% KOH was added. The tube was flushed with nitrogen and capped. Then the contents inside the tube were thoroughly mixed and saponified at 70°C for 20 min. After cooling on

ice, the tube was added 2 ml of 2% NaCl. The tocopherols and tocotrienols were extracted with 5 ml of hexane containing ethyl acetate (v:v = 9:1). 100 μl of aliquots of the hexane layer was injected into the HPLC.

Fish muscle, liver, intraperitoneal fat and skin samples (0.5 g) were homogenized in 2 ml of absolute ethanol containing 0.2% BHT using an Ultra-Turrax tissue disrupter (IKA, Malaysia). The tissue homogenate was wholly transferred to a centrifuge tube and 0.2 ml of 60% KOH was added. The tube was flushed with nitrogen, capped, vortexed and saponified at 70°C for 20 min. After cooling on ice, the tube was added 2 ml of 2% NaCl. The tocopherols and tocotrienols were extracted with 3 ml of hexane containing ethyl acetate (v:v = 9:1). 100 μl of aliquots of the hexane layer was injected into the HPLC.

For vitamin E determination of fish plasma in Experiment 2, 0.15 ml of fish plasma was pipetted into a centrifuge tube, to which 2 ml of ethanol containing 0.2% BHT and 0.1 ml of 60% KOH was added. The tube was flushed with nitrogen, capped, vortexed and saponified at 70°C for 20 min. After cooling on ice, the tube was added 2 ml of 2% NaCl. The tocopherols and tocotrienols were extracted with 3 ml of hexane containing ethyl acetate (v:v = 9:1). The upper hexane layer was removed to a 5-ml vial and dried under N_2 stream. The residue in the vial was reconstituted into 250 μl of mobile phase, 100 μl of it was injected into the HPLC.

2.10 Determination of fatty acid profile of diets and fish muscle

Fatty acids were analysed using gas chromatography (GC) on experimental diets, initial and final fish muscle in Experiment 1.

2.10.1 Chemicals and solvents

Analytical grade methanol, chloroform, sodium chloride (ACS-graded, NaOH), potassium hydroxide (ACS-graded, KOH), anhydrous sodium sulphate (Na_2SO_4), methanolic boron trifluoride (BF_3), heptane were all purchased from Fisher Scientific Ltd.

2.10.2 GC equipments and fatty acid methyl ester standards

Shimadzu GC-14A GC is composed of a flame ionization detector and a Shimadzu C-R6A Chromato-Integrator. The column used was a 30 m × 0.32 mm (L × ID) Omegawax™ 320 fused silica capillary column (Supelco, Bellafonte, PA). Column temperature was set at 50°C for the first 2 min, then increased to 220°C at 4°C/min and held at this temperature for 15 min. An SPL-14 injector (split ratio 1:100) was used. Injector port and detector temperatures were 250 and 260°C, respectively. Helium was used as the carrier gas. Fatty acids were identified by comparing retention time with those of known standards (Supelco 37 Component FAME Mix; Supelco) and areas beneath the identified chromatographic peaks were calculated by integration.

2.10.3 Samples preparation

Lipids were extracted from diets and fish muscle samples with the chloroform and methanol (Bligh and Dyer, 1959). Appropriate amount of sample (1 g for diet and 5 g for fish muscle) was accurately weighed into the homogenization tube. The sample was extracted using 25 ml chloroform : methanol (2:1, v/v) for three times. The combined extract was transferred to the separating funnel, to which 15 ml of 0.88% KCl was added immediately and the mixture was vigorously shaken and then allowed to settle. The resultant lower layer was directly drawn into the evaporator flask and evaporated under reduced pressure at 75°C in a water bath until a residue was achieved.

The lipids obtained were processed to be methylated and transestificated with methanolic BF_3 (AOAC, 1997). The sample was boiled for 10 minutes at 75°C in a water bath after added 4 ml of 0.5 M NaOH and 3 ml of methanolic BF_3 until the droplets of fat disappeared. Before an appropriate volume of saturated NaCl was added to the flask, 4 ml of heptane was added to the boiling mixture and continued to boil for 1 minute. Then 1 ml of upper heptane layer was transferred to a test tube and added a little anhydrous Na_2SO_4 to remove traces of moisture. The heptane layer was transferred to a vial and kept -20°C until analyzed by GC.

2.11 Determination of TBARS concentrations in fish muscle, liver and plasma

Malondialdehyde (MDA) which was formed during the breakdown of PUFA was used as an index for determining the extent of lipid peroxidation. To determine the

oxidative stability of the fish muscle, liver (in Experiment 1 & 2) and plasma (in Experiment 2), the TBARS test was employed according to the procedure outlined in Baker and Davies (1996a). Briefly, 0.1 ml of 100 mg/ml homogenized muscle and liver sample, plasma or 11.5 g/L KCl (for the reagent blanks) was added to the screw-capped glass tubes containing 500 µl tris-maleate buffer (80 mM, Ph 7.4), 200 µl ascorbic acid (2 mM) and 200 µl $FeSO_4 \cdot 7H_2O$ (2 mM). Tubes were incubated for 30 minutes at room temperature. After incubation, 2 ml of TBA reagent (150 g TCA and 3.75 g 2-thiobabituric acid dissolved in 1 L of 0.25 M HCl) was added to each tube. The tubes were incubated in a boiling water-bath for 15 minutes. The color intensity of the MDA-TBA adduct was measured spectrophotometrically at 535 nm. The MDA concentration (express as nmol MDA/g tissue or nmol MDA/ml plasma) calculated from a calibration curve using MDA standards.

2.12 Calculations of bioavailability of α-tocopherol

Ng et al. (1998) developed a bioassay procedure to estimate the bioavailability of niacin originating from different dietary ingredients. This method was applied to calculate the bioavailability of α-T in different diets in Experiment 1. The standard linear regression equation of available α-T in diet (y in mg/kg) as a function of liver α-T concentration (x in µg/g) was derived, using Statistica 6.0 software (StatSoft, Tulsa, USA) from diets containing graded levels of α-tocopheryl acetate which were assumed to be 100% available. Assuming that the response of the observed liver α-T concentration for tilapia fed the test vitamin E sources fit into the regression equation, calculations of the amount of available α-T in the diet could be performed. The α-T

bioavailability estimate was then calculated as the ratio of available α-T (by liver α-T assay) to total α-T (by chemical method) in the diet.

2.13 Statistical Analysis

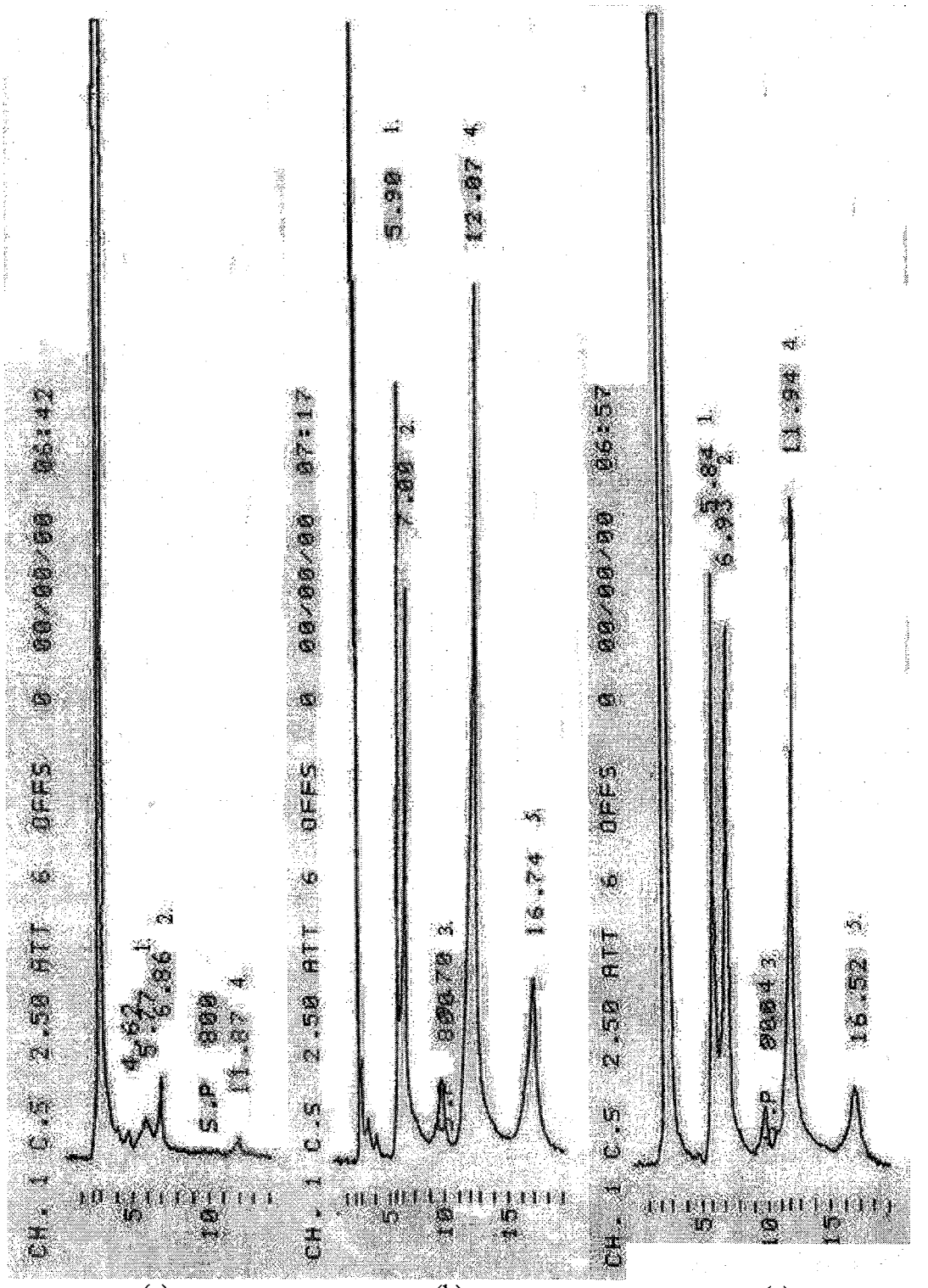
Final fish weight, percent weight gain, daily weight gain, special growth rate, feed conversion rate, feed efficiency, protein efficiency ratio, HSI, IPI, hematocrit, tissue vitamin E, fatty acids and TBARS were all subjected to one-way analysis of variance (ANOVA) using SPSS 11.5 for Windows (SPSS, Chicago, IL). Multiple comparison among means were made using the Duncan's new multiple range test (Duncan, 1955) when the analysis of variance value was significant. Statistical significance was set at the level $P<0.05$. All linear regression analysis used in both Experiment 1 and Experiment 2 were performing on Statistica 6.0 (StatSoft, Tulsa, USA).

Chapter 3. Results

3.1 Experiment 1

3.1.1 Vitamin E concentrations and composition of dietary oils

Assayed concentrations of vitamin E constituents in dietary CPKO, PFAD and CPO are summarized in Table 3.1. The percentage of individual tocopherol and tocotrienol, and their respective total percentage in oils are also present. Very low vitamin E was detected inn CPKO. Its constituents were α-T, α- T3 and γ-T3 with the concentrations of 3.11, 20.69 and 19.19mg/kg oil, respectively. Other vitamin E isoforms were not detected. The percentage of total tocotrienols accounted for up to 92.8% of total vitamin E. PFAD and CPO, both richest in γ-T3, were higher in vitamin E, amounting to 3889.22 mg total vitamin E /kg for PFAD which was about 4 times higher than CPO at 983.28 mg/kg, but their total percentages of tocopherols and tocotrienols were almost similar, with 20.6% versus 19.6% and 79.4% versus 80.4%, respectively. Figure 3.1 shows vitamin E profiles of the analyzed oils from their respective HPLC chromatograph.



(a) (b) (c)

Figure 3.1 Vitamin E profiles of HPLC chromatograph of CPKO (a); PFAD(b); CPO(c); peaks (1): α-T; (2): α-T3, (3): γ-T; (4): γ-T3; (5): δ-T3.

Table 3.1 Total tocopherols and tocotrienols in CPKO, PFAD and CPO used in Experiment 1[1].

Vitamin E	Oils		
	CPKO	PFAD	CPO
Concentration (mg/kg)			
α-T	3.11	745.52	176.45
β-T	ND	13.65	N D
γ-T	ND	41.47	16.41
α-T3	20.69	638.33	236.73
γ-T3	19.19	1676.08	452.51
δ-T3	ND	774.17	101.13
Total T + T3	42.99	3889.23	983.29
Composition (%)			
α-T	7.2	19.2	18.0
β-T	-	0.4	-
γ-T	-	1.1	1.7
α-T3	48.1	16.4	24.1
γ-T3	44.6	43.1	46.0
δ-T3	-	19.9	10.3
Total T	7.2	20.6	19.6
Total T3	92.8	79.4	80.4

[1]Values were expressed as means of three replicate analysis. ND = Not detected.

3.1.2 Experimental diets

3.1.2.1 Proximate composition

Table 3.2 summarizes the analysed results of proximate composition for the eight experimental diets. Moisture content of the eight pelleted diets ranged from 14.00 to 16.17%. Protein content ranged from 36.37 to 37.00%, somewhat higher than the level of 35% as set during dietary formulations. Dietary lipid levels remained at 9.20 to 9.57%, except for Diet 7 at 8.82%, probably resulting from the PFAD getting stuck within the mixing bowl during the mixing process due to the nature of PFAD that is easily solidificate. Ash and fiber contents were constant at 3.96 to 4.18% and 3.82 to 4.00%, respectively. Calculated metabolizable energy (kJ/g) and protein energy (%) of these eight experimental diets were similar at 17.27 to17.41 kJ/g and 35.08 to 35.86 %, respectively. Ratios of protein to energy ranged from 20.96 to 21.43 mg protein per kJ energy.

3.1.2.2 Dietary Vitamin E composition

Table 3.3 summarizes the HPLC results of individual vitamin E components for the eight experimental diets. Diets 1 to 5 supplemented with 0, 25, 50, 100 mg/kg α-ToAc, and 50 mg/kg α-ToSc, respectively, were detected after saponification to contain 2.98, 28.41, 54.59, 103.39, 46.03 mg α-T / kg dry diet, respectively. In these diets α-T is the dominant analysed dietary vitamin E component, accounting for more than 94%, except for Diet 1 in which although β-T was detected in trace amounts, as in all eight

Table 3.2 Proximate composition (g/100 g dry diet) and dietary energy contents of the eight semi-purified diets used in Experiment 1[1].

[1]	Diets							
	1	2	3	4	5	6	7	8
	ToAc-0	ToAc-25	ToAc-50	ToAc-100	ToSc	TRF	PFAD	CPO
Moisture	14.00 ± 0.08	15.16 ± 0.04	14.97 ± 0.04	15.91 ± 0.10	15.04 ± 0.05	16.17 ± 0.14	15.91 ± 0.07	15.04 ± 0.01
Protein	36.88 ± 0.08	36.62 ± 0.17	36.37 ± 0.03	36.50 ± 0.22	36.87 ± 0.19	36.83 ± 0.13	37.00 ± 0.13	36.83 ± 0.03
Lipid	9.24 ± 0.10	9.33 ± 0.10	9.27 ± 0.07	9.57 ± 0.10	9.20 ± 0.09	9.35 ± 0.13	8.82 ± 0.11	9.36 ± 0.04
Ash	4.09 ± 0.03	4.18 ± 0.03	3.97 ± 0.02	3.96 ± 0.02	4.03 ± 0.03	4.03 ± 0.01	4.03 ± 0.02	4.01 ± 0.05
Fiber	3.90 ± 0.07	3.93 ± 0.33	4.00 ± 0.36	3.96 ± 0.17	3.90 ± 014	4.09 ± 0.16	3.82 ± 0.08	3.95 ± 0.16
NFE[2]	45.89 ± 0.18	45.94 ± 0.06	46.39 ± 0.06	46.02 ± 0.18	45.99 ± 0.13	45.70 ± 0.01	46.33 ± 0.19	45.85 ± 0.03
Metabolizable energy (kJ/g)	17.33 ± 0.02	17.33 ± 0.02	17.34 ± 0.01	17.41 ± 0.02	17.33 ± 0.02	17.33 ± 0.03	17.27 ± 0.03	17.36 ± 0.02
Protein energy (%)	35.61 ± 0.06	35.37 ± 0.20	35.10 ± 0.06	35.08 ± 0.24	35.61 ± 0.23	35.56 ± 0.18	35.86 ± 0.12	35.51 ± 0.00
Protein : Energy ratio	21.28 ± 0.04	21.13 ± 0.12	20.97 ± 0.03	20.96 ± 0.14	21.28 ± 0.14	21.24 ± 0.11	21.43 ± 0.07	21.22 ± 0.00

[1]Values are means ± s.e. of three analysis, except fibre with only two replicates
[2]NFE: Nitrogen Free Extract.

diets, made up 20.7%. Vitamin E composition in Diets 6, 7 and 8 fundamentally reflected characteristics of their individually added TRF, PFAD, and CPO as different dietary vitamin E sources had substantial tocotrienols detection, accounting for around 75% of total vitamin E. Concentrations of γ-T3 was the highest (average 42.8%), followed by those of α-T3 (average 22.6%) and δ- T3 (average 9.2%). The content of α-T in Diets 6, 7 and 8 significantly dropped from more than 94.4 % in synthetic vitamin E added diets (Diets 2 to 5) to only 22%-24% of total vitamin E. Their actual levels varied between 27.15 and 34.20 mg/kg and were close to the measured level of 28.41 mg/kg in Diet 2. Diets 6 to 8 had trace amounts of β-T and γ-T detected.

3.1.2.3 Dietary fatty acid composition in Experiment 1.

Gas chromatography analyses were performed on the eight experimental diets, and their respective fatty acid profiles were summarized in Table 3.4. Dietary fatty acid compositions were basically reflected by the fatty acid profiles of their respective lipid source. Major fatty acid constituents (those >10%) in Diets 1 to 6, which used 10% CPKO as sole dietary lipid source, consisted of more than 76% of total fatty acids. In these diets, myristic acid (14:0) concentrations were the highest at 45.5 to 48.9%; lauric acid (12:0) and oleic acid (18:1n9) had somewhat lower concentrations of 14.6 to 15.7%, and 15.3 to 17.2%, respectively. Total minor fatty acid composition (those < 9% total fatty acid composition) of these six diets were less than 20%. Diet 7 was supplemented with a blend of 7% CPKO supplemented with 3% PFAD as dietary lipid source. Palmitic acid (16:0), one of minor fatty acids in the previous six diets (average 8.1%), has now become one of the major fatty acids, constituting a percentage of 19.6%

Table 3.3 Vitamin E concentrations and composition of the eight semi-purified experimental diets in Experiment 1[1].

Vitamin E	Diets							
	1	2	3	4	5	6	7	8
	ToAc-0	ToAc-25	ToAc-50	ToAc-100	ToSc-50	TRF	PFAD	CPO
Concentration (mg/kg diet)								
α-T	2.98	28.41	54.59	103.39	46.03	27.15	34.20	25.65
β-T	0.88	0.91	0.88	0.90	0.88	0.85	0.83	1.02
γ-T	ND	ND	ND	ND	ND	2.54	2.32	1.62
α-T3	0.38	0.78	0.56	0.54	0.78	28.55	29.10	27.24
γ-T3	ND	ND	ND	ND	0.00	52.08	60.69	48.92
δ-T3	ND	ND	ND	ND	ND	11.78	16.63	7.27
Total T + T3	4.24	30.10	56.04	104.82	47.68	122.96	143.77	111.71
Composition (%)								
α-T	70.2	94.4	97.4	98.6	96.5	22.1	23.8	22.9
β-T	20.7	3.0	1.6	0.9	1.8	0.7	0.6	0.9
γ-T	-	-	-	-	-	2.1	1.6	1.4
α-T3	9.0	2.6	1.0	0.5	1.6	23.2	20.2	24.4
γ-T3	-	-	-	-	-	42.4	42.2	43.8
δ-T3	-	-	-	-	-	9.6	11.6	6.5
Total T	91.0	97.4	99.0	99.5	98.4	24.8	26.0	25.3
Total T3	9.0	2.6	1.0	0.5	1.6	75.2	74.0	74.7

[1]Values were expressed means of three replicate analyses. ND = Not detected

of the total fatty acids. Other major fatty acids were myristic acid (10.8%), lauric acid 31.2%, and oleic acid (22.0%). For Diet 8, CPO was used as the sole dietary lipid source. Its palmitic acid, oleic acid and linolenic acid (18:2n-6) accounted for 43%, 37.5% and 11.6% of the total fatty acids, respectively. Minor fatty acids composition in Diets 7 and 8 were not more than 12% and 6.0% of the total fatty acids, respectively. Total saturates decreased from a mean of 78.0% of the total fatty acids of previous six diets to 67.6% in Diet 7 and 49.0% in Diet 8. For total monoenes, on the contrary, they increased from a mean of 16.0% of the total fatty acids of previous six diets to 22.0% in Diet 7 and 37.6% in Diet 8, which somewhat increased dietary unsaturated degree. All eight diets had a total PUFA consisting of exclusively n-6 fatty acids with no detectable n-3 fatty acids, resulting in a ratio of n-3/n-6 of zero. Contents of n-6 PUFA increased from a mean at 3.1% of in the first six diets to 5.9% in Diet 7 and 11.6% in Diet 8.

