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Relationship of Total Phenolic Contents, DPPH Activities and Anti-Lipid-Oxidation Capabilities of Different Bioactive Beverages and Phenolic Antioxidants

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RELATIONSHIP OF TOTAL PHENOLIC CONTENTS, DPPH ACTIVITIES AND ANTI-LIPID-OXIDATION CAPABILITIES OF DIFFERENT BIOACTIVE BEVERAGES AND PHENOLIC ANTIOXIDANTS

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ABSTRACT

Polyphenol compounds are widely used as antioxidants in food processing. The conventional phenolic content, scavenging DPPH free radical assays have been widely used for antioxidant evaluation. This research aimed to study the correlation between scavenging DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical activity, total phenolic content and anti-lipid-oxidation capability of natural and synthetic antioxidants and four beverages drinks. Synthetic phenol antioxidants, BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), PG (propyl gallate), TBHQ (tert-butylhydroquinone) and naturally occurring phenolics α-T(α-tocopherol), CA(Chlorogenic acid), GA(Gallic acid), QCT(Quercetin), RSV(Resveratrol), and SA(Syringic acid) were examined for their total phenolic content (TPC) and scavenging DPPH free radical activity. The anti-lipid-oxidation capability of these antioxidants was determined by using a fish oil emulsion which imitated the blood serum environment. CA and GA had great phenolic content, while they were in the group of lowest anti-lipid-oxidation capability. Oppositely, BHA was in the lowest group of phenolic content but provided the greatest anti-lipid-oxidation capability. The DPPH annihilation activity of GA was equivalent to PG and QCT and among the top three performances in the assay. However, its anti-lipid-oxidation capability was four times lower than PG or QCT. In general, most of the synthetic antioxidants had poor phenolic content but demonstrated better anti-lipid-oxidation capability, while, the phenolic acids such as GA and CA, were totally reverse. Only QCT and PG exhibited great performance in all the three assays. Thus, the fish oil emulsion developed in this study could be an efficient and reliable model for the evaluation of various antioxidants which overcomes the unilateral reaction of conventional spectrophotometric assays.

Tea, coffee, red wine and white wine were also investigated to assess the correlations of TPC, DPPH free radical scavenging activity and anti-lipid-oxidation capability in a fish oil emulsion model.

Diversities and concentrations of the major phenolics in the four beverages were also determined. With the most abundant phenolics, red wine dominated in TPC, DPPH scavenging activity, and anti-lipid-oxidation capability. However, white wine which had better performance in the TPC and DPPH assays showed the lowest capability in preventing fish lipid oxidation. The anti-lipid-oxidation capability of tea or coffee was much higher than white wine, although the three beverages had similar TPC. Therefore, the results from TPC and DPPH assays may not always correspond to the actual anti-lipid-oxidation capabilities. As the fish oil emulsion model was designed to imitate the human serum, the obtained anti-lipid-oxidation capability could closely reflect the antioxidant activity in stabilizing lipids and reducing harmful lipid oxidation products in the serum.

CHAPTER 1
LITERATURE RIVEW

1.1 Introduction

Lipid oxidation is initiated with active free radicals under oxidative stress (Carocho, 2013). The free radicals could be internally generated by normal metabolism of a biological system or by external factors such as smoking, pollution, and radiation. They result in the homeostasis of lipid metabolism and acceleration of lipid oxidation in the human body (Lobo et al. 2010). Lipid oxidation has been considered as a major problem in the storage of fatty food products. Oxidative changes can result in repugnant flavors, nutrition substances damage and toxic compounds generation (J. Kanner et al., 1992). Antioxidants are compounds that prolong the shelf life of foods by protecting them against deterioration caused by oxidation. They are usually classified into two groups - natural antioxidants and synthetic antioxidants. Naturally existing phenolics are reported to be capable of scavenging the free radical and stabilizing normal lipid metabolism (Xu, 2012). The phenolics are a group of phytochemicals and biosynthesized as the secondary metabolites from all kinds of plants. For example, as a group of tocopherols and tocotrienols, vitamin E is lipophilic antioxidants and rich in cereals such as rice bran, nuts, or corns (Xu, 2012). Other common phenolics such as quercetin (QCT), chlorogenic acid (CA), gallic acid (GA) or syringic acid (SA) are widely found in a variety of fruits and vegetables including apples, peppers, and culinary herbs etc. (Xu, 2012). These phenol-based antioxidants are reported to be effective modulators of redox homeostasis under oxidative stress and reduce the risks of the diseases involved by lipid oxidation (Robards et al. 1999). Therefore, by retarding the lipid oxidation, antioxidants play an important role on promoting health status and preventing the