Table 3.4 Fatty acid composition (% of total fatty acids) of the eight semi-purified experimental diets used in Experiment 1.

Fatty acids	Diets							
%	1	2	3	4	5	6	7	8
	ToAc-0	ToAc-25	ToAc-50	ToAc-100	ToSc-50	TRF	PFAD	CPO
C8:0	3.5	2.8	2.8	3.3	2.9	2.6	1.9	ND[1]
C10:0	3.2	2.6	2.8	3.1	2.8	2.7	1.8	ND
C12:0	47.5	45.4	45.9	47.4	48.9	46.9	31.2	1.0
C14:0	14.6	14.8	15.4	14.9	15.7	15.7	10.8	1.3
C16:0	8.0	8.2	8.2	7.7	8.2	8.5	19.6	43.0
C16:1n7	ND	ND	ND	ND	ND	ND	ND	ND
C18:0	1.9	2.0	2.1	1.9	1.9	2.0	2.4	3.7
C18:1n9	15.6	16.4	17.2	15.3	15.5	15.7	22.0	37.5
C18:1n7	ND	ND	ND	ND	ND	ND	ND	ND
C18:2n6	3.0	3.4	3.2	2.8	3.1	3.1	5.9	11.6
C18:3n3	ND	ND	ND	ND	ND	ND	ND	ND
C18:4n3	ND	ND	ND	ND	ND	ND	ND	ND
C20:1n9	ND	ND	ND	ND	ND	ND	ND	ND
C20:3n6	ND	ND	ND	ND	ND	ND	ND	ND
C20:4n6	ND	ND	ND	ND	ND	ND	ND	ND
C20:3n3	ND	ND	ND	ND	ND	ND	ND	ND
C20:4n3	ND	ND	ND	ND	ND	ND	ND	ND
C20:5n3	ND	ND	ND	ND	ND	ND	ND	ND
C22:1n11	ND	ND	ND	ND	ND	ND	ND	ND
C22:5n6	ND	ND	ND	ND	ND	ND	ND	ND
C22:5n3	ND	ND	ND	ND	ND	ND	ND	ND
C22:6n3	ND	ND	ND	ND	ND	ND	ND	ND
Total saturates	78.6	75.7	77.2	78.2	80.4	78.4	67.6	49.0
Total monoenes	15.6	16.4	17.2	15.3	15.5	15.7	22.0	37.6
Total PUFA	3.0	3.4	3.2	2.8	3.1	3.1	5.9	11.6
Total n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total n-6	3.0	3.4	3.2	2.8	3.1	3.1	5.9	11.6
n-3 / n-6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[1]ND = not detected.

3.1.3 Tilapia

3.1.3.1 Growth performance and feed efficiency

Weekly accumulated percent weight gain, parameters of growth performance and feed utilization efficiencies of tilapia fed with eight experimental diets for 8 weeks, are shown in Figure 3.2 (the data were present in Appendix 2) and Table 3.5, respectively. As shown in Figure 3.2, from week 4, there was a trend that fish weekly accumulated percent weight gain began to deviate, but even at the termination of the experiment, the extent of deviation was still not high. No statistical significance was detected among mean weights of initial fish receiving their assigned diets. -Mean final weight and final fish weight gains were both not statistically significantly different ($P>0.05$), with the values ranging from 22.39-25.90 g and 438.87-525.07%, respectively (Table 3.5). Fish daily weight gain, SGR were not significantly different ($P>0.05$) among fish fed with the eight experimental diets, with values 0.34 to 0.40 g/day and 3.11 to 3.45 %/day. With respect to feed conversion ration (FCR) and protein efficiency rate (PER), values between treatments were not statistically significant. Fish fed Diet 5 (ToSc-50) had the highest FCR (1.01) and the lowest PER (2.71); whereas, fish fed Diet 8 had the lowest FCR (0.88) and the highest PER (3.08). As to survival rates, no statistical significance was detected, but 72.2% of fish receiving Diet 1 (ToAc-0) and 77.8% for Diet 5, respectively, were apparently lower than survival of fish receiving other diets. Among them, fish fed Diet 8 (CPO) had the highest survival rate of 94.44%. FCR, PER, SGR, and survival rates were all not significantly different ($P>0.05$).

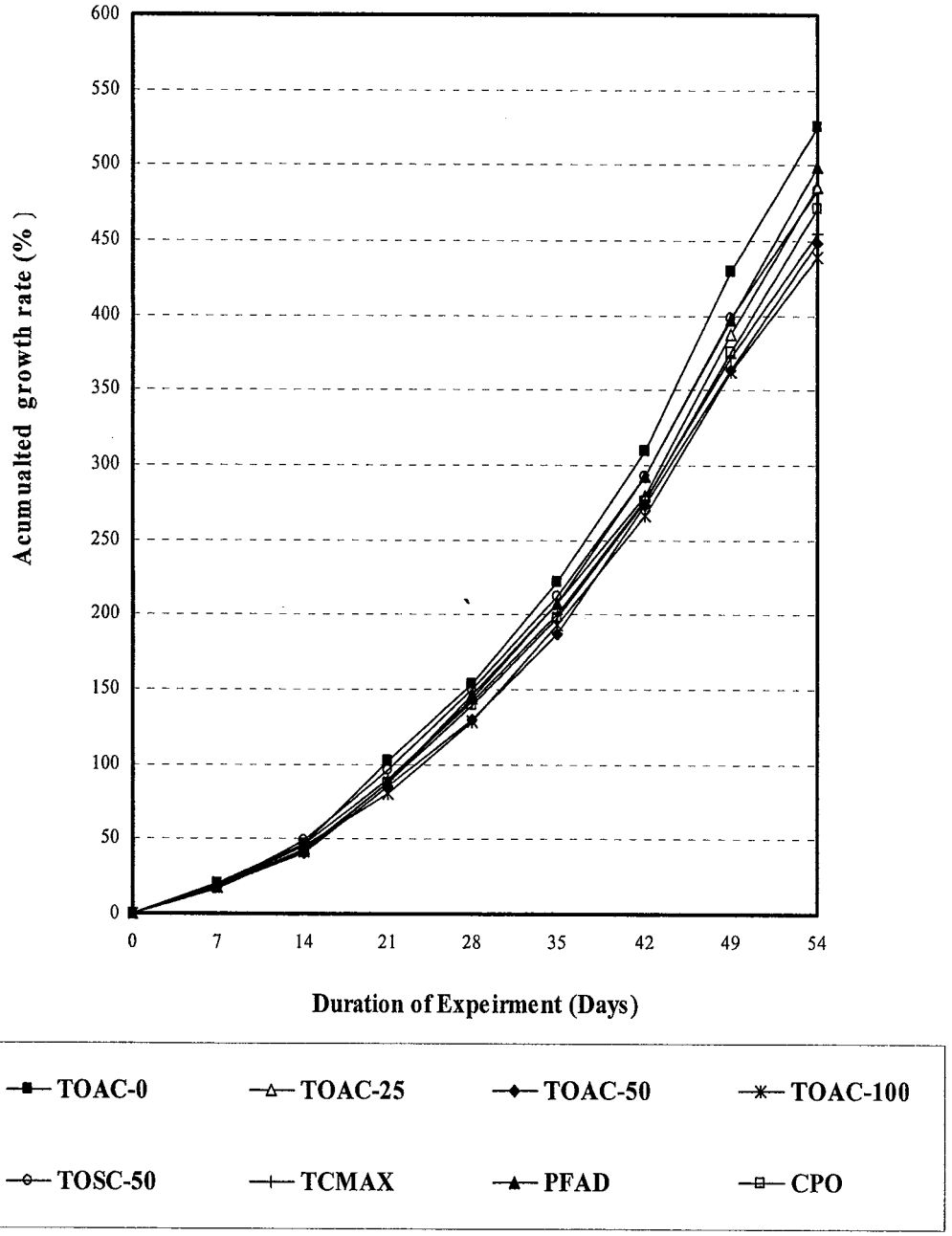


Figure 3.2 Weekly percent weight gain of red hybrid tilapia fed the eight semi-purified experimental diets for 8 weeks in Experiment 1.

Table 3.5 Growth performance, feed efficiency and survival rates of red hybrid tilapia given the eight semi-purified experimental diets for 8 weeks in Experiment 1[1].

	Diets	Mean initial fish weight (g)	Mean final fish weight (g)	Weight gain (%)	Specific growth rate (SGR) (% / day)	Daily weight gain (g/day)	Feed conversion rate (FCR)	Protein efficiency rate (PER)	Survival Rate (%)
1	ToAc-0	4.15 ± 0.03	25.90 ± 2.14	525.07 ± 55.28	3.38 ± 0.16	0.40 ± 0.04	1.00 ± 0.11	2.77 ± 0.28	72.2 ± 10.0
2	ToAc-25	4.11 ± 0.04	24.04 ± 1.35	485.64 ± 37.95	3.27 ± 0.12	0.37 ± 0.03	0.95 ± 0.04	2.87 ± 0.13	80.6 ± 7.4
3	ToAc-50	4.13 ± 0.03	22.68 ± 1.44	449.35 ± 36.22	3.15 ± 0.12	0.34 ± 0.03	0.99 ± 0.04	2.78 ± 0.10	80.6 ± 12.1
4	ToAc-100	4.16 ± 0.04	22.39 ± 1.67	438.87 ± 39.96	3.11 ± 0.14	0.34 ± 0.03	0.94 ± 0.03	2.92 ± 0.09	91.7 ± 4.8
5	ToSc- 50	4.15 ± 0.05	24.22 ± 1.51	483.44 ± 31.98	3.26 ± 0.10	0.37 ± 0.03	1.01 ± 0.05	2.71 ± 0.12	77.8 ± 10.0
6	TRF	4.10 ± 0.04	22.72 ± 0.58	454.48 ± 18.69	3.17 ± 0.06	0.34 ± 0.01	0.95 ± 0.05	2.89 ± 0.16	86.1 ± 7.4
7	PFAD	4.16 ± 0.04	24.96 ± 1.28	499.71 ± 25.37	3.45 ± 0.08	0.39 ± 0.02	0.91 ± 0.03	2.98 ± 0.09	80.6 ± 10.0
8	CPO	4.16 ± 0.03	23.77 ± 0.49	472.07 ± 11.05	3.16 ± 0.04	0.36 ± 0.01	0.88 ± 0.00	3.08 ± 0.02	94.4 ± 2.8

[1]Values were expressed as means ± s.e., all parameters had no statistical significance detected (P>0.05).

3.1.3.2 Somatic indices

Hepatosomatic index (HSI) were significantly ($P<0.05$) lower in fish fed with the CPO diet, compared to fish fed with the ToAc-0 (Diet 1) and ToAc-50 (Diet 3), but were not significantly different ($P>0.05$) compared to fish fed with the other diets (Table 3.6). Intraperitoneal fat index (IPI) among fish fed with various diets were not significantly different ($P>0.05$), with its values remaining somewhat constant at a mean of about 0.32.

Table 3.6 Hepatosomatic Index (HSI) and Intraperitoneal fat Index (IPI) of red hybrid tilapia given the eight semi-purified experimental diets for 8 weeks in Experiment 1[1].

	Diets	Hepatosomatic Index (HSI)	Intraperitoneal fat Index (IPI)
1	ToAc-0	1.94 ± 0.12^{a}	0.34 ± 0.06
2	ToAc-25	1.32 ± 0.08^{ab}	0.26 ± 0.05
3	ToAc-50	1.78 ± 0.18^{a}	0.38 ± 0.06
4	ToAc-100	1.69 ± 0.43^{ab}	0.26 ± 0.05
5	ToSc-50	1.62 ± 0.20^{ab}	0.44 ± 0.06
6	TRF	1.22 ± 0.06^{ab}	0.30 ± 0.07
7	PFAD	1.53 ± 0.30^{ab}	0.32 ± 0.15
8	CPO	0.98 ± 0.07^{b}	0.29 ± 0.02

[1]Values were expressed as means ± s.e. from three replicates, results in columns bearing different superscripts were significantly different ($P<0.05$).

3.1.3.3 Hematological results

Results of hematocrits and 10-hr incubation hemolysis are summarized in Table 3.7. Hematocrits were significant higher ($P<0.05$) in fish fed Diet 5 compared to fish fed with Diet 8 and Diet 2, but was not significantly different compared to fish fed with other diets. There was no significant difference ($P>0.05$) for hemolysis after a 10-hr incubation at 37 °C, but values of 25.30% and 28.68% from blood of fish fed with Diets 1 and 5, respectively, were apparently higher than those of fish fed the other six diets.

Table 3.7 Hematocrits and 10-hr incubated blood hemolysis under 37 °C of red hybrid tilapia given the eight semi-purified experimental diets after 8 weeks for Experiment 1[1].

	Diets	Hematocrits (%)	10-hr Blood Hemolysis (%)
1	ToAc-0	41.04 ± 3.09^{ab}	25.30 ± 7.26
2	ToAc-25	35.32 ± 1.67^{b}	17.41 ± 0.34
3	ToAc-50	36.90 ± 2.75^{ab}	16.29 ± 1.35
4	ToAc-100	39.56 ± 2.47^{ab}	16.17 ± 1.35
5	ToSc-50	44.36 ± 4.05^{a}	28.68 ± 10.18
6	TRF	41.83 ± 1.00^{a}	19.95 ± 2.75
7	PFAD	41.08 ± 2.07^{a}	16.76 ± 1.40
8	CPO	35.83 ± 0.43^{b}	23.92 ± 2.03

[1]Values were expressed as means ± s.e. from three replicates, results in columns bearing different superscripts were significantly different ($P<0.05$).

3.1.4 Fish muscle, liver, adipose tissue and skin

3.1.4.1 Vitamin E distribution in various tilapia tissues

After an 8-week feeding trial, tilapia were sacrificed and sampled for vitamin E analysis. Figure 3.3 (a), (b), (c) to (d) showed the distribution of various vitamin E isoforms in the fish muscle, liver, adipose tissue and skin, respectively, and the corresponding data were presented in Appendix 3A, B, C and D, respectively. All of the vitamin E isoforms detected in dietary lipids were deposited into the tissues at varying amounts.

3.1.4.1.1 Vitamin E concentration and composition in fish muscle

Only α- and β-T were detected in the muscle of initial fish (Appendix 3A). Their respective concentration were 8.47 and 0.39 μg/g, respectively, accounting for 95.6 and 4.4% of the total vitamin E. After tilapia were fed with the respective experimental diets for 8 weeks, vitamin E profile of fish muscle significantly changed (Figure 3.3 (a)). However, the major vitamin E isoform deposited was still α-T. It was observed that only the concentration (9.06 μg/g) of α-T of fish fed Diet 4 (100 mg/kg α-ToAc) was higher than that of initial fish. Muscle concentrations of α-T of fish fed Diet 1 (without vitamin E added) and Diet 5 (50 mg/kg α-ToSc) were 1.32 and 2.19 μg/g, respectively. They were significantly ($P<0.05$) lower than those of fish fed with other diets. α-T concentrations of muscle from fish fed with Diets 2, 3, and 6 to 8 varied from 4.18 to 6.58 μg/g. A constant low concentration (average 0.34 μg/g) of β-T was

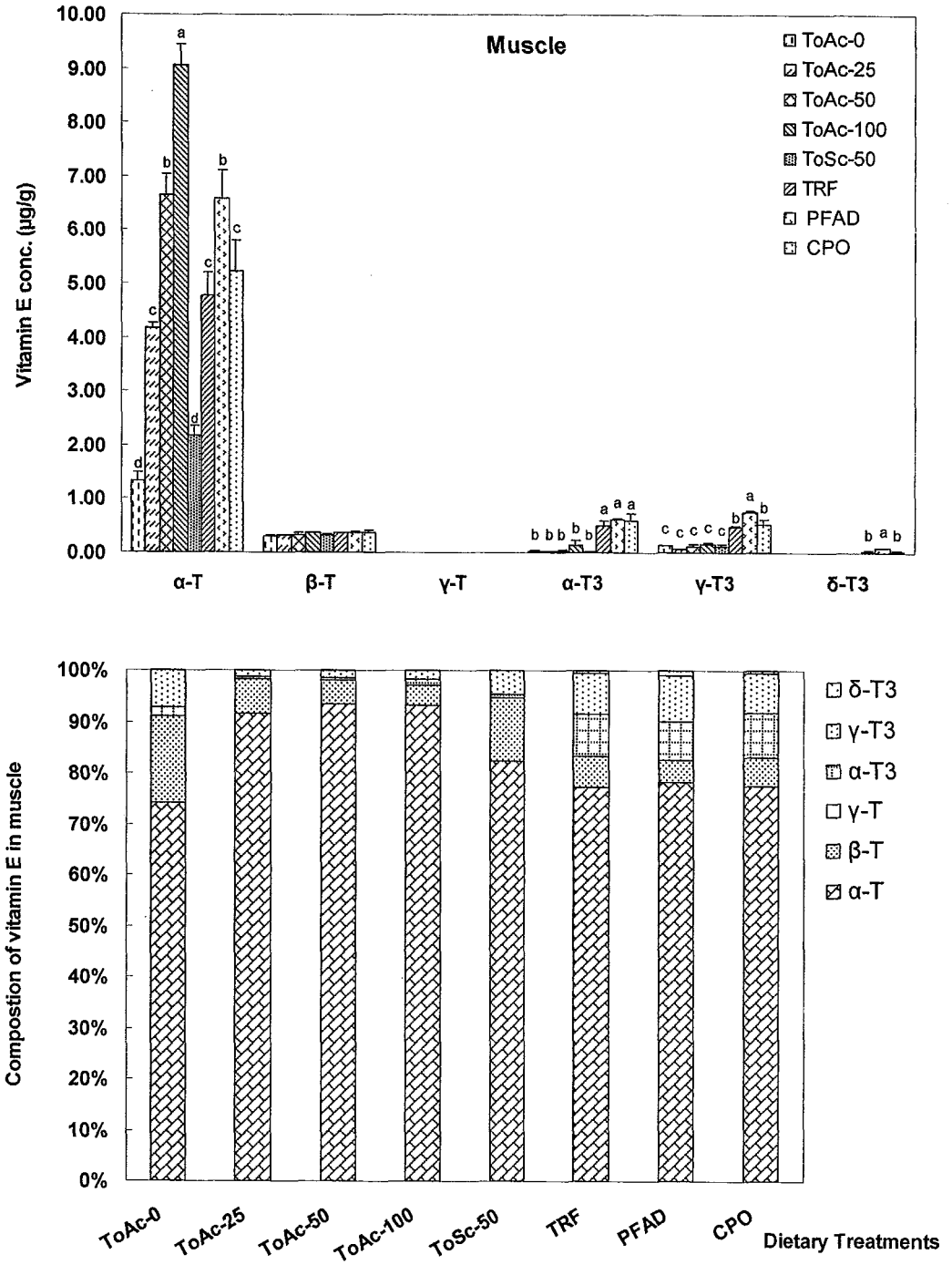


Figure 3.3 (a) Vitamin E concentrations and composition in muscle of red hybrid tilapia fed with eight different experimental diets for 8 weeks in Experiment 1. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

detected in muscle of fish fed with different experimental diets, but no γ- and δ-T was detected. Total tocopherols accounted for 91.1 to 98.2% of total vitamin E in the muscle of fish given Diets 1 to 5, which was significantly higher than 82.6 to 83.2% of total vitamin E in the muscle of fish given Diets 6 to 8.

α- and γ-T3 were deposited in the muscle of experimental tilapia, but their concentrations were very low (Appendix 3A), irrespective of dietary tocotrienols concentrations. Muscle concentrations of α- and γ-T3 of fish fed Diets 6 to 8, varied from 0.51 to 0.61 μg/g and 0.49 to 0.75 μg/g, corresponding to 7.3 to 8.7% and 7.7 to 9.0% of total vitamin E in muscle, respectively. Concentrations of muscle α- and γ-T3 were 0.02 to 0.13 μg/g and 0.06 to 0.16 μg/g from muscle of fish fed with Diets 1 to 5, respectively. With respect to δ-T3, only traces (0.03 to 0.09 μg/g) were detected in fish fed Diets 6 to 8. Total tocotrienols accounted for 16.8 to 17.1% of total vitamin E in the muscle of fish given tocotrienols-rich Diets 6 to 8, which were significantly ($P<0.05$) higher than 1.8 to 9.8% in the muscle of fish given Diets 1 to 5.

3.1.4.1.2 Vitamin E concentration and composition in fish liver

Vitamin E distribution of tilapia liver was shown in Figure 3.3 (b), and the data were given at Appendix 3B. Liver concentrations of α-T of fish fed with Diet 1 (without vitamin E added) and Diet 5 (50 mg/kg α-ToSc) were 2.39 and 5.56 μg/g, respectively. They were significantly ($P<0.05$) lower than those of fish fed with other diets. The two highest liver α-T concentrations of 74.40 and 40.72 μg/g were obtained from fish fed with Diet 4 (100 mg/kg α-ToAc) and Diet 3 (50 mg/kg α-ToAc),

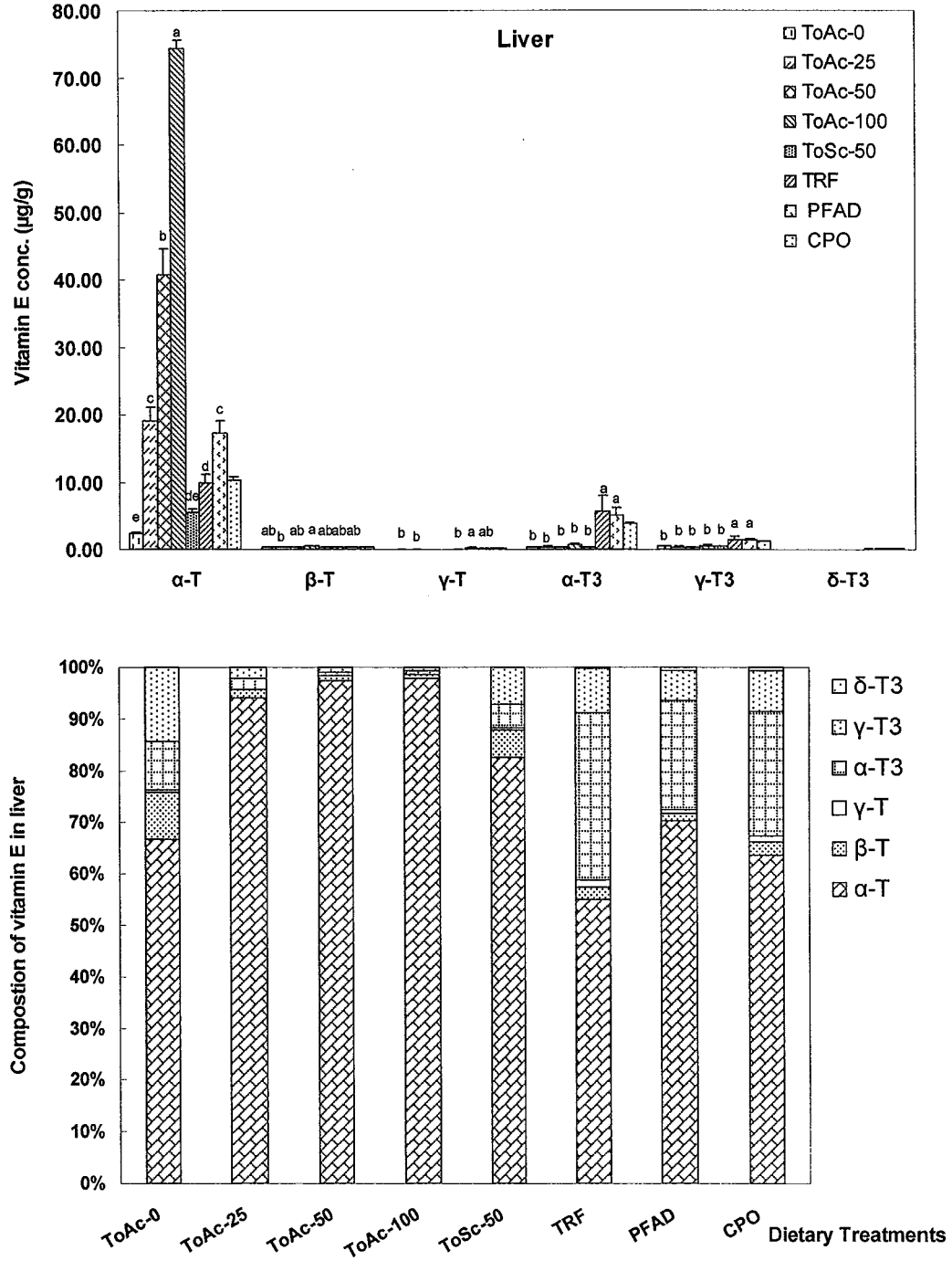


Figure 3.3 (b) Vitamin E concentrations and composition in liver of red hybrid tilapia fed with eight different experimental diets for 8 weeks in Experiment 1. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

respectively. Liver concentrations of α-T of fish fed with the remaining diets (Diets 2, 6, 7 and 8) varied from 9.82 to 19.01 μg/g. A constant low concentration (average 0.39 μg/g) of β-T was detected in liver of fish fed with different experimental diets, and only traces of γ-T were deposited in some fish. Total tocopherols accounted for 88.1 to 98.2% of total vitamin E in the livers of fish given Diets 2 to 5, which was significantly ($P<0.05$) higher than 61.5 to76.3% of total vitamin E in the livers of fish given Diets 1, 6 to 8.

Similar levels of α-T3 (0.32 to 0.66 μg/g) and γ-T3 (0.40 to 0.52 μg/g) were deposited in the liver of tilapia fed with synthetic vitamin E supplemented diets (Diets 1 to 5) (Appendix 3B). They was significantly ($P<0.05$) lower than liver concentrations of α-T3 (3.89 to 5.74 μg/g) and γ-T3 (1.26 to 1.54 μg/g), respectively, of tilapia fed with TRF, PFAD, or CPO-based diets (Diets 6 to 8). α-T3 concentrations of liver of tilapia fed with TRF, PFAD, and CPO-based diets were found to be two times higher than γ-T3 concentrations despite dietary γ-T3 being twice as much as the amounts of α-T3. Total tocotrienols accounted for 27.2 to 38.5% of total vitamin E in the livers of fish given tocotrienols-rich, Diets 6 to 8, which were significantly ($P<0.05$) higher than 1.6 to 23.7% in the livers of fish given Diets 1 to 5.

3.1.4.1.3 Vitamin E concentration and composition in fish adipose tissue

Vitamin E distribution of tilapia adipose tissue was shown in Figure 3.3 (c), and the data were given in Appendix 3C. Adipose concentration of α-T of fish fed Diet 5 (50 mg/kg α-ToSc) were 4.53 μg/g, which was significantly ($P<0.05$) lower than 24.55,

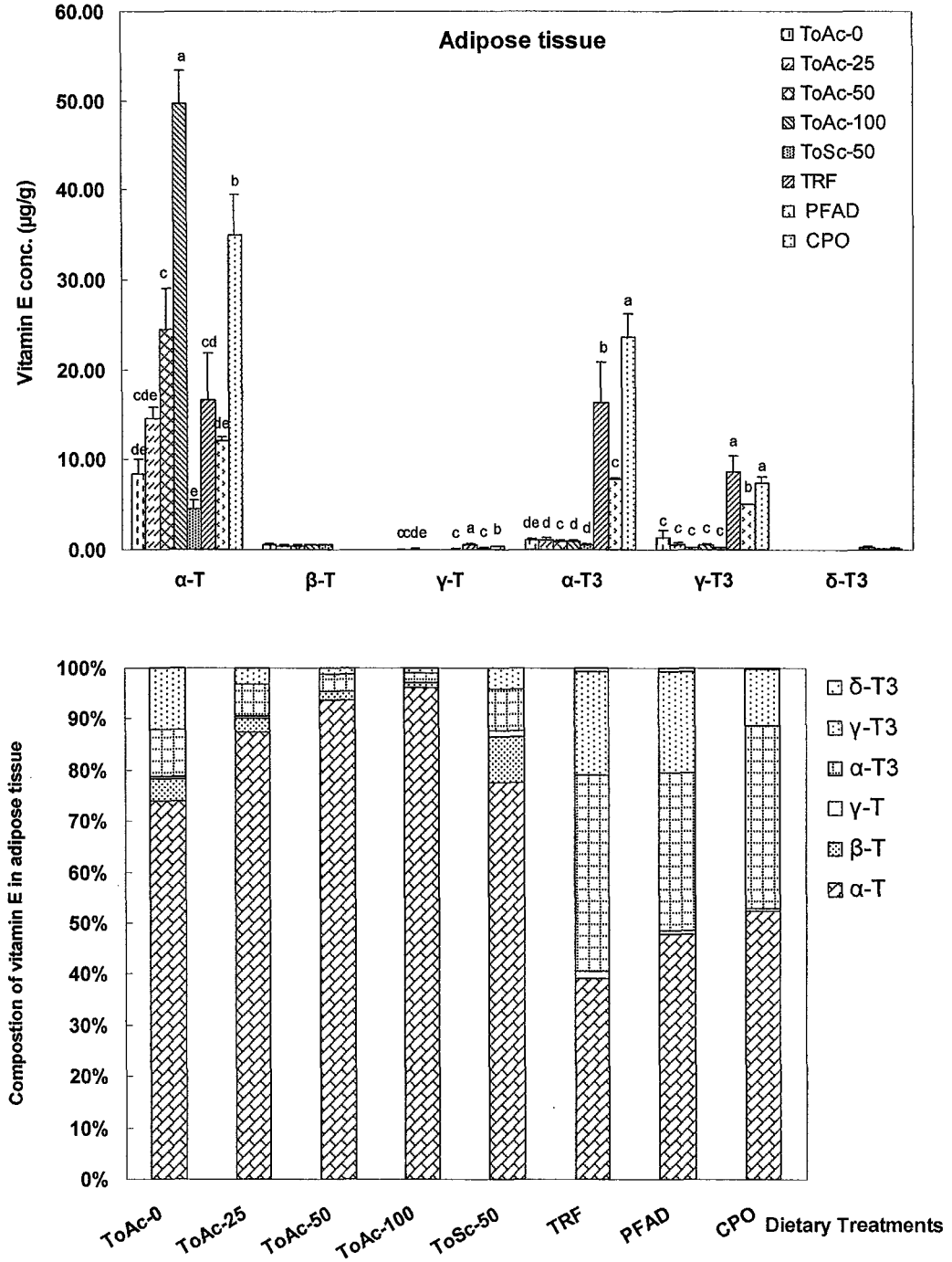


Figure 3.3 (c) Vitamin E concentrations and composition in adipose tissue of red hybrid tilapia fed with eight different experimental diets for 8 weeks in Experiment 1. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

49.70, 16.63, and 34.97 μg/g of fish fed Diets 3, 4, 6 or 8, respectively; but not significantly different from 8.37, 14.62 and 12.13 μg/g of fish fed given Diets 1, 2 and 7, respectively. Only adipose tissue of fish given synthetic vitamin E diets (Diet 1 to 5) had β-T (average 0.50 μg/g) detected. Adipose tissue of most of fish had low levels of γ-T (0.04 to 0.61 μg/g). Total tocopherols accounted for 80.8 to 97.1% of total vitamin E in the adipose tissue of fish given Diets 1 to 5, which were significantly ($P<0.05$) higher than 40.1 to 52.9% of total vitamin E in the livers of fish given Diets 6 to 8.

Substantial levels of 7.83 to 23.71 μg/g of α-T3 and 5.05 to 8.63 μg/g of γ-T3 were deposited into the adipose tissue of tilapia fed tocotrienols-rich diets (Diets 6 to 8) (Figure 3.3 c and Appendix 3C), the former were much higher than the latter despite dietary γ-T3 being twice as much as the amounts of α-T3. Low levels of 0.49 to 1.06 μg/g of α-T3 and 0.23 to 1.37 μg/g of γ-T3 were deposited in the adipose tissue of fish fed with synthetic vitamin E supplemented diets (Diets 1 to 5). As to adipose δ-T3, only traces were deposited in fish fed with Diets 6 to 8. Total tocotrienols accounted for 40.1 to 52.9% of total vitamin E in the adipose tissue of fish given tocotrienols-rich Diets 6 to 8, which were significantly ($P<0.05$) higher than 2.9 to 19.2% in the adipose tissue of fish given Diets 1 to 5.

3.1.4.1.4 Vitamin E concentration and composition in fish skin

Vitamin E distribution of tilapia skin was shown in Figure 3.3 (d), and the data were given in Appendix 3D. Skin concentration of α-T of fish fed Diet 4 (100 mg/kg α-ToAc) was 20.46 μg/g, which was significantly ($P<0.05$) higher than those of fish fed

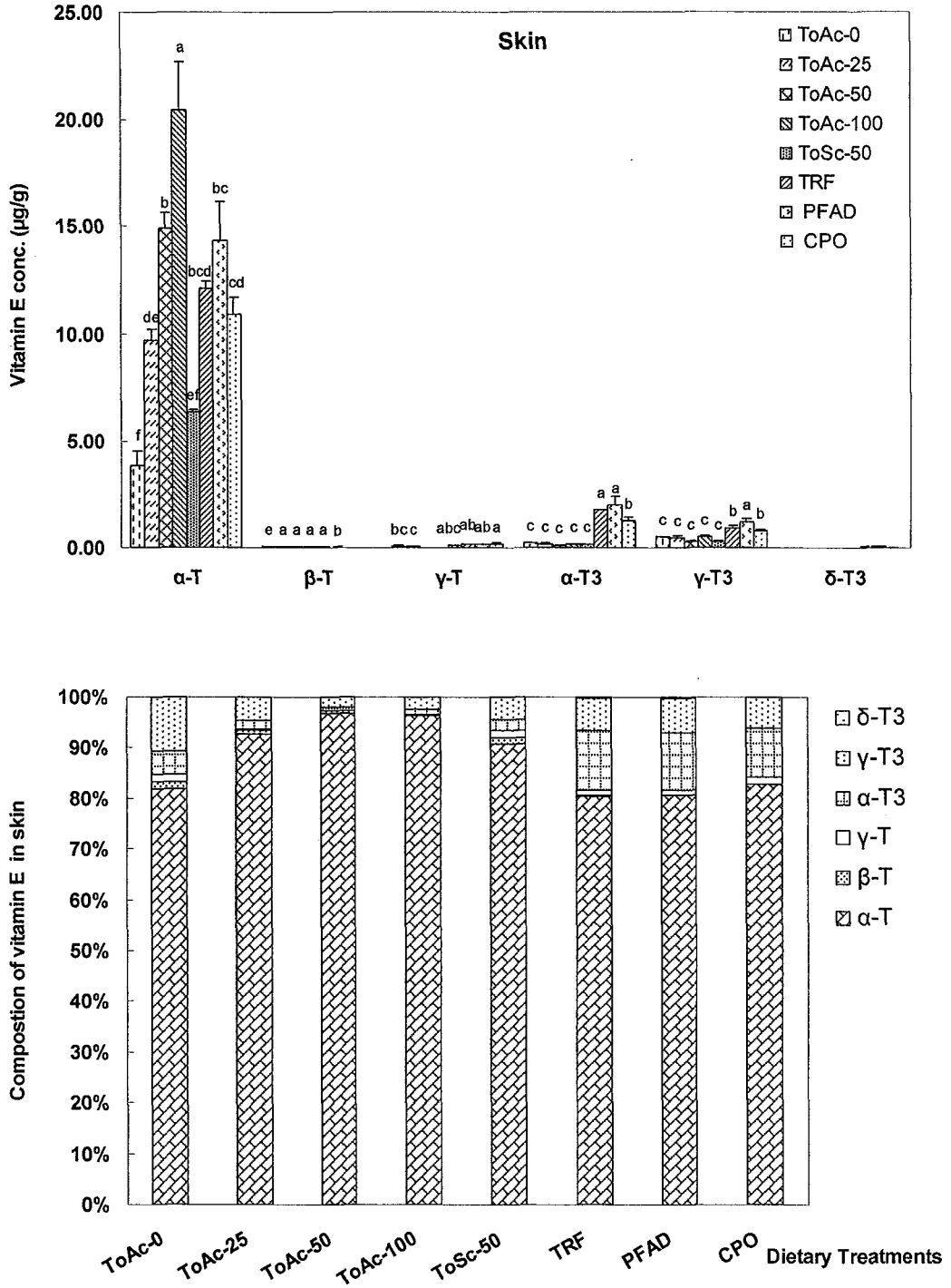


Figure 3.3 (d) Vitamin E concentrations and composition in skin of red hybrid tilapia fed with eight different experimental diets for 8 weeks in Experiment 1. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

with other diets. Fish fed with Diet 1 (without vitamin E supplementation) had the lowest concentration of 3.84 μg/g of α-T detected. Other skin concentrations of α-T ranged from 9.69 to 14.93 μg/g. Traces of β- and γ-T were detected in some of the fish skin samples. Total tocopherols accounted for 93.5 to 97.3% of total vitamin E in the skin of fish given Diets 2 to 5, which were significantly higher ($P<0.05$) than 81.6 to 84.4% found in the skin of fish given Diets 1, 6 to 8.

α- and γ-T3 were also deposited in the skin of experimental tilapia. Their concentrations in fish fed with tocotrienols rich Diets 6 to 8 were 1.28 to 2.02 μg/g and 0.79 to 1.22 μg/g, respectively. Lower levels of 0.12 to 0.21 μg/g of α-T3 and 0.31 to 0.50 μg/g of γ-T3, respectively, were detected in fish fed with synthetic vitamin E supplemented Diets 1 to 5. Only traces of δ-T3 were found in fish fed Diet 6 and Diet 7. Total tocotrienols accounted for 15.6 to 18.4% of total vitamin E in the skin of fish given Diets 1, 6 to 8, which were significantly ($P<0.05$) higher than 2.7 to 6.5% in the skin of fish given Diets 2 to 5.

3.1.4.2 Bioavailability of α-tocopheryl succinate and palm α-tocopherol

Liver α-T concentrations increased linearly ($r^2 = 0.99861$) in fish fed graded levels of supplemented α-ToAc from 0 to 100 mg/kg diet (Diets 1 to 4). The linear regression equation (Figure 3.4) for the standard α-T response curve was $y = 1.37851\chi + 0.29467$, where y = available α-T in diet (mg/kg) and χ = liver α-T (μg/g). Liver α-T levels of fish fed on diets supplemented with test α-ToSc in Diet 5, or α-T in Diets 6 to 8 fell within this linear portion of the response curve. The available α-T in the test diets (Table 3.8) was then calculated from the regression equation for the standard α-T response curve.

The total α-T content was used to calculate the amount of total α-T contributed by the test ingredients in the experimental diets. Synthetic α-ToSc was found to be only 17.31% available to fingerling tilapia, which was significantly lower ($P<0.05$) than the 50.95, 71.72 and 56.30% of biological availability of α-T to tilapia from TRF, PFAD, and CPO, respectively. Figure 3.4 also showed estimated available α-T in diets

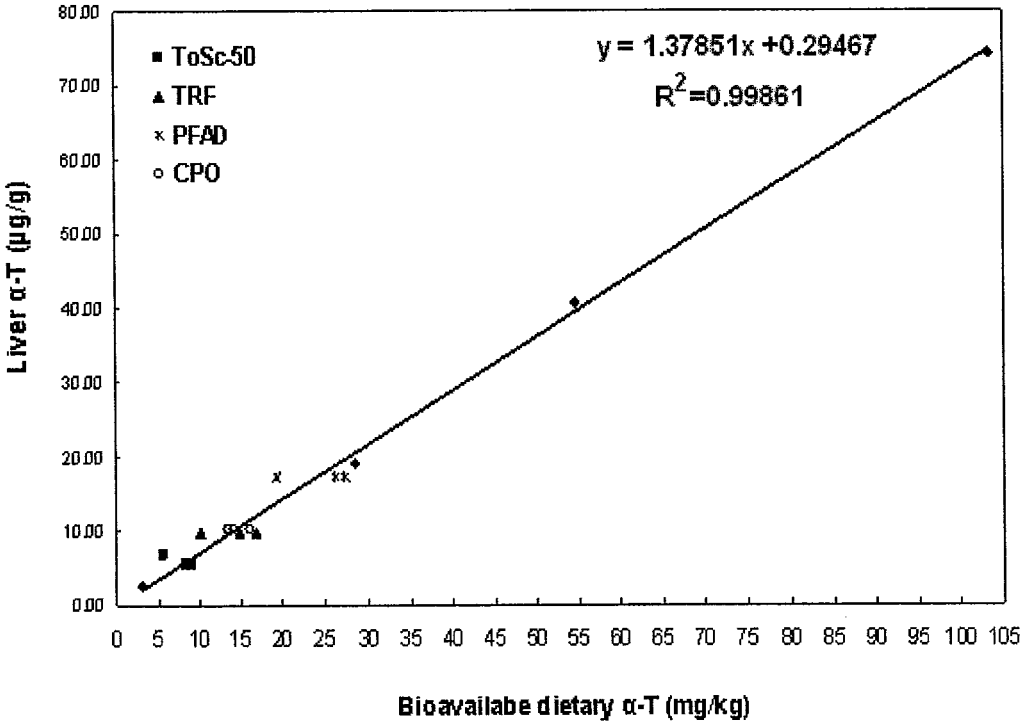


Figure 3.4 Linear regression equation for the standard α-T response curve y = 1.37851χ +0.29467 (R^2 = 0.99861), where y = available α-T in diet (mg/kg) and χ = liver α-T (µg/g) was derived from the fish fed the first four diets in Experiment 1. Estimated bioavailability of α-T originating from α-ToSc, TRF, PFAD or CPO was also plotted.

Table 3.8 Bioassay of available α-tocopherol in the test diet using liver α-tocopherol concentrations of red hybrid tilapia fingerlings as the response measure in Experiment 1[1].

α-T Source[2]	Total α-T in diet (mg/kg diet)[3]	Liver α-T (μg/g)	Available α-T in diet (mg/kg diet)[4]	Available α-T : Total α-T (%)
ToAc-0	2.98	2.39^{e}	3.59^{e}	120.63^{a}
ToAc-25	28.41	19.01^{c}	26.50^{c}	93.27^{b}
ToAc-50	54.59	40.72^{b}	56.42^{b}	103.36^{ab}
ToAc-100	103.39	74.40^{a}	102.86^{a}	99.48^{b}
ToSc-50	46.02	5.56^{de}	7.97^{de}	17.31^{d}
TRF	27.15	9.82^{d}	13.83^{d}	50.95^{c}
PFAD	34.20	17.33^{c}	24.18^{c}	70.72^{c}
CPO	25.65	10.26^{d}	14.44^{d}	56.30^{c}

[1]Values were expressed as the mean of three replicate groups of pooled livers from live fish, results in columns bearing different superscripts were significantly different ($P<0.05$).
[2]α-tocopherol sources came from α-tocopheryl acetate at 0, 25, 50, and 100 mg/kg diet (ToAc-0, ToAc-25, ToAc-50, and ToAc-100, respectively), α-tocopheryl succinate at 50 mg/kg (ToSc-50), tocotrienol-rich fraction (TRF), palm fatty acid distillate (PFAD) and crude palm oil (CPO).
[3]Based on the amount of α-tocopherol determined in the test diets as shown in Table 3.3.
[4]Based on the equation $y = 1.37851\chi + 0.29467$, where y = available α-T in diet (mg/kg) and χ = liver α-T (μg/g).

3.1.4.3 Fatty acid profile of fish muscle

Table 3.9 summarized the fatty acid profiles in the muscle lipids of red hybrid tilapia obtained from Experiment 1. They were strongly reflected by dietary fatty acid composition. Generally, the fatty acids found in high concentrations in the diets were also the most prevalent in the muscle and the converse was true of the abundant fatty acids. The lowest amounts of 12:0 and 14:0 (0.3% and 1.0%, respectively) were found in the muscle of fish fed Diet 8 using CPO as dietary lipid source. Moderate amounts of 12:0 (5.5-7.4%) were deposited in the muscle of fish fed with CPKO-containing

diets (Diets 1 to 6, 10% CPKO; Diet 7, 7% CPKO + 3% PFAD) with concentrations of 12:0 varying from 31.2% to 48.9%. The concentrations of 14:0 in the muscle of fish fed diets with CPKO (Diets 1 - 6) as the sole lipid source remained constant at a mean of about 11% (Table 3.9), significantly higher ($P<0.05$) than 7.3% of fish fed Diet 7, in which contained a blend of CPKO and PFAD. The palmitic acid (16:0) was generally high in muscle lipid regardless of diets. Palmitic acid in Diets 1 to 6 was maintaining at an equilibrium of 7.7 % - 8.5% and at higher concentrations in Diets 7 and 8 at 19.6% and 43.0% (Table 3.4), respectively. A relatively higher concentration of 16:0 was found in the muscle of fish fed with Diet 8 at 23.0% of total fatty acids.

Oleic acid (18:1n-9) was present at the highest concentrations found in all the muscle samples. Concentrations of oleic acid in muscle of fish (26.2%) fed with Diets 1-6 were significantly lower than those of fish fed with Diets 7 and 8 (29.4% and 32.1%, respectively). Concentrations of linoleic acid (18:2n-6) in muscle of fish had the same pattern with those of oleic acid. Fish fed with the CPO diet contained a significantly higher concentration (7.5%) of linoleic acid compared to concentrations of this fatty acid in muscle of fish fed other diets (Table 3.4). Though fatty acids with longer than 18-carbon chain were not detected in the experimental diets, the muscle of tilapia fed with the CPO diet had the highest concentrations of 20:4n-6 and 22:5n-6, with their value being 2.7% and 4.0%, respectively. There were only traces or non-detectable levels of other dietary long chain fatty acids in tilapia muscle (Table 3.4).

Table 3.9 Fatty acid composition (% of total fatty acids) from muscle of tilapia fed with the eight experimental diets for 8 weeks in Experiment 1[1].

Fatty acid	Initial sample	Diet 1	2	3	4	5	6	7	8
		ToAc-0	**ToAc-25**	**ToAc-50**	**ToAc-100**	**ToSc**	**TRF**	**PFAD**	**CPO**
C12:0	1.9	6.7 ± 1.0^{a}	7.1 ± 0.3^{a}	6.9 ± 0.7^{a}	7.3 ± 0.6^{a}	7.4 ± 0.9^{a}	7.3 ± 0.7^{a}	5.5 ± 0.9^{a}	0.3 ± 0.1^{b}
C14:0	3.3	11.1 ± 1.0^{a}	11.4 ± 0.2^{a}	10.8 ± 0.5^{a}	11.6 ± 0.5^{a}	11.2 ± 0.7^{a}	10.9 ± 1.1^{a}	7.3 ± 0.7^{b}	1.0 ± 0.0^{c}
C16:0	17.5	16.7 ± 1.1^{bc}	16.9 ± 0.5^{bc}	16.4 ± 0.1^{bc}	17.5 ± 0.2^{b}	16.5 ± 0.3^{bc}	15.3 ± 0.9^{c}	18.6 ± 0.8^{b}	23.0 ± 0.7^{a}
C16:1n7	4.8	6.1 ± 0.5^{ab}	6.1 ± 0.2^{ab}	6.6 ± 0.2^{a}	6.5 ± 0.1^{ab}	6.3 ± 0.2^{ab}	5.6 ± 0.1^{bc}	5.5 ± 0.4^{bc}	4.8 ± 0.3^{c}
C18:0	5.7	4.1 ± 0.2^{b}	4.1 ± 0.2^{b}	3.8 ± 0.1^{b}	3.9 ± 0.3^{b}	3.8 ± 0.1^{b}	3.7 ± 0.4^{b}	3.9 ± 0.1^{b}	4.8 ± 0.2^{a}
C18:1n9	28.1	26.8 ± 1.1^{c}	27.2 ± 0.5^{bc}	23.2 ± 0.2^{d}	26.7 ± 0.5^{c}	26.7 ± 0.2^{c}	26.4 ± 0.6^{c}	29.4 ± 1.2^{b}	32.1 ± 1.2^{a}
C18:1n7	ND	ND	ND	ND	ND	ND	ND	ND	ND
C18:2n6	9.7	3.8 ± 0.1^{cd}	4.4 ± 0.2^{cd}	4.7 ± 0.2^{bc}	3.4 ± 0.3^{d}	4.0 ± 0.2^{cd}	4.8 ± 0.6^{bc}	5.8 ± 0.7^{b}	7.5 ± 0.1^{a}
C18:3n3	0.3	ND	0.1 ± 0.1	0.1 ± 0.0	ND	ND	ND	ND	ND
C18:4n3	0.0	ND	ND	ND	ND	ND	ND	ND	TR[1]
C20:1n9	1.9	1.1 ± 0.1	0.8 ± 0.4	0.7 ± 0.4	0.9 ± 0.4	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.4	1.4 ± 0.1
C20:3n6	0.9	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.9 ± 0.1
C20:4n6	2.3	2.0 ± 0.1^{abc}	2.0 ± 0.1^{abc}	2.3 ± 0.3^{ab}	1.9 ± 0.3^{bc}	1.7 ± 0.1^{bc}	1.5 ± 0.4^{c}	2.2 ± 0.1^{abc}	2.7 ± 0.2^{a}
C20:3n3	0.0	ND	ND	ND	ND	ND	ND	ND	ND
C20:4n3	0.3	ND	ND	ND	ND	ND	ND	ND	ND
C20:5n3	0.3	ND	ND	ND	ND	ND	ND	ND	ND
C22:1n11	0.0	ND	ND	ND	ND	ND	ND	ND	ND
C22:5n6	2.2	2.4 ± 0.3^{b}	2.3 ± 0.2^{b}	2.3 ± 0.4^{b}	1.8 ± 0.3^{b}	2.5 ± 0.8^{b}	1.8 ± 0.3^{b}	2.7 ± 0.2^{b}	4.0 ± 0.6^{a}
C22:5n3	1.0	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	ND	0.5 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.0
C22:6n3	5.3	1.5 ± 0.3	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.0	1.6 ± 0.4	1.1 ± 0.3	1.2 ± 0.2	1.6 ± 0.2
Total saturates	28.4	38.6 ± 2.9^{a}	39.4 ± 0.9^{a}	38.0 ± 1.0^{a}	40.3 ± 1.0^{a}	38.9 ± 1.3^{a}	37.1 ± 2.1^{a}	35.2 ± 2.3^{a}	29.1 ± 0.8^{b}
Total monoenes	34.8	34.0 ± 1.7^{bc}	34.1 ± 0.8^{bc}	30.6 ± 0.5^{c}	34.0 ± 1.0^{bc}	34.3 ± 0.4^{bc}	33.2 ± 0.6^{bc}	35.8 ± 1.5^{b}	38.3 ± 1.5^{a}
Total PUFA	22.4	10.6 ± 0.6^{bc}	10.9 ± 0.7^{bc}	11.3 ± 0.8^{bc}	9.1 ± 0.9^{c}	10.8 ± 1.1^{bc}	9.8 ± 0.9^{bc}	12.6 ± 0.9^{b}	17.1 ± 1.0^{a}
Total n-3	7.3	1.8 ± 0.3	1.5 ± 0.2	1.3 ± 0.2	1.2 ± 0.0	2.1 ± 0.6	1.1 ± 0.3	1.4 ± 0.3	1.9 ± 0.2
Total n-6	15.2	8.9 ± 0.4^{c}	9.4 ± 0.5^{bc}	9.9 ± 0.6^{bc}	7.9 ± 0.9^{c}	8.7 ± 0.6^{c}	8.7 ± 0.8^{c}	11.2 ± 0.7^{b}	15.2 ± 0.8^{a}
n-3 / n-6	0.5	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

[1]Values were expressed as mean ± s.e. of three replicate groups of pooled muscle from live fish, results in rows bearing different superscripts were significantly different ($P<0.05$). ND=not detected; TR=trace detection <0.05%.

In spite of a wide range of values found in the various diets (67.6 - 80.4%), total saturated fatty acids in the muscle of tilapia were somewhat constant at 35.2 - 40.9%, with the exception of fish fed with the CPO diet. CPO-sourced Diet 8, which had the lowest dietary saturated fatty acids (49.0%), attained a total saturate concentration of only 29.1% in the muscle. The highest composition of total monounsaturated fatty acids (38.3%) and PUFA (17.1%) were found in the muscle of fish fed with Diet 8, reflecting the highest dietary amounts of these fatty acids found in this diet. Fish fed with PFAD diet had the second highest relative concentrations of total monoenes (35.8%) and PUFA (12.6%). The ratios of n-3 to n-6, however, were not significantly different from each other, ranging from 0.1 to 0.2%.

3.1.4.4 Oxidative stability of tilapia muscle and liver

The TBARS concentrations (expressed as nmol MDA/g tissue) in the muscle and liver of tilapia fed with different dietary vitamin E regimes were illustrated in Figure 3.5 and the data given in Appendix 4. As shown, both iron-vitamin C induced TBARS concentrations of muscle and liver of tilapia fed with Diet 1 (without added vitamin E) were significantly higher ($P<0.05$) than those of fish fed with all other vitamin E added diets. TBARS concentrations in the muscle and liver of fish fed with Diets 1 to 4, decreased concomitant with increasing α-ToAc level up to 50mg/kg. Further increase in dietary α-ToAc did not see apparent TBARS dropping. In the muscle of fish fed with Diet 5 (ToSc-50) and Diet 8 (CPO), TBARS levels were significantly higher ($P<0.05$) than those of fish fed with either ToAc or TRF or PFAD-based diets. The lowest TBARS found in the muscle of tilapia fed with Diet 6 (TRF). In the liver,

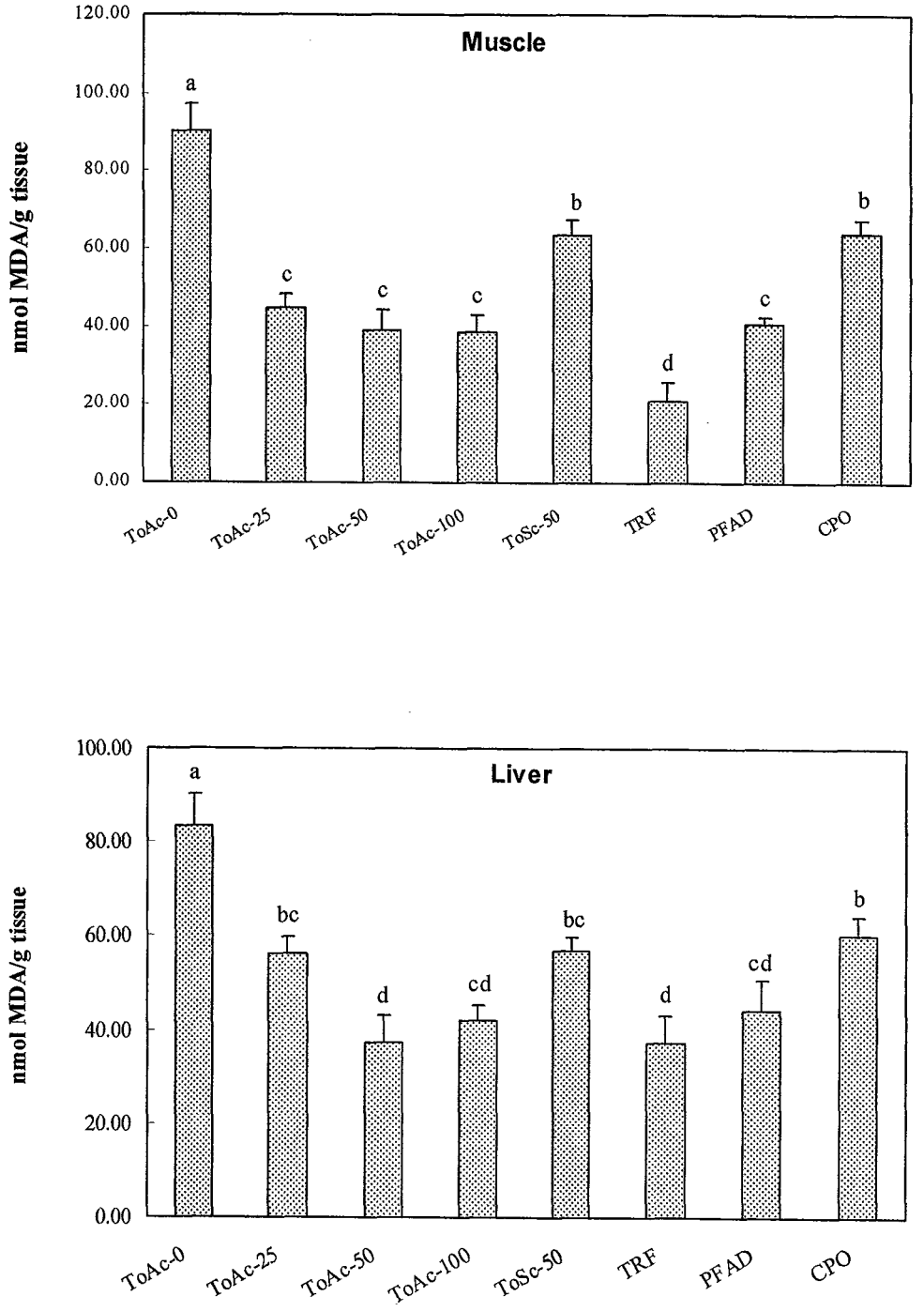


Figure 3.5 Effects of different vitamin E sources on thiobarbituric acid-reactive substances (TBARS) from iron-vitamin C induced lipid peroxidation in the muscle and livers of tilapia fed with eight different experimental diets for 8 weeks in Experiment 1. Values were mean + s.e of three replicates of one dietary treatment. For each tissue, values not sharing a letter were significantly different, $P<0.05$. MDA, malondialdehyde.

almost the same trend was observed except that TBARS in fish fed with ToAc-25 had a similar level with fish fed with ToSc and CPO diets. The muscle and liver from fish fed with TRF diet had significantly enhanced oxidant stabilities (Figure 3.5).

3.1.4.4.1 Relative potency of total vitamin E in experimental diets

Induced TBARS concentrations of livers (Appendix 4) linearly (r = -0.9948) decreased from 83.17 to 37.28 nmol MDA/g within tilapia fed with the standard Diets 1 to 3, which were supplemented with α-ToAc from 0 to 50 mg/kg (Table 3.3). 42.07 nmol MDA/g of induced TBARS was not seen significantly dropping in fish fed with Diet 4, compared to 37.28 nmol MDA/g of fish fed with Diet 3. Thus, a linear regression equation for the standard TBARS response curve was constructed using dietary α-T concentrations of Diet 1 to Diet 3 and induced TBARS levels of liver in fish fed these three diets. The equation was $y = -0.08859\chi + 85.57184$, where y = theoretical amounts of α-ToAc equivalent in diet (mg/kg) and χ = TBARS liver concentration (nmol MDA/g). All induced-TBARS levels of livers from fish fed α-ToSc or other palm-based vitamin E fell within this linear portion of the response curve. The theoretical amounts of α-ToAc equivalent in diets (Table 3.10) were then calculated from the regression equation for the standard TBARS curve.

In the study, potency was defined as the potential of a certain amount of α-ToSc (in Diet 5) or total vitamin E in TRF, PFAD or CPO (in Diets 6 to 8, respectively) needed to inhibit TBARS occurrence equaled to that of 1 mg α-ToAc. The potency of 1 mg α-

ToAc relative to α-ToSc, total vitamin E in TRF, PFAD and CPO was found at 1.56, 2.47, 3.54, and 4.38 mg (Table 3.10), respectively.

It was observed that concentrations of induced TBARS in the muscle of fish fed with Diet 2 (ToAc-25) were significantly ($P<0.05$) higher than that of fish fed with Diet 6 (TRF) (Figure 3.5 and Appendix 4). Similar levels of α-T were deposited in the muscle of fish fed with Diet 2 and Diet 6, 4.18 and 4.77 μg/g, respectively. But significantly lower tocotrienols level of 0.08 μg/g were found in the muscle of fish fed with Diet 2, compared to 1.03 μg/g of fish fed with Diet 6. Therefore, the significant reduction of induced TBARS in the muscle of fish fed with Diet 6 was contributed by the deposition of tocotrienols to this tissue. The same findings were seen in the livers of tilapia fed with Diet 2 and Diet 6. Significantly ($P<0.05$) higher α-T level of 19.01 μg/g were observed in the liver of fish fed with Diet 2 compared to 9.82 μg/g of fish fed with Diet 6. Inversely, significantly ($P<0.05$) lower tocotrienols level of 0.86 μg/g was found in the liver of fish fed with Diet 2 compared to 7.34 μg/g of fish fed with Diet 6. However, similar levels of induced TBARS were found in the livers of fish fed with the two diets (Figure 3.5 and Appendix 4). Hence, the conclusion that palm based-tocotrienols are actually more potent antioxidants than α-T can be drawn.

Table 3.10 Relative potency of α-ToAc to α-ToSc or total vitamin E derived from TRF, PFAD or CPO[1].

Diet code	Total Vitamin E in diet (mg/kg)[2]	Liver TBARS (nmol/g)	Theoretical ToAc equivalent diet (mg/kg)[3]	Potency[4]
ToAc-0	4.24	83.17^{a}	6.31^{d}	0.94^{d}
ToAc-25	30.10	56.20^{bc}	33.12^{bc}	0.96^{d}
ToAc-50	56.04	37.28^{d}	54.27^{a}	1.17^{d}
ToAc-100	104.82	42.07^{cd}	-	-
ToSc-50	47.68	56.87^{bc}	32.38^{bc}	1.56^{cd}
TRF	122.96	37.38^{d}	54.15^{a}	2.47^{bc}
PFAD	143.77	44.32^{cd}	46.40^{ab}	3.54^{ab}
CPO	111.71	60.21^{b}	28.64^{c}	4.38^{a}

[1]Values were expressed as the mean of three replicate groups of pooled livers from live fish, results in columns bearing different superscripts were significantly different ($P<0.05$).
[2]Based on the amount of total vitamin E determined in the test diet as shown in Table 3.3.
[3]Based on the equation $y = -0.08859\chi + 85.57184$, where y = theoretical amounts of ToAc in diet (mg/kg) and χ = TBARS liver concentration (nmol/g).
[4]Potency = Total vitamin E in diet (mg/kg)/ Theoretical α-ToAc in diet (mg/kg).

3.2 Experiment 2

3.2.1 Experimental diets

3.2.1.1 Dietary vitamin E composition

Table 3.11 showed the vitamin E composition of the five experimental diets analysed after being pelleted. Concentration of individual vitamin E component of each diet increased concomitant with graded tocotrienol-rich fraction (TRF) added. Diet 1 contained total vitamin E concentration of 5.20 mg/kg originating from CPKO's limited endogenous vitamin E, with tocopherols accounting for 22.9% and tocotrienols 77.1%. From Diets 2 to 5, dietary concentrations of various vitamin E components substantially increased in a graded manner, whilst their respective vitamin E composition remained almost constant with α-T from 17.0-18.9%, α-T3 22.7-24.2%, γ-T3 44.7-45.0%, and δ-T3 18.6-21.0%. Diet 2, 3, 4 and 5 contained 37.70, 66.38, 127.82 and 247.60 mg total vitamin E/kg diet, respectively. ToVe-0, ToVe-30, ToVe-60, ToVe-120 and ToVe-240 were the assigned codes to Diets 1 to 5 , respectively, according to the amounts of total vitamin E supplemented to them.

3.2.1.2 Proximate composition

Table 3.12 showed the analyzed results of proximate composition of the five experimental diets used in Experiment 2. Moisture content of the five pelleted diets were constant from 15.52 - 16.26%. Diets were inertially isonitrogeous with protein

content ranging from 36.45 - 37.14. Dietary lipid levels remained at 9.71 - 9.97%. Ash and fiber content were constant at 3.94 - 3.95% and 5.55 -5.78%, respectively. Calculated metabolizable energy (kJ/g) and protein energy (kJ/g) of these five experimental diets were between 17.15 - 17.23 kJ/g and 35.56 - 36.16 kJ/g, respectively. Ratios of protein to energy ranged from 21.25 - 21.61 mg protein per kJ metaboliszable energy.

Table 3.11 Vitamin E concentrations and composition of the five semi-purified experimental diets used in Experiment 2[1].

Vitamin E	Diets				
	1	2	3	4	5
	ToVe-0	ToVe-30	ToVe-60	ToVe-120	ToVe-240
Concentration (mg/kg diet)					
α-T	1.20	6.42	12.28	24.10	46.93
γ-T	ND	0.59	1.19	2.49	5.40
α-T3	1.70	9.14	16.03	29.74	56.23
γ-T3	2.30	16.92	29.85	57.17	111.14
δ-T3	ND	4.63	6.94	14.32	27.89
Total T + T3	5.20	37.70	66.38	127.82	247.60
Compositions (%)					
α-T	22.9	17.0	18.5	18.9	18.9
γ-T	-	1.6	1.8	2.0	2.2
α-T3	32.7	24.2	24.2	23.3	22.7
γ-T3	44.4	44.9	45.0	44.7	44.9
δ-T3	-	12.3	10.5	11.2	11.3
Total T	22.9	18.6	20.3	20.9	21.0
Total T3	77.1	81.4	79.7	79.1	79.0

[1]Values were expressed as means of three replicate analyses. ND = Not detected

Table 3.12 Proximate composition (g/100 g dry diet) and dietary energy contents of the five semi-purified experimental diets used in Experiment 2[1].

	Diets				
	1	2	3	4	5
	ToVe-0	ToVe-30	ToVe-60	ToVe-120	ToVe-240
Moisture	15.57 ± 0.17	15.68 ± 0.23	15.52 ± 0.32	16.26 ± 0.40	15.83 ± 0.12
Protein	37.00 ± 0.17	36.71 ± 0.32	36.78 ± 0.13	37.14 ± 0.10	36.45 ± 0.34
Lipid	9.73 ± 0.05	9.97 ± 0.10	9.94 ± 0.09	9.91 ± 0.09	9.77 ± 0.07
Ash	3.94 ± 0.01	3.95 ± 0.01	3.94 ± 0.01	3.94 ± 0.02	3.94 ± 0.02
Fiber	5.61 ± 0.79	5.55 ± 0.83	5.61 ± 0.92	5.73 ± 0.34	5.78 ± 0.56
NFE[2]	43.72 ± 0.13	43.82 ± 0.23	43.73 ± 0.09	43.28 ± 0.13	44.06 ± 0.40
Metaboliszable energy (kJ/g)	17.17 ± 0.01	17.23 ± 0.02	17.22 ± 0.02	17.19 ± 0.02	17.15 ± 0.01
Protein energy (%)	136.06 ± 0.18	35.65 ± 0.35	35.75 ± 0.15	36.16 ± 0.12	35.56 ± 0.32
Protein : Energy ratio	21.54 ± 0.11	21.30 ± 0.21	21.36 ± 0.09	21.61 ± 0.07	21.25 ± 0.19

[1]Values are means ± s.e. of three analysis, except fibre with only two replicates
[2]NFE: Nitrogen Free Extract.

3.2.2 Tilapia

3.2.2.1 Growth performance and feed efficiency

Figure 3.6 (the detailed data given in Appendix 5) shows the weekly accumulated weight percent gain of experimental tilapias, while Table 3.13 illustrates parameters of fish growth performance and feed efficiency. Mean initial weights of experimental tilapias were not significantly different. It was observed that growth deviation appeared after feeding on diets from the fifth week till the termination of the experiment. Final fish weight, weight percent gain, specific growth rate (SGR) and daily weight gain seemed to decline in line with total vitamin E inclusion level, with values of 273.33-313.15%, 2.16-2.33%/day, and 0.27-0.37 g/day, respectively, but no significant differences ($P>0.05$) were detected. As to feed utilization, measured parameters such as feed efficiency ratio (FER) and protein efficiency ratio (PER) also were no significance detected ($P>0.05$). Both of these two parameters possessed by fish given Diets 2 (ToVe-30) and 3 (ToVe-60) were slightly higher than those of fish fed Diets 1 (ToVe-0), 4 (ToVe-120) and 5 (ToVe-240).

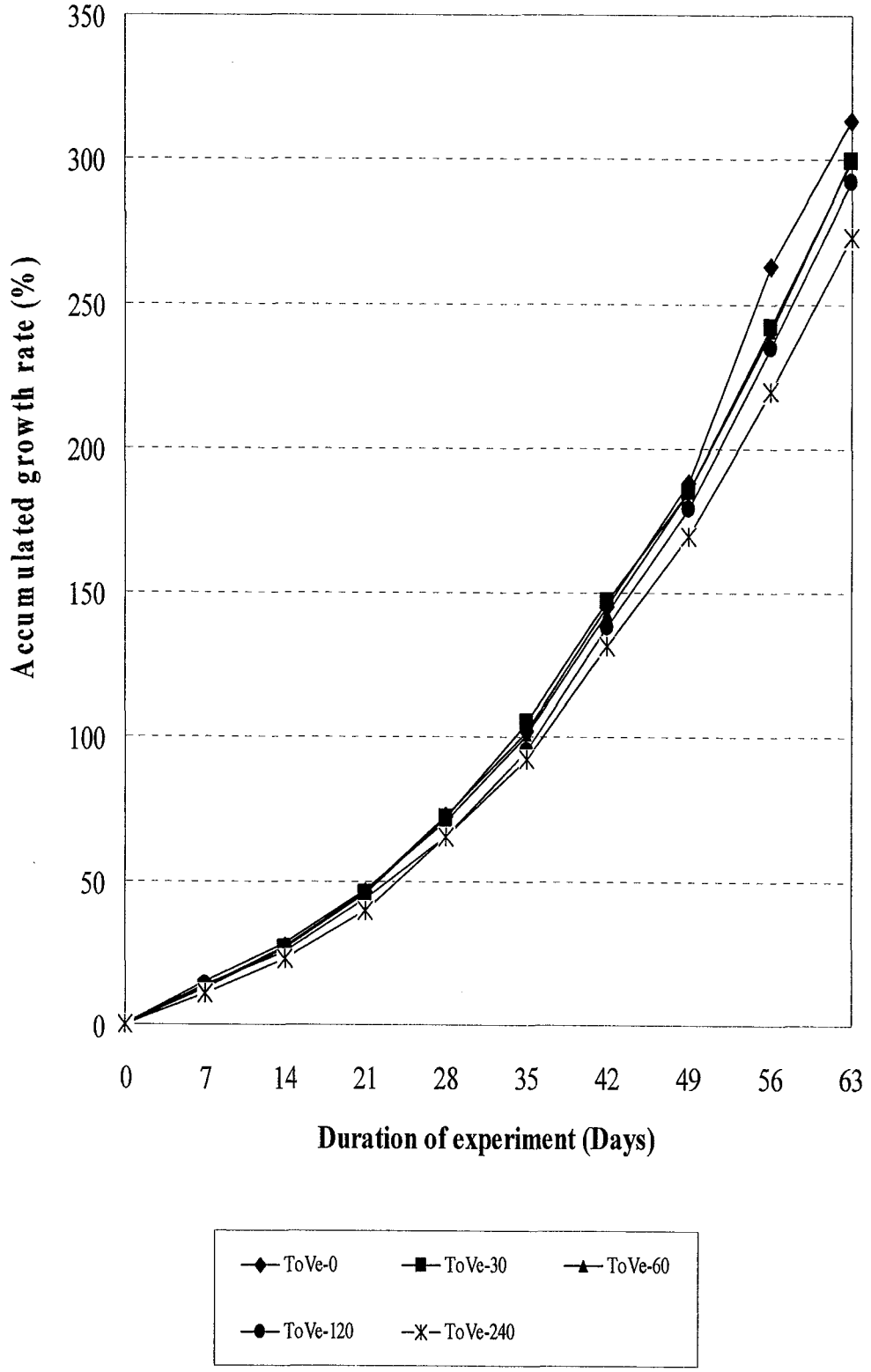


Figure 3.6 Weekly percent weight gain of red hybrid tilapia fed the five semi-purified experimental diets for 9 weeks in Experiment 2.

Table 3.13 Growth performance and feed utilization efficiency of red hybrid tilapia given the five semi-purified experimental diets for 9 weeks in Experiment 2[1].

	Diets				
	ToVe-0	ToVe-30	ToVe-60	ToVe-120	ToVe-240
Mean initial fish weight (g)	6.00 ± 0.05	5.99 ± 0.03	6.03 ± 0.04	6.01 ± 0.06	5.98 ± 0.04
Mean final fish weight (g)	24.77 ± 0.44	23.94 ±1.15	24.17 ± 0.54	23.60 ± 1.66	22.31 ± 0.61
Weight gain (%)	313.15 ± 5.71	299.77 ± 19.58	300.41 ± 6.10	292.57 ± 25.49	273.33 ± 9.53
Specific growth rate (SGR) (%/day)	2.33 ± 0.02	2.27 ± 0.08	2.27 ± 0.03	2.23 ± 0.11	2.16 ± 0.04
Daily weight gain (g/day)	0.31 ± 0.01	0.29 ± 0.02	0.30 ± 0.01	0.29 ± 0.03	0.27 ± 0.01
Feed efficiency ratio (FER)	0.82 ± 0.05	0.85 ± 0.04	0.86 ± 0.02	0.82 ± 0.04	0.81 ± 0.02
Protein efficiency rate (PER)	2.25 ± 0.11	2.31 ± 0.10	2.31 ± 0.05	2.20 ± 0.10	2.20 ± 0.05

[1]Values were expressed as means ± s.e.. All parameters in the same row were not significant different (P>0.05).

3.2.2.2 Fish body proximate composition

Table 3.14 summarizes the whole body proximate composition before and after the 9-week feeding trial in Experiment 2. Moisture, protein and lipid content in fish body apparently changed, moisture from 78.49% in the initial fish decreased to 73.6-74.1% when fish were given Diets 1- 5, while protein from 14.01% and lipid from 2.87% increased to 14.4-15.5% and 6.1-7.0%, respectively. In the mean time, ash content in fish before and after the experiment were almost unchanged maintaining at 4.1 - 4.4% in final fish, compared with 4.24% in the initial fish. As in Table 3.14, results of body

proximate composition, except moisture, of fish given Diets 1 to 5, they all had statistical significances detected by Duncan multiple range test (1959), but did not differ too much.

Table 3.14 Whole body composition (% wet weight) of red hybrid tilapia given the five semi-purified experimental diets for 9 weeks in Experiment 2[1]

Diets		Moisture	Ash	Protein	Lipid
	Initial Fish	78.49 ± 0.38	4.24 ± 0.04	14.01 ± 0.07	2.87 ± 0.03
1	ToVe-0	73.55 ± 0.70	4.23 ± 0.07^{ab}	15.5 3± 0.38^{a}	6.72 ± 0.32^{ab}
2	ToVe-30	73.84 ± 0.23	4.37 ± 0.02^{a}	15.28 ± 0.13^{a}	6.24 ± 0.23^{ab}
3	ToVe-60	73.87 ± 1.01	4.17 ± 0.03^{b}	15.05 ± 0.29^{a}	6.73± 0.19^{ab}
4	ToVe-120	73.57 ± 1.19	4.13 ± 0.05^{b}	14.36 ± 0.11^{b}	7.02 ± 0.12^{a}
5	ToVe-240	74.09 ± 0.81	4.21 ± 0.03^{b}	15.17 ± 0.20^{ab}	6.13 ± 0.25^{b}

[1]Values were expressed as means ± s.e. of three replicates of four fish per group. Mean values in columns with different superscripts were significantly different ($P < 0.05$).

3.2.2.3 Somatic indices

Hepatosomatic and intraperitoneal fat indices (HSI and IPI, respectively) of tilapia are summarized in Table 3.15. There were no statistical significances detected ($P>0.05$) in HSI and IPI, with values ranging from 1.36 - 1.63 and 0.78-1.08,

respectively. However,d it was observed there was a trend that HSI slightly increased concomitant with the dietary inclusion levels of total vitamin E.

Table 3.15 Hepatosomatic Index (HSI) and Intraperitoneal fat Index (IPI) of red hybrid tilapia given the five semi-purified experimental diets for 9 weeks in Experiment 2[1].

	Diets	Hepatosomatic Index (HSI)	Intraperitoneal fat Index (IPI)
1	ToVe-0	1.36 ± 0.07	0.85 ± 0.06
2	ToVe-30	1.38 ± 0.09	1.08 ± 0.20
3	ToVe-60	1.63 ± 0.11	0.78 ± 0.08
4	ToVe-120	1.47 ± 0.20	0.88 ± 0.10
5	ToVe-240	1.52 ± 0.14	0.85 ± 0.08

[1]Values are expressed as means ± s.e. from three replicates of total sampled fish per group, results in columns were not significantly different (P<0.05).

3.2.2.4 Hematological results

Table 3.16 shows the results for hematocrits and blood hemolysis analysis of tilapia. Hematocrit of fish (38.46%) given Diet 5 (ToVe-240) was significantly lower (P<0.05) than those (40.08 - 42.83%) of fish given Diets 2 (ToVe-30), 3 (ToVe-60) and 4 (ToVe-120). Hematocrit (39.11%) of fish given no TRF added Diet 1 (ToVe-0) was not significantly different (P>0.05) when compared to fish given TRF supplemented

diets (ToVe-30 to ToVe-240). With respect to blood hemolysis, it was observed that it correlated well with dietary TRF inclusions, where the values decreased with increasing level of dietary vitamin E supplement, however, no significances were detected ($P>0.05$) among treatment. Hemolysis of fish given Diet 1 no vitamin E added was 50.35% which was apparently higher than those of fish fed the other four vitamin E administered diets (values ranging from 36.72 to 48.02%).

Table 3.16 Hematocrits and 15-hr incubated blood hemolysis under 24°C of red hybrid tilapia given the five semi-purified experimental diets for 9 weeks in Experiment 2[1].

	Diets	Hematocrits (%)	15-hr Blood Hemolysis (%)
1	ToVe-0	39.11 ± 0.90^{ab}	50.35 ± 6.87
2	ToVe-30	42.43 ± 1.14^{a}	48.02 ± 11.33
3	ToVe-60	40.08 ± 0.70^{a}	47.28 ± 4.85
4	ToVe-120	42.83 ± 1.94^{a}	36.72 ± 7.2
5	ToVe-240	38.46 ± 1.19^{b}	38.46 ± 6.57

[1]Values were expressed as means ± s.e. of three replicates of four fish per group. Mean values in columns with different superscripts were significantly different ($P < 0.05$).

3.2.3 Fish muscle, liver, skin, adipose tissue and plasma

3.2.3.1 Vitamin E distribution in various tilapia tissues and plasma

After the 9-week feeding trial, tilapia were sacrificed and sampled for vitamin E analysis. Figure 3.7 (a), (b), (c), (d) and (e) shows the distribution of various vitamin E isoforms in the fish muscle, liver, adipose tissue, skin, and plasma, respectively, and the corresponding detailed data are presented in Appendix 6A, B, C, D and E, respectively. All of the vitamin E isoforms, irrespective of minor or major components, detected in experimental diets were deposited into the tissues at varying amounts.

3.2.3.1.1 Vitamin E concentration and composition in fish muscle

α-T was exclusively detected in the muscle of initial tilapia, with a concentration of 6.79 μg/g (Appendix 6A). After tilapia were given the five experimental diets, muscle vitamin E profile significantly changed (Figure 3.7 (a)). The major vitamin E isoform deposited was α-T. Its concentrations were significantly ($P<0.05$) different among the muscle of tilapia fed the various diets, with the lowest (1.42 μg/g) and the highest (13.66 μg/g) concentrations found in fish fed with Diet 1 (without added vitamin E) and Diet 5 (240 mg/g total vitamin E), respectively. It was observed that α-T concentrations (2.55 and 6.09 μg/g, respectively) of the muscle in fish fed with Diets 2 to 3 were lower than that of initial fish. There was a good correlation between muscle α-T and dietary α-T concentrations. The former linearly increased concomitant with the increasing level of the latter ($y = 0.27\chi + 1.61$, $r = 0.98$). Low levels of γ-T (0 to

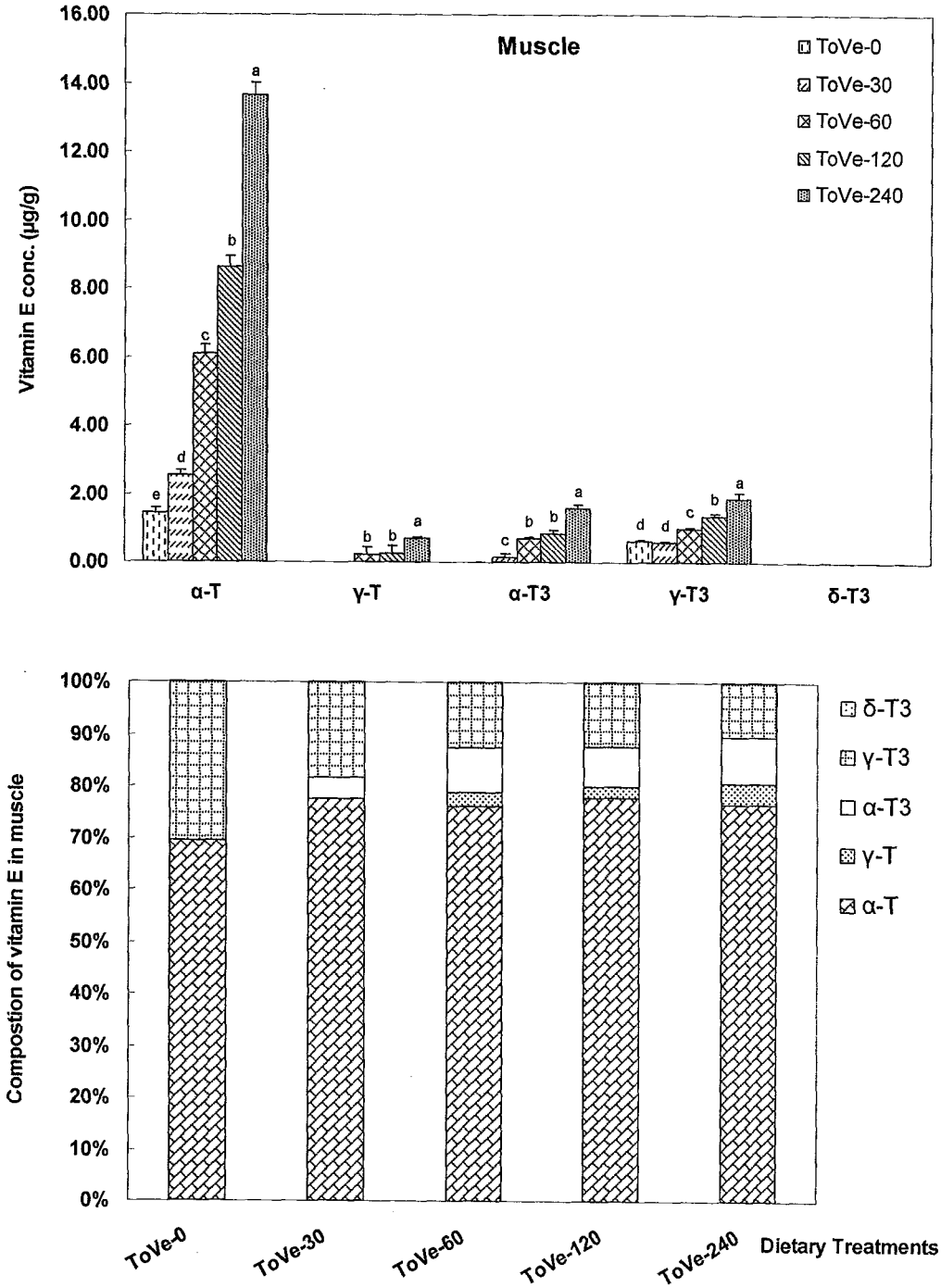


Figure 3.7 (a) Vitamin E concentrations and composition in muscle of red hybrid tilapia fed with five different experimental diets for 9 weeks in Experiment 2. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

0.71 μg/g) were deposited to the muscle of fish, which were reflective of γ-T concentrations in the experimental diets. Total tocopherols remained constant at 77.9 to 80.8% of total vitamin E in the muscle of fish fed with TRF supplemented diets (Diets 2 to 5), which were significantly ($P<0.05$) higher than 69.2% of total vitamin E in the muscle of fish given Diet 1.

The α- and γ-T3 concentrations in tilapia muscle had a direct and linear relationship with their respective dietary concentration ($y = 0.03\chi - 0.02$, $r = 0.92$; and $y = 0.12\chi + 0.56$, $r = 0.94$, respectively) (Appendix 6A). Muscle concentrations (0.62 to 1.86 μg/g) of γ-T3 were higher than those (0 to 1.58 μg/g) of α-T3 in the fish fed within the same dietary treatment. All muscle had no δ-T3 detected, irrespective of its dietary concentrations. Total tocotrienols increased linearly from 0.62 to 3.44 μg/g in the fish when fed with the experimental diets supplemented with 0 to 1111.11 mg/kg of TRF. Tocotrienols composition accounted for 19.2 to 22.1% of total vitamin E in the muscle of fish fed with TRF supplemented diets (Diets 2 to 5), which was significantly lower ($P<0.05$) than the level of 30.8% in the muscle of fish given no vitamin E added Diet 1.

3.2.3.1.2 Vitamin E concentration and composition in fish liver

Only α-T was detected in the liver of initial tilapia, with a concentration of 17.64 μg/g (Appendix 6B). After tilapia were given the five experimental diets, liver vitamin E profile significantly changed (Figure 3.7 (b)). The major vitamin E isoform deposited was α-T. Liver α-T concentration linearly increased concomitant with dietary α-T concentrations ($y = 0.60\chi - 0.46$, $r = 0.94$). The lowest (2.06 μg/g) and the

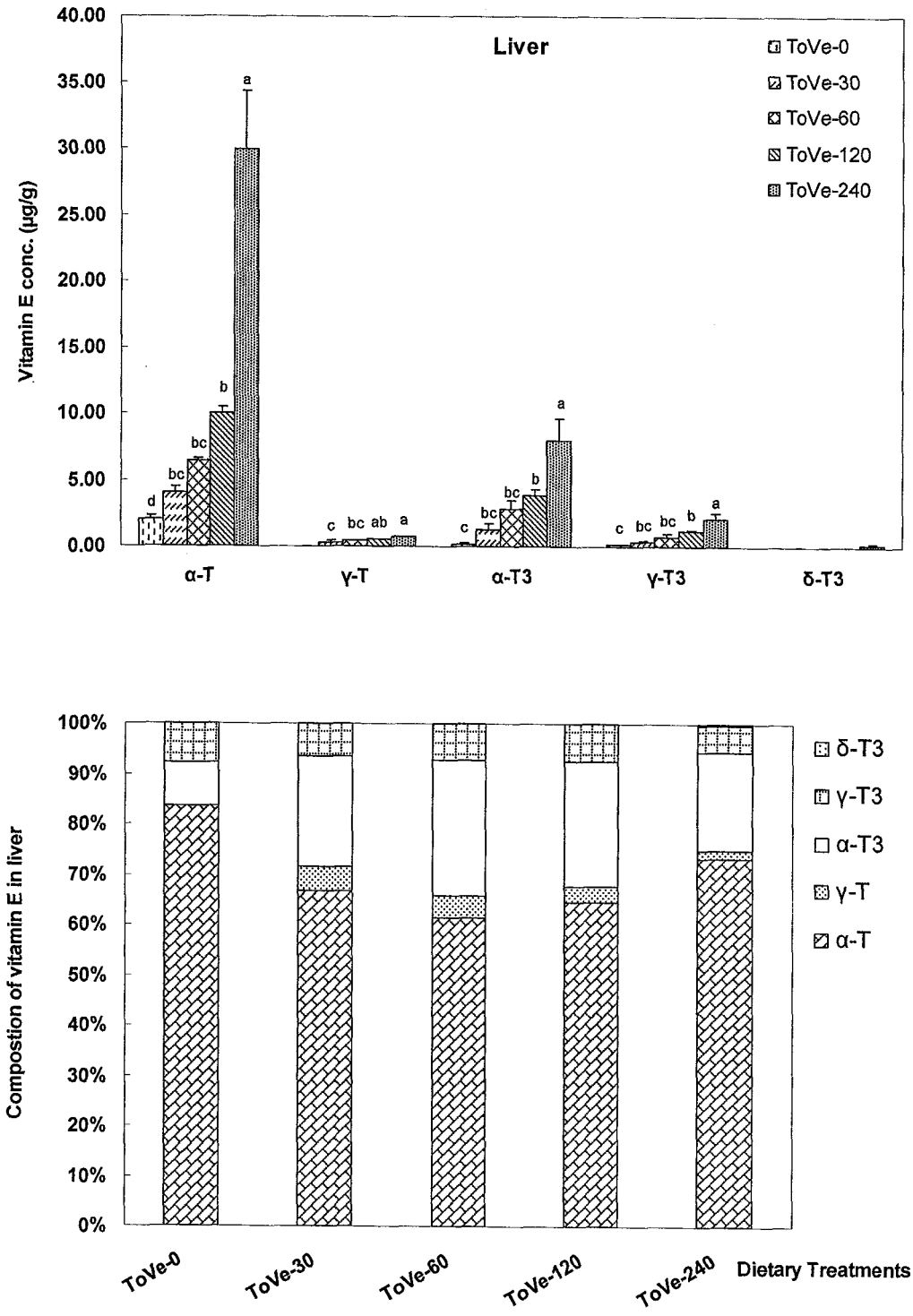


Figure 3.7 (b) Vitamin E concentrations and composition in livers of red hybrid tilapia fed with five different experimental diets for 9 weeks in Experiment 2. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

highest (29.92 μg/g) concentrations were found in the liver of fish of fed Diet 1 (without added vitamin E) and Diet 5 (240 mg/kg total vitamin E), respectively. Low levels of γ-T (0 to 0.69 μg/g) were deposited to the liver of fish fed with the experimental diets, reflective of γ-T concentrations in these diets. Total tocopherols remained somewhat constant at 67.9 to 75.3% of total vitamin E in the liver of fish fed with TRF supplemented diets (Diets 2 to 5), which were significantly lower than the level of 83.4% in the liver of fish given Diet 1.

The α- and γ-T3 concentrations in tilapia liver had a direct and linear relationship with their respective dietary concentrations ($y = 0.04\chi - 0.14$, $r = 0.91$; and $y = 0.02\chi + 0.14$, $r = 0.91$, respectively)(Appendix 6B). The significant increase in liver concentration of α-T3 occurred in fish fed with Diet 2 (30 mg/kg of total vitamin E) compared to fish fed Diet 1 (without vitamin E added). Liver concentrations of γ-T3 (0.19 to 2.11 μg/g) were generally lower than those (0.21 to 7.58 μg/g) of α-T3 in the fish fed with the same diet. Only liver of fish fed with Diet 5 (240 mg/kg of total vitamin E) had a very low level (0.14 μg/g) of δ-T3 detected. Total liver tocotrienols increased linearly from 0.40 to 10.07μg/g in the fish when fed on the experimental diets with supplemented with 0 to 1111.11 mg/kg of TRF. Tocotrienols composition accounted for 24.7 to 33.1% of total vitamin E in the liver of fish fed with TRF supplemented diets (Diets 2 to 5), which were significantly higher than 16.6% in the fish given no vitamin E added Diet 1.

3.2.3.1.3 Vitamin E concentration and composition in fish adipose tissue

Figure 3.7 (c) showed the distribution of vitamin E isoforms in tilapia adipose tissue, and the detailed data were given in Appendix 6C. The major isoform deposited was α-T. Its adipose concentration linearly increased concomitant with dietary α-T concentrations ($y = 1.36\chi - 2.49$, $r = 0.98$). The highest α-T concentration (66.66 μg/g) in the adipose of fish fed with Diet 5 (240 mg/kg total vitamin E) was nearly 18 times higher than that (3.79 μg/g) found in the adipose of fish fed with Diet 1 (without added vitamin E). Low levels of γ-T (0 to 2.24 μg/g) were deposited to the adipose tissue of fish fed with the experimental diets which were reflective of γ-T concentrations in these diets. Total tocopherols remained constant at 51.1 to 53.3% of total vitamin E in the adipose tissue of fish fed with TRF supplemented diets (Diets 2 to 5), which were significantly ($P<0.05$) lower than the level of 71.8% in the adipose tissue of fish given Diet 1.

The α- and γ-T3 concentrations in tilapia adipose tissue had a direct and linear relationship with their respective dietary concentrations ($y = 0.70\chi + 0.38$, $r = 0.98$; and $y = 0.20\chi - 0.18$, $r = 0.99$, respectively)(Appendix 6C). The significant increase in adipose concentration of α- and γ-T3 both occurred in fish fed with Diet 2 (30 mg/kg of total vitamin E) when compared to fish fed Diet 1 (without vitamin E added). Adipose tissue concentrations of α-T3 and γ-T3 increased 36 and 61 times, respectively. α-T3 concentrations of adipose tissue ranging from 1.10 μg/g of fish fed with Diet 1 to 66.66 μg/g of fish fed with Diet 5 , which were generally higher than γ-T3 concentrations of 0.37 to 22.72 μg/g in fish fed with the same dietary treatment. All fish, except those fed Diet 1, had low levels (0.09 to 1.37 μg/g) of δ-T3 detected. Total tocotrienols

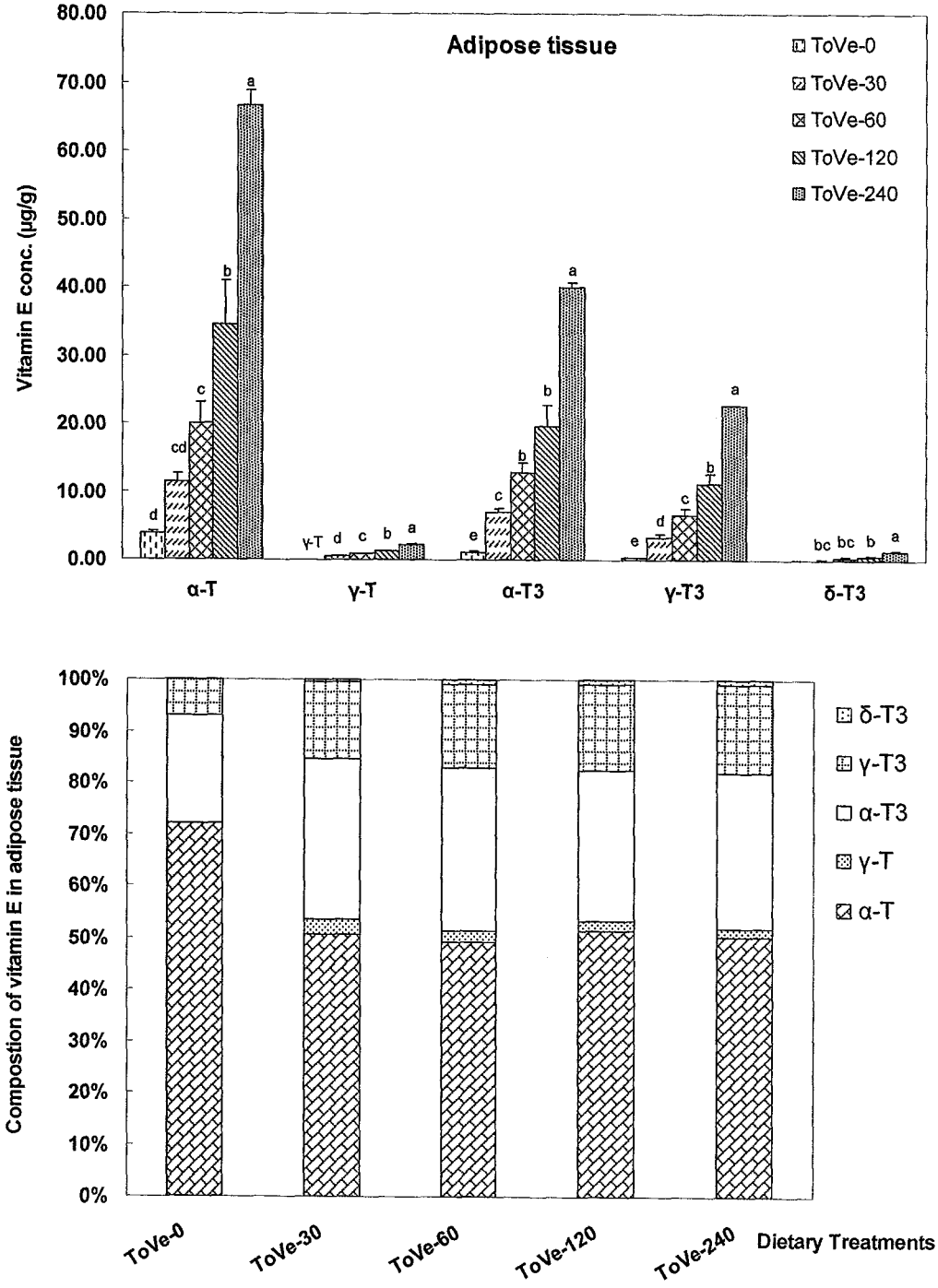


Figure 3.7 (c) Vitamin E concentrations and composition in adipose tissue of red hybrid tilapia fed with five different experimental diets for 9 weeks in Experiment 2. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

increased linearly from 1.47 to 63.06 μg/g in fish when fed with the experimental diets supplemented with 0 to 1111.11 mg/kg of TRF. Tocotrienols composition accounted for 46.7 to 48.9% of the total vitamin E in the adipose tissue of fish fed with TRF supplemented diets (Diets 2 to 5), which was significantly higher than the level of 28.2% in the fish given no vitamin E added diet (Diet 1).

3.2.3.1.4 Vitamin E concentration and composition in fish skin

Figure 3.7 (d) showed the distribution of vitamin E isoforms in tilapia skin, and the detailed data were given in Appendix 6D. α-T was the exclusive vitamin E isoform detected in the initial skin. After an 9-week feeding trial, vitamin E profile of fish skin significantly changed. Although the major isoform deposited was still α-T, its skin concentrations were significantly ($P<0.05$) different among fish fed with various diets. They linearly increased from 4.29 μg/g to 17.77 μg/g in line with graded levels of dietary α-T supplementation ($y = 0.26\chi + 5.03$, $r = 0.96$). Very low levels of γ-T (0 to 0.51 μg/g) were deposited to the skin of fish fed with the experimental diets, which were reflective of γ-T concentrations in these diets. Total tocopherols remained constant at 74.0 to 79.1% of total vitamin E in the skin of fish fed with Diets 1 to 5.

Low levels of α- and γ-T3 concentrations were deposited in tilapia skin, with values varying at 0.66 to 3.67 μg/g and 0.51 to 2.38 μg/g (Figure 3.7 (d) and Appendix 6D), respectively. Both had a direct and linear relationship with their respective dietary concentrations ($y = 0.54\chi + 0.68$; $r = 0.94$ and $y = 0.15\chi + 0.62$, $r = 0.92$, respectively). Skin concentrations of γ-T3 were generally lower than those of α-T3 in the fish fed

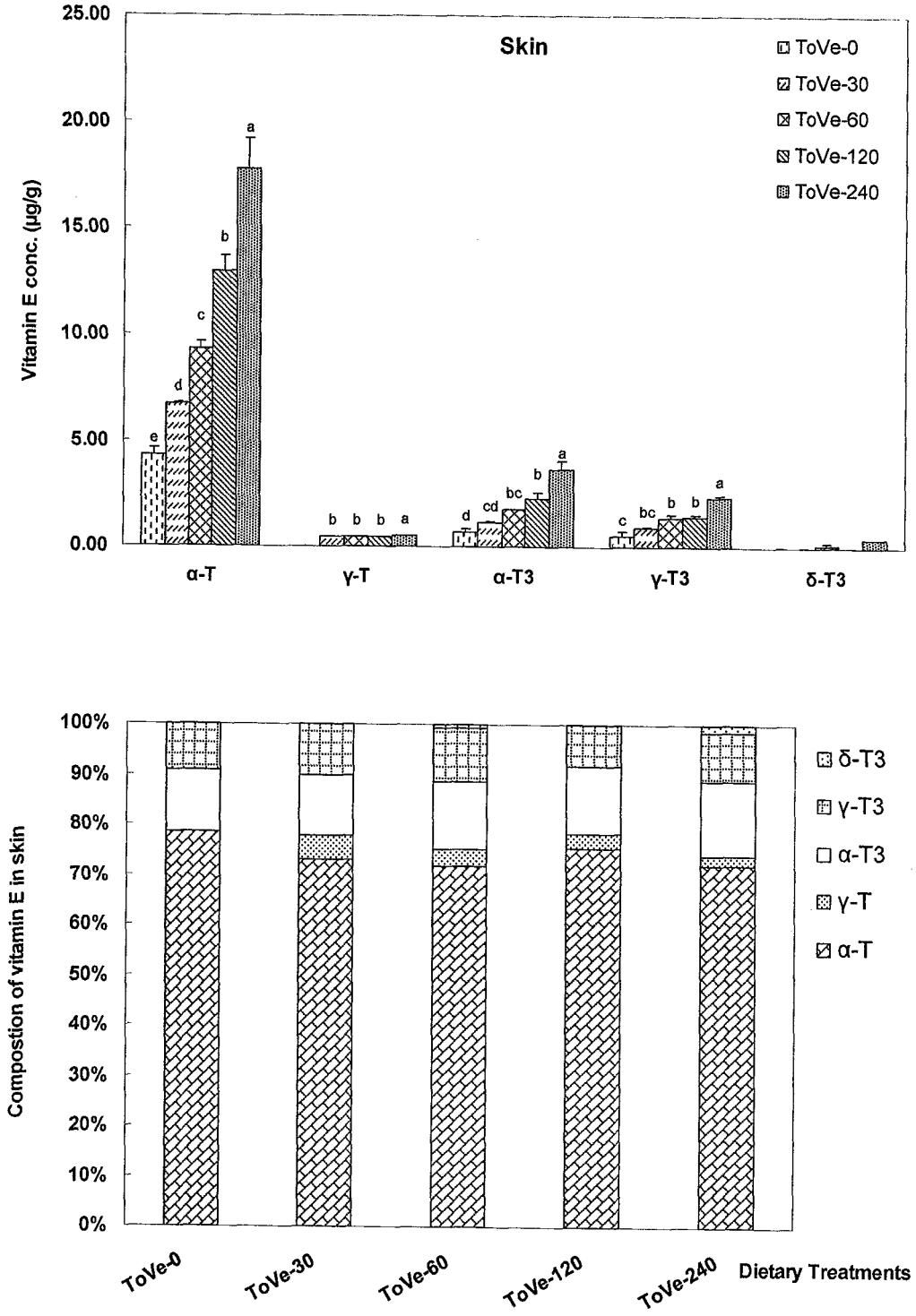


Figure 3.7 (d) Vitamin E concentrations and composition in skin of red hybrid tilapia fed with five different experimental diets for 9 weeks in Experiment 2. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

with the same dietary treatment. Negligible amounts of δ-T3 were occasionally detected in the skin of fed with Diet 3 and Diet 5. Tocotrienols composition accounted for 20.9 to 24.8% of total vitamin E in the skin of fish fed with Diets 1 to 5, with no significant differences detected.

3.2.3.1.5 Vitamin E concentration and composition in fish plasma

On the final samplings, fish plasma was obtained through immediately spinning fish blood once collected into the vacutainer tube from all the fish in each aquarium. Figure 3.7 (e) shows the distribution of vitamin E in tilapia plasma, and the detailed data were given in Appendix 6E. Plasma α-T was the predominant vitamin E isoform detected. Its concentrations were significantly ($P<0.05$) different among the plasma of fish fed with the experimental diets, linearly increasing from 0.89 μg/ml in the plasma of fish fed with Diet 1 (without vitamin E supplemented) to 7.04 μg/ml of in the plasma of fish fed with Diet 5 (240 mg/g total vitamin E) ($y = 0.12\chi + 1.65$, $r = 0.93$). α-T composition accounted for 87.1 to 94.1% of total vitamin E in fish plasma. The concentrations of all non-α-T such as γ-T, α- and γ-T3 in the plasma were very low (<0.4 μg/ml), irrespective of their respective dietary concentrations. The percentage of non-α-T contributed 5.9 to 12.9% of total vitamin E in the plasma of fish fed with various diets.

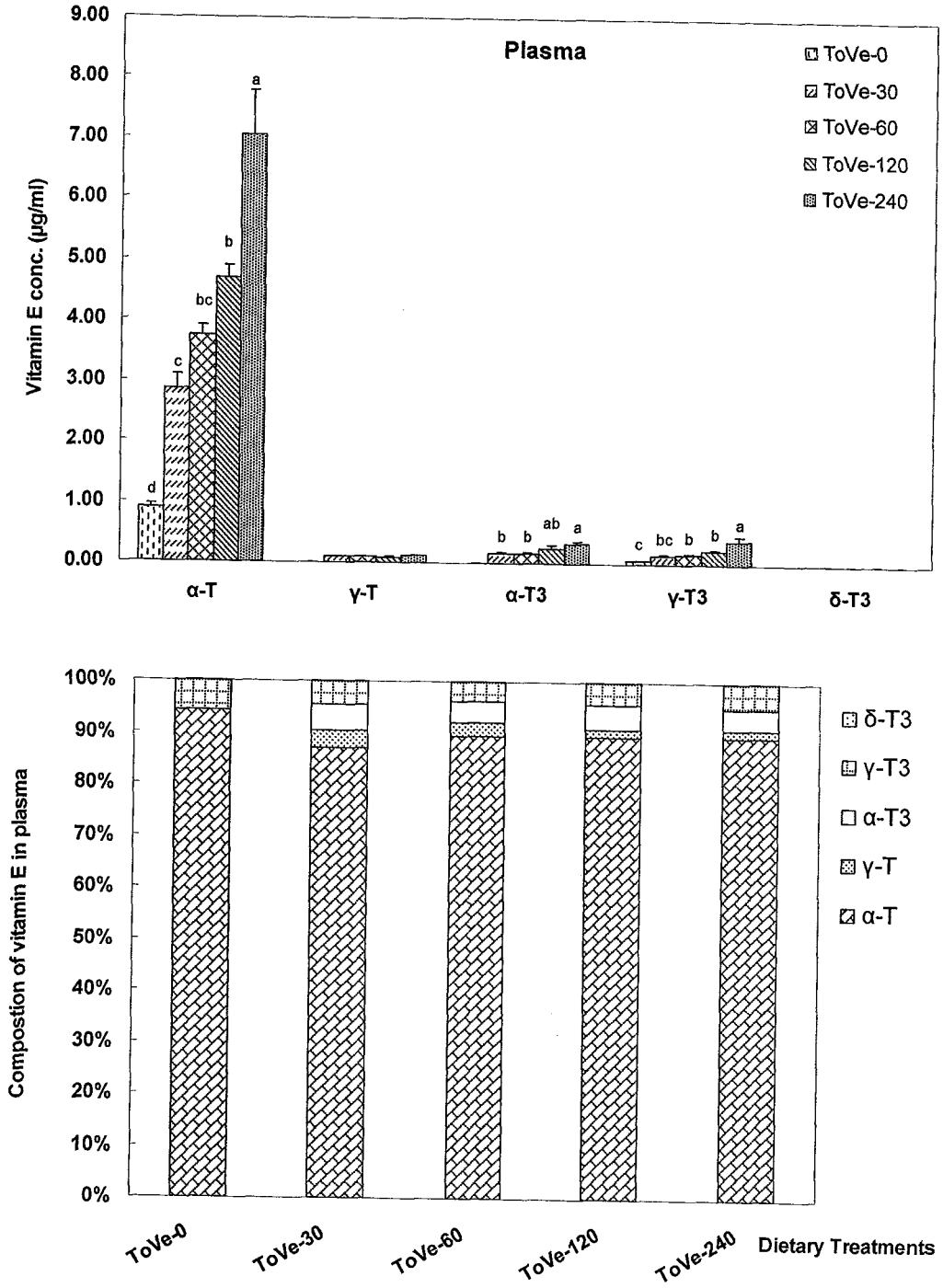


Figure 3.7 (e) Vitamin E concentrations and composition in plasma of red hybrid tilapia fed with five different experimental diets for 9 weeks in Experiment 2. Concentrations were expressed as means + s.e. of three replicates. For each vitamin E isoform, means not sharing the same letter were significantly different, $P<0.05$.

3.2.3.1.6 Comparison of vitamin E concentration and composition of various fish tissues and plasma

Interestingly, the percentage (69.2%) of tocopherols in the muscle of tilapia fed no TRF added diet (Diet 1) was found significantly lower ($P<0.05$) than those (76.8 to 78.1%) in the muscle of tilapia fed the TRF added diets (Diets 2 to 5). However, this pattern was reversed in the liver, adipose tissue, skin and plasma, that is, tocopherols composition in these tissues of fish fed with Diet 1 were significantly higher than those of fish fed with Diets 2 to 5 (Appendix 6B,C, D and E). The higher levels of α-T3 than γ-T3 of tilapia given the same dietary treatment were generally found in liver, adipose tissue, skin and plasma in the fish fed with the TRF supplemented Diets 2 to 5 (Appendix 6B,C, D and E). However, this is not true of muscle α- and γ-T3 concentrations, where γ-T3 concentrations is higher than α-T3 in fish fed with Diet 1 (Appendix 6 A).

Total vitamin E concentration in tilapia tissues and plasma mirrored the dietary concentration, but the vitamin E composition did not (Figure 3.7 (a) to (e) and Appendix 6). Percentages of dietary tocotrienols were at 77.1 to 81.4% in the five experimental diets (Table 3.11), which were about 4 times higher than the levels (22.9 to 18.6%) of tocopherols, regardless of increasing dietary supplementation of TRF levels from 0 to 1111.11 mg/kg. Inversely, higher percentages (66.9 to 94.1%) of tocopherols (α-T is the major isoform) were found in plasma and various tissues (with the only exception of adipose) of tilapia, compared to 5.9 to 23.1% of total tocotrienols. A ratio of adipose tocopherols / tocotrienols was found to be about 1:1 in fish fed with TRF supplemented diets (Diets 2 to 5), indicating that adipose tissue was an unique tissue with an ability to accumulate tocotrienols. The results obtained showed that the

capacity of various tilapia tissues to accumulate tocotrienols decreased in the order : adipose tissue > liver > skin > muscle > plasma.

3.2.3.2 TBARS of tilapia muscle, liver and plasma

The TBARS concentrations in the muscle□liver (expressed as nmol MDA/g tissue) and plasma (expressed as nmol MDA/ml) of tilapia fed TRF at all levels of dietary inclusion were significantly lower ($P < 0.05$) than those of fish fed the control diet without TRF supplementation (Figure 3.8 and Appendix 7). The oxidative stability of muscle, liver and plasma of tilapia were apparently improved up to tilapia fed 60 mg/kg total vitamin E derived from TRF supplementation. Further increasing dietary total vitamin E levels (120 and 240 mg/kg) exerted no further enhanced oxidative stability, but they were significantly better compared to fish fed the 30 mg/kg diet.

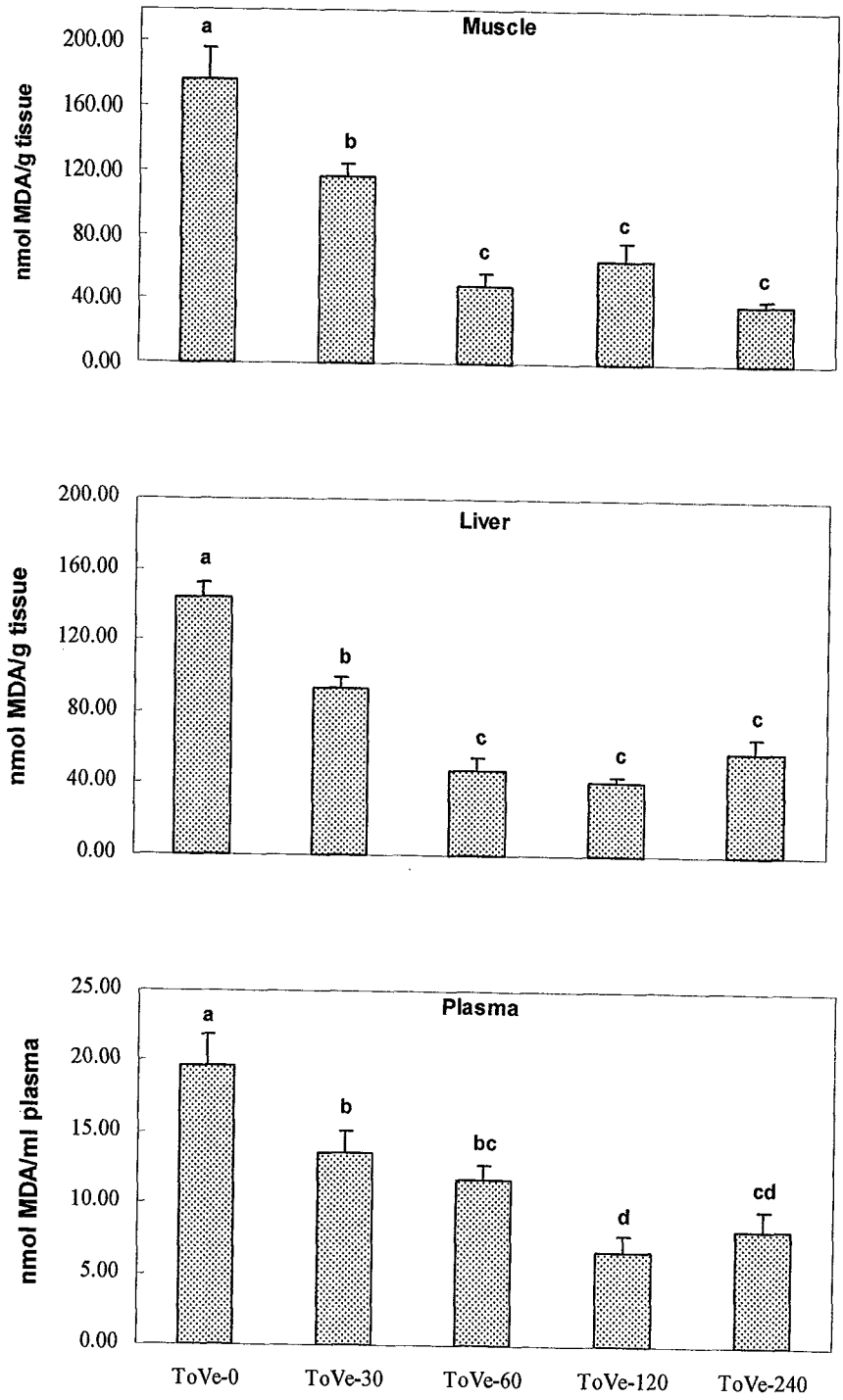


Figure 3.8 Effects of graded levels of total vitamin E derived from TRF on thiobarbituric acid-reactive substances (TBARS) from iron-vitamin C induced lipid peroxidation in tissues and plasma of tilapia fed with five experimental diets for 9 weeks in Experiment 2. Values were means + s.e of three replicates of one dietary treatment. For each tissue and plasma, values not sharing a letter were significantly different, $P<0.05$. MDA, malondialdehyde.

Chapter 4. General Discussion

4.1 Growth performances and feed efficiency

In Experiment 1, the eight semi-purified diets used in the study were supplemented with different vitamin E regimes, Diets 1to 4 supplemented with graded level of 0, 25, 50, and 100 mg α-tocopheryl acetate /kg dry diet; Diet 5 supplemented with 50 α-tocopheryl succinate mg/kg; Diet 6-8, supplemented with palm-based vitamin E deriving from TRF, PFAD or CPO, respectively. While in Experiment 2, only TRF was added as the sole vitamin E source, supplemented with graded levels of 0, 30, 60, 120, and 240 mg total vitamin E/ kg diet. After feeding experimental diets to fish for 8 and 9 weeks in Experiment 1 and Experiment 2, respectively, growth performances and feed utilization efficiency of tilapia fed different dietary vitamin E inclusion levels were not significantly different in both separate experiment, similar to reported findings in rainbow trout (Hung et al., 1981; Cowey et al., 1981), channel catfish (Wilson et al., 1984), and Nile tilapia (Satoh, 1987). However, Roem et al. (1990) found blue tilapia, *Oreochromis aureus*, receiving diets deficient in vitamin E grew at a lower rate than those receiving complete diets in a 12-week feeding trial. In Experiment 1, tilapia fed diets without vitamin E supplement (Diet 1) or with α-ToSc (Diet 5) had higher mortalities (27.8% and 22.2%, respectively) than fish fed other diets, though not significantly different. Atlantic salmon (*Salmo salar*) fries when fed unsupplemented *all-rac*-α-ToAc, had apparently higher mortalities than those fed with supplemented diets during the course of two 24-week feeding trial, and to the end got extinct (Hamre et al., 1994, Hamre & Lie, 1995). Feed utilization efficiency was not significantly affected in the two present experiments, which were similar to those findings in

rainbow trout (Cowey et al., 1981 & 1983), channel catfish (Wilson et al., 1984), African catfish (Baker & Davies, 1997) and hybrid tilapia (Huang et al., 2003).

4.2 Vitamin E deficiency signs

Fish pathologies that have been associated with vitamin E deficiency include: exudative diathesis, depigmentation in Atlantic salmon (Poston et al., 1976), muscular dystrophy in carp (Watanabe et al., 1977), channel catfish (Lovell et al., 1984) and rainbow trout (Cowey et al., 1984), and exophthalmia in Korea rock fish (Bai & Lee, 1998). By contrast, there were no gross pathologies detected among all harvested fish in the present two experiment. However, erythrocyte fragility of fish receiving Diet 1 unsupplemented with vitamin E (in Experiment 1 and Experiment 2) and Diet 5 supplemented with 50 mg/kg α-ToSc (in Experiment 1), measured spectrophotometrically through incubation of blood for 10 hours at a temperature of 37°C (Experiment 1) and 15 hours in a water bath of 24°C (Experiment 2), was clearly higher than those of fish fed with the other diets though there were no significant differences detected ($P>0.05$). Some researchers found hemolysis from blood obtained from vitamin E deficient fish were significantly higher than those receiving vitamin E supplemented diets in rainbow trout (Cowey et al., 1981, 1983; Pearce et al., 2003) and channel catfish (Wilson et al., 1984).

As to hematocrits of experimental tilapia blood, values (44.36%) obtained from fish fed with Diet 5 (ToSc-50) in Experiment 1 were significantly higher ($P<0.05$) than those of fish fed with Diet 2 (ToAc-25) and Diet 8 (CPO), with values of 35.32 and

35.83% (Tale 3.6), respectively, but not significantly different (P>0.05) when compared to those of fish fed with the remaining diets. Among values obtained from fish fed diets supplemented with α-ToAc, it was evident that hematocrit values increased slightly either side of that obtained for the fish fed 25 mg α-ToAc per kg dry diet. A similar observation was made in African catfish receiving either lower or higher than 15 mg α-ToAc per kg dry diet (Baker & Davies, 1997). In Experiment 2, hematocrit increased from 39.11% of fish fed Diet 1 (no added vitamin E) to 42.83% (Table 3.16) of fish fed with Diet 4 supplemented with 120 mg total vitamin E per kg dry diet. Fish receiving Diet 5 supplemented with 240 mg total vitamin E per kg dry diet had a significant lower (P<0.05) value of 38.46% when compared to fish fed with Diet 4, but not significantly different (P>0.05) from those obtained form fish fed with other diets. Based on the observation by Roem et al. (1990) of impaired erythropoiesis in vitamin-E deficiency tilapia, *Oreochromis aureus* (Steindachner), Baker & Davies (1996a & 1997) excluded the possibility that the increased hematocrit in vitamin E deficient fish may be due to the increased erythropoiesis in fish. They proposed the possible mechanism that the phenomenon might have resulted from increased erythrocyte volume which was caused by an elevated fluid infiltration without compromising red blood cell membrane integrity.

4.3 Somatic Index

In Experiment 1, hepatosomatic indices (HSI) of tilapia fed with Diets 1 (1.94%) and 3 (1.78%) were significantly higher than that of tilapia fed with Diet 8 (0.98%) (Table 3.6), but not significant with those of fish fed with the remaining diets. Fish fed with Diet 1 deficient in vitamin E had the highest HSI probably because fatty

infiltration into liver cells may account for an enlarged liver, as pointed by Baker & Davies (1996b). In Experiment 2, HSI obtained from tilapia fed different dietary treatments were not significantly different, but a trend of increase with the increasing dietary total vitamin E, which is similar to those findings of Sagaguchi & Hamaguchi (1969) in yellowtail and Baker & Davies (1996a) in African catfish. Sagaguchi & Hamaguchi (1969) demonstrated that oxidized oil caused decreased HIS, but this could be prevented by vitamin E supplementation. Baker & Davies (1996a) also showed that elevated dietary α-tocopherol levels resulted in a larger liver.

Intraperitoneal fat indices (IPI) of tilapia obtained from Experiment 1 were not significantly different ($P>0.05$) among each other, so as in Experiment 2. The IPI values originating from Experiment 1 varied from 0.26-0.44% were generally lower than those from Experiment 2 ranging from 0.78-1.08%. Differences in prior nutritional history, fish stress duration of the experiments might have caused differences in IPI values between similar sized fish from these two experiments.

4.4 Vitamin E distribution in various fish tissues

In the past several decades, studies of vitamin E as an essential nutrient as well as a potent antioxidant have been performed on a wide variety of fish species, including Chinook salmon (Woodall et al., 1964), Atlantic salmon (Hamre & Lie, 1995), rainbow trout (Cowey et al., 1981 & 1983; Lovell et al., 1984), carp (Watanabe et al., 1970a,b), channel catfish (Wilson et al., 1984), African catfish (Baker & Davies, 1997), Korea rockfish (Bai & Lee, 1998), hybrid striped bass (Kocabas & Gatlin, 1999), tilapia

(Satoh, 1987; Roem et al., 1990). However, almost all of these studies used *all-rac*-α-tocopheryl acetate (α-ToAc) as the sole dietary vitamin E source and occasionally d-α-tocopherol. Only limited work were done using non-α-tocopherols (γ- or δ-T) as dietary vitamin E sources on salmonids (Sigurgisladottir et al., 1994; Hamre & Lie, 1997; Hamre et al., 1998; Parazo et al., 1998). There is almost no information available on fish tocotrienols nutrition, with the only exception of the work were recently published (Ng et al. 2004). The present study would further add to our knowledge on the distribution of tocotrienols in various tissues of tilapia fed diets supplemented with palm-based tocotrienols.

To the best knowledge of the author, the study of bioavailability of α-tocopheryl succinate on animals has not yet been reported on fish, but some work has really been done on broilers (Jensen et al, 1999) and ruminants (Hidiroglou and Singh, 1991; Hidiroglou et al., 1992). Thus in Experiment 1, the bioavailability of α-tocopheryl succinate (α-ToSc) as dietary vitamin E source by fish was investigated, employing red hybrid tilapia as the experimental model. The results obtained from the present study indicated that the succinate ester of vitamin E is an inferior vitamin ester, compared to α-tocopheryl acetate as a source of vitamin E for fish nutrition.

Tocotrienols can be deposited in various tissues of tilapia, which seems very tissue-specific. Ng et al. (2004) had shown that dietary tocotrienols were deposited into the muscle of catfish fed diets containing PFAD. In that study, despite an increasing percentage of tocotrienols presented in the diets (1.8-58.2%), tocotrienols composed of only 13.4-26.7% of the total vitamin E deposited in the catfish muscle. In both of the current Experiment 1 and Experiment 2, dietary tocotrienols were detected in all the

measured tissues of tilapia, even among those fed with diets containing only traces of tocotrienols originating from CPKO. However these vitamin E isoforms were certainly significantly lower than their corresponding α-tocopherol counterpart (Figure 3.3 (a) to (d); Figure 3.7 (a) to (e)). The tocotrienols percentage composition of muscle, liver, skin and adipose of tilapia fed diets containing tocotrienols in the present two experiments were very close, and somewhat constant at equilibriums of 16.8-22.1%, 24.7-38.5%, 15.6-26.0%, and 46.7-60.0%, respectively. Apparently, adipose tissue of tilapia was the only tissue to accumulate more preferentially tocotrienols compared to tocopherols. This was similar to findings reported by Ikeda et al. (2001 & 2003). In contrast, we did not find high deposit of tocotrienols in tilapia skin, unlike skin of rats which was also a specifically tocotrienol-depositing tissue (Ikeda et al., 2001 & 2003). The precise mechanism of the accumulation of tocotrienols in adipose tissues is unknown. With the exception of adipose tissue, all other fish in both the present experiments, 61.5-92.0% of the total vitamin E deposited was present as α-T. Similar high percentages of α-T to the sum of other vitamin E isoforms in tissues have also been reported for laboratory land animals such as rabbit (Goh et al., 1992) and rat (Ikeda et al., 2001 & 2003).

Active research is currently being performed to explore the causes for the preferential deposition of α-T in animal tissues. Based on current knowledge, there is a consensus among scientists that biodiscrimination takes place largely in the liver where an α-tocopherol transfer protein (α-TTP) has been identified in rats. α-TTP has a high affinity for α-T and preferentially incorporates this vitamin E isoform into lipoproteins that transport α-T to the various tissues. This study seems to suggest that a similar α-T binding protein may exist in tilapia liver, although the isolation of such a protein has

not been reported in fish. Studies on dietary γ-tocopherols and δ-tocopherols in Atlantic salmon have shown the preferential deposition of α-T in fish muscles over the non-α-T isoforms (Hamre and Lie, 1997; Parazo et al., 1998). The existence of a mechanism similar to that present in mammals for biodiscrimination between the tocopherols mediated through competition for a α-TTP in salmon liver was suggested by the authors. Other mechanisms of biodiscrimination at the cellular membranes of specific tissues and other routes of vitamin E delivery also have been proposed to account for the varying deposition of vitamin E isoforms in various tissues (Parazo et al., 1998; Ikeda et al., 2003).

Ng et al. (2004) have assumed the existence of an α-TTP which expresses different relative affinities of the three tocotrienols (α-, γ- and δ-) in African catfish liver. The results from the present two experiments strongly reflected this assumption again. For instance, tocotrienols supplemented diets (Diets 6-8 in Experiment 1, Diets 2-5 in Experiment 2), had dietary concentrations of γ-T3 nearly 2 times more than α-T3, similar concentrations of γ-T3 and α-T3 were detected in respective tilapia tissues examined. This indicated that the relative affinity of γ-T3 for α-TTP is lower than that of α-T3. Similar conclusions were made in African catfish by Ng et al. (2004) and in rats by Ikeda et al. (2003). By measuring urinary excretions of the carboxyethyl-hydrochromans (CEHC) that are tocotrienols metabolites, Ikeda et al. (2003) further suggested that γ-T3 might be preferentially metabolized. Even though tocotrienols supplemented diets contained certain concentration of δ-T3, however negligible amounts of it, far lower than those of α- and γ-T3 was occasionally deposited in some tissues of tilapia. Indirect evidence from the present two experiments seemed to indicate that the relative affinity of α-T3 for α-TTP is the highest, followed by γ-T3 and

then δ-T3, which is similar to the conclusion made by Ng et al. (2004). Dietary supplementation of α-T has been reported to suppress the retention of γ-T and δ-T in human serum (Huang & Appel, 2003) and also α-T3 in rat tissues (Ikeda et al., 2003).

Low concentrations of β- and γ-T were both present in the experimental diets in Experiment 1, and only γ-T in Experiment 2, but similar low levels were detected in various tissues of tilapia in both experiments. The two vitamin E isoforms could not compete for the α-TTP especially since the dietary concentrations of α-T were overwhelmingly higher. With the exception of Sigurgisladottir et al. (1994) who found γ-T and α-T deposited to the same degree in Atlantic salmon muscle, other authors (Hamre and Lie, 1997; Parazo et al., 1998) have reported significantly low deposition of γ-T, β-T, and δ-T compared to α-T in salmon tissues.

4.5 Bioavailability of dietary α-T

Ng et al. (1998) developed a liver NAD assay, which provided a simple, accurate and straightforward method to estimate the bioavailability of niacin from dietary ingredients tested. This method offered a greater control of variability because it eliminated many influencing factors affecting growth assays, compared to those previously existing methods based on weight gain as the response measure reported in land animals such as rats (Carter and Carpenter, 1982) or chickens (Oduho & Baker, 1993). Liver α-T concentration represented a direct measurement of the utilization of dietary α-ToAc, α-ToSc, or palm α-T, and would give a close approximation of the true availability of these vitamin E derivatives in the test diets. Using the same bioassay

concept, the bioavailability of dietary α-tocopheryl succinate, and α-tocopherol present in the TRF, PFAD, and CPO in red hybrid tilapia was measured in Experiment 1.

Results obtained from the study indicated that α-ToSc is an inferior vitamin E source for tilapia. A bioavailability of only about 17% (Table 3.8) in tilapia supplied with this vitaminer displayed a good coincidence with low concentration of α-T in various fish tissues. Similar observations were also made in sheep (Hidiroglou and Signh, 1991) and lambs (Hidiroglou et al., 1992) when being administrated orally α-ToSc in diets. Moreover, Jensen et al. (1999) reported the apparent absorption coefficient of 58.0 ± 5.4 for all-rac-α-ToSc versus 70.8 ± 5.6 for all-rac-α-ToAc when supplying these two tocopheryl esters to broilers, irrespective of their dietary inclusion levels. *In vitro* hydrolysis of α-tocopheryl esters by porcine pancreatic carboxyl ester hydrolase (CEL),showed that the capacity of this enzyme to hydrolyze α-ToSc was only 1.5% of the corresponding capacity to hydrolyze α-ToAc. Furthermore, they speculated that this phenomenon may close to a lower affinity of the less hydrophobic *all-rac*-α-ToSc to intestinal hydrolysis by CEL. The possibility may also exist in red hybrid tilapia.

The bioavailability of free dietary d-α-T present in three palm-based vitamin E sources (TRF, PFAD and CPO) was not as high as expected. The calculated α-T bioavailabity of TRF, PFAD and CPO were 50.95, 70.72, and 56.30%, respectively. Since it is well known that d-α-T is the most active one among the vitamin E isoforms, these values may be underestimated. The requirement for α-T of fish may be affected by the level of polyunsaturated fatty acids in the diet (Watanabe et al., 1977 & 1981; Cowey et al., 1981 & 1983; Lovell et al., 1984). Satoh et al. (1987) reported that tilapia

receiving elevated dietary PUFA (from 5% to 15% fish oil methyl esters) in the diet containing 50 mg of α-T significantly reduced the whole body α-T content from 47.3 to 11.8 μg/g. It is generally believed that the higher inclusion of unsaturated lipids in the diets, the higher concentration of α-T is needed to meet dietary needs. In Experiment 1 (the study of α-T bioavailability), PFAD and CPO supplemented diets had higher concentrations of high unsaturated fatty acids (consisted of only n-6 series) and monounsaturated (Table 3.4) than those of other diets added CPKO as dietary sole lipid source. In Table 3.9, it was seen that the fatty acid profiles of tissues of fish feed the PFAD or CPO diets reflected those of diets. Thus fish fed these two diets would be somewhat more susceptible to lipid peroxidation than those fed CPKO-based diets. To combat this physiological stress, tilapia fed PFAD and CPO diets would need more *in vivo* antioxidants to trap lipid oxidation products in tissues (e.g. in liver), resulting in lower liver concentrations of α-T compared to that of fish fed ToAc-25 (Diet 2) with a similar level of dietary α-T. When using these two values of liver α-T levels as a measure of dietary available α-T, one can easily get lower values of bioavailability in the PFAD and CPO fed tilapia. However this explanation could not explain the low bioavailability of α-T in TRF by tilapia, since the TRF diet was supplemented with CPKO. It is possible that the high dietary concentrations of tocotrienols present in the TRF diet may have contributed to a lower deposition of α-T in the liver through competition of vitamin E receptor sites. Further research needs to be conducted to elucidate this.

4.6 Oxidative stability of fish tissues

Elevated concentrations of α-T in tilapia (Satoh et al., 1987; Shiau and Shiau, 2001; Huang et al., 2003) and many other fish species (O'Keefe & Noble, 1978; Frigg et al., 1990; Baker & Davies, 1996a & 1997, Gatlin et al., 1992; Gatta et al., 2000; Scaife et al., 2000; Chaiyapechara et al., 2003) has been reported to improve oxidative stability of fish fillets. To date, all studies on the role of vitamin E in decreasing lipid peroxidation in fish tissues had relied on the use of synthetic *all-rac*-α-ToAc as the sole dietary source of vitamin E. In Experiment 1, we evaluated oxidative ability of tissues of tilapia fed with either α-ToAc (Diets 2 - 4), α-ToSc (Diet 5), or palm-based natural tocopherols and tocotrienols originating from TRF (Diet 6), PFAD (Diet 7) or CPO (Diet 8), while in Experiment 2 only graded levels of TRF were fed. The iron-ascorbic acid induced thiobarbituric acid-reactive substances (TBARS) of tilapia muscle and livers showed similar trends (Figures 3.5 and 3.8). We found TBARS of muscle or livers of fish fed with α-ToAc decreased with increasing dietary levels until 50 mg α-ToAc /kg dry diet (Figure 3.5), which is in good agreement with the findings of Shiau & Shiau (2001). Based on the fact that induced TBARS of liver microsomes of juvenile tilapia would not decrease further once the vitamin E requirement was met, they re-evaluated the requirement of tilapia for 42 to 44 and 50 to 66 mg α-ToAc /kg dry diet when feeding tilapia test diets supplied at 5% or 12% of dietary lipid, respectively. Muscle and liver from fish supplied with α-ToSc were found to generally have higher amounts of TBARS than those of fish supplied with α-ToAc. This reflected the low α-T levels deposited in these two tissues. This further demonstrated that vitamin E in α-ToSc form was not easily bioavailable to red hybrid tilapia.

In Experiment 1, muscle concentration of α-T of fish fed with TRF supplemented diet was 4.77 μg/g tissue close to 4.18 μg/g of fish fed with 25 mg/kg α-ToAc diet, but significantly ($P<0.05$) lower than 6.66 and 9.06 μg α-T /g deposited in fish fed the 50 and 100 mg/kg α-ToAc diet, respectively. However, induced muscle TBARS of fish fed these two latter higher levels of ToAc were significantly ($P<0.05$) higher (39.08 and 38.75 nmol MDA/g, respectively) than that of 21.01 nmol MDA /g from fish fed the TRF supplemented diet. Similar trends were found in induced-TBARS of livers of these fish. It was noticeable that other than α-T, 1.03 and 7.34 μg total T3 per g tissue, respectively, was present in the muscle and livers of fish fed diets supplemented with TRF. Fatty acids profiles of muscle of fish fed with α-ToAc or TRF supplemented diets for 8 weeks were similar (Table 3.9). Although fatty acids compositions of livers of these fish were not determined in the study, they were expected to possess similar profiles of their corresponding muscles. The influence of fatty acids on induced-TBARS of tissues (muscle or liver) was believed equivalent among the fish fed diets with CPKO as sole dietary lipid source. Hence, tocotrienols in the muscle and liver of fish fed TRF clearly contributed to the substantial reduction of TBARS in these two tissues, and conclusively demonstrated them more potent antioxidants than α-T. Kamat and Davasagayam from India in 1995 had reported that tocotrienols from TRF, extracted from crude palm oil, could significantly inhibit higher oxidative damage *in vitro* to both lipids and protein in rat brain mitochondria induced by three different initiative matrices (ascorbate-Fe^{2+}, the free radical initiator of azobis(2-amidoprapane)dihydrochloride (AAPH) and photosentitisation) than the dominant form of vitamin E, α-T did. In 1997, Kamat et al. again demonstrated that tocotrienols from TRF were more potent antioxidants when employing rat liver microsomes as the biological model.

In Experiment 2, induced TBARS of muscle and livers of tilapia decreased significantly until fish receiving the 60 mg total vitamin E / kg supplemented diet (Figure 3.8). TBARS in the muscle and livers of tilapia fed with diets of beyond 60 mg/kg of total vitamin E were not be further suppressed. It would therefore seem that a dietary concentration of 0.23 mg/kg TRF extracted from CPO that gave a total vitamin E concentration of 60 mg/kg was sufficient to prevent tissue peroxidation in tilapia fed a 10% saturated dietary lipid source.

The unsaturation degree of fatty acids plays a much more important role in affecting oxidative stability of tissues than vitamin E does. The rates of forced lipid peroxidation of muscle from fish fed TRF, PFAD and CPO supplemented diets in Experiment 1 were significantly ($P<0.05$) different from each other (Figure3.4). The value (40.81 μg MDA/g) of induced-TBARS of muscle from fish fed with PFAD were almost same to those (38.75-44.82 μg MDA/g) of fish fed α-T supplemented diets. These values were significantly ($P<0.05$) lower than the values (63.76 and 63.36 μg MDA/g, respectively) from muscle of fish fed with α-ToSc and CPO supplemented diets. With regard to induced-TBARS of livers from fish fed with the PFAD and CPO supplemented diets, there were very similar trends found. These findings were in agreement with tissue fatty acid profiles observed as shown in Table 3.9. According to this table, it was not difficult to find that muscle contents of unsaturated fatty acids (total monoenes + total PUFA) of fish fed with TRF diet increased from 43.0% to 48.4%, and 55.4% of fish received PFAD and CPO diets, respectively. Taking into account the fact of the deposition of vitamin E in muscle and livers of fish fed with these three palm-based tocotrienol-rich diets, it can be deducted that the extent of lipid peroxidation of tissues depend mainly on their unsaturated lipid composition rather

than vitamin E. Leibovitz et al (1990) demonstrated that the liver and heart from rats fed highly unsaturated menhaden-oil containing diets were more susceptible to induced lipid peroxidation than those from less unsaturated corn oil-lard (1:1 mixture) fed rats when studying the effects of dietary vitamin E on inhibition of lipid peroxidation. In a study published recently by Huang et al. (2003), they found that the extent of lipid peroxidation of tilapia liver were generally higher than that of muscle from tilapia receiving the same level of dietary α-ToAc. They pointed out that liver normally contains much higher amount of lipids which would be more susceptible to lipid peroxidation than muscle.

The potency of various vitamin E isoforms were extensively reported in human and animal nutrition, but not in fish. Historically, the calculation of potency of vitamin E of a food has been based on summing the contribution of the various tocopherol and tocotrienol isomers in a manner that accounts for each isomer's presumed biological activity in comparison to α-T (Mag, 2002). In Experiment 1, using the standard TBARS response curve derived from tilapia fed with 0, 25, and 50 mg α-ToAc/kg diets, we calculated theoretical α-ToAc equivalent in diets (Table 3.10) and further obtained the potency of α-ToSc and total vitamin E of TRF, PFAD and CPO in comparison to α-ToAc. In this study, the potency of 1 mg α-ToAc obtained was equivalent to 1.56 mg α-ToSc, and 2.47, 3.54, 4.38 mg total vitamin E in TRF, PFAD, CPO, respectively. The potency of 1.56 of α-ToSc / α-ToAc was very close to the ration of 1.67 in mammalian (Papas, 1999). No other data is currently available to compare the potencies of total vitamin E in three palm-based matters to α-ToAc. Their values were significantly higher when compared to that of α-ToSc. The inefficient bioavailability on tocotrienols in tilapia may partially explain this kind of difference. However, when

the actual deposited concentrations of palm vitamin E was compared with that of deposited α-T from α-ToAc, it was evident that palm tocotrienols were the more potent as an antioxidant than α-T in tilapia tissues.

Chapter 5. Conclusions and Recommendations

5.1 Conclusions

The following conclusions could be made based on the results obtained from the current two experiments:

1. α-tocopherol was the predominant vitamin E isoform present in various tissues of red hybrid tilapia with the possible exception of adipose tissue, irrespective of dietary vitamin E source or level.

2. Red hybrid tilapia could only utilize about 18% of α-T present in α-tocopheryl succinate when used as a dietary vitamin E source, and is not as effective as α-tocopheryl acetate. Even though TRF, PFAD and CPO possessed substantial amounts of free α-tocopherol, only about 50.95%, 70.72% and 56.30%, respectively, were bioavailable to tilapia when using liver as the bioassay tissue .

3. Tilapia tissues varied in their ability to accumulate tocotrienols. Adipose tissue had the highest composition of tocotrienols (47-60%), followed by liver (24-38%), skin (16-27%), muscle (17-22%) and plasma (8-10%).

4. Palm tocotrienols were more potent *in vivo* antioxidants than α-tocopheryl acetate and α-tocopheryl succinate. TRF supplementation in diets could markedly enhance the palm tocopherols and tocotrienols concentration in red tilapia tissues and provide higher protection of these tissues against lipid peroxidation.

5.2 Recommendations

Reasons for lower than expected bioavailability of α-tocopherol in TRF, PFAD, and CPO obtained by tilapia in this study need to be further investigated. Research on the accurate mechanisms to discriminate various vitamin E isoforms within the fish body remain to be explored. α-TTP which is a protein playing important role in the absorption and transportation of vitamin E has already been isolated and purified from mammalian animals, but has not yet demonstrated in fish or in red hybrid tilapia. A further study should also be conducted to investigate whether the abundance of carotenoids present in the TRF or CPO could be deposited into tilapia tissues and subsequently cause a synergistic role with accumulated tocopherols and tocotrienols in preventing *in vivo* or *in vitro* lipid peroxidation of fish tissues.

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