development of chronic diseases in human body. Compared with the natural antioxidants, synthetic phenol antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) are well known as food preservers or antioxidants and have been widely used in food industry for extending the shelf-life of various food products (Xiu-Qin et al. 2009). However, the performances of the synthetic and natural antioxidants in different antioxidant assays have not been fully explored and compared.

Consumptions of fruits, vegetables, cereals, and grains are beneficial for the health status and antioxidant level in the body. The health promoting function of the beverages made from natural antioxidant-rich plants and fruits have also been widely recognized (Wootton, 2011). For example, tea is well known for the anti-lipidemic, anti-inflammatory and anti-thrombogenic activities (Di Castelnuovo, Giuseppe, Iacoviello, and Gaetano, 2012). Coffee rich in a variety of phenolics could also effectively counteract against chronic diseases (Higdon and Frei, 2006). The presence of anthocyanins and hydroxycinnamate derivatives in grape wines was reported to contribute to the decrease of serum cholesterol level, neuroprotective and anti-aging activities (Fernández, Mateos, García-Parrilla, Puertas, and Cantos, 2012).

Total phenolic content (TPC) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays are the traditional assays of evaluating the antioxidant activity of food products. The two in vitro assays can evaluate implicit correlation with the health promoting antioxidant potential in most antioxidant studies. However, the unilateral evaluation by those single chemical-based spectrophotometric assays may not totally reflect the real antioxidant performance in a

lipid-related environment, especially in human serum. In fact, the mechanism of lipid oxidation involved in a biological system is complicated. In this study, a fish oil emulsion model was used to simulate the human serum and examine the actual anti-lipid-oxidation capability of the selected antioxidants and drinks. The results in this study could demonstrate the relationship of the performances obtained from different antioxidant assays. However, the conventional phenolic content, scavenging DPPH free radical assays may not be correlated to the actual antioxidant capability of protecting lipid oxidation in an emulsion model associated with the anti-lipid-oxidation activity in human serum or body. Therefore, the results could provide useful information for understanding the difference between phenolic content, scavenging DPPH free radical activity, and real anti-lipid-oxidation or health promoting capability in an emulsion.

1.1.1 Lipid Oxidation

Oxidation is defined as the loss of electrons or an increase in oxidation state by a molecule, atom, or ion. Simply, it can be explained as the gaining of bonds to oxygen (Hudlický, 1990). Lipid oxidation is a common oxidation phenomenon that occurs in food processing and storing affects shelf-life and quality loss of foods (Jin et al., 2012). The oxidation in food lipids is generally associated with fats and oils or in oil phases of emulsions and primarily involves the reaction of oxygen with unsaturated fatty acids. It usually occurs in triacylglycerols, phospholipids, free fatty acids and fatty acids esterified to other molecules such as sterols and alcohols (Christie, 1982).

Generally, lipid oxidation is cataloged into three types, autoxidation, photooxiation and

enzymatic oxidation. Among them, autoxidation is confirmed as the primary oxidation, which is based on mechanism of a sequential free radical chain-reaction. This mechanism includes several fundamental steps. As shown in Figure 1.1, the oxidation process is divided into initiation (start), propagation, branching and termination stages. First of all, autoxidation is initiated by free radicals. This initiation is started by abstraction of an H-atom from a fatty acid molecule [reaction step 2 (Step-2 in Fig 1.1)]. The rate of H-abstraction process is the slowest. By monomolecular degradation mechanism, the radicals generated from the degradation of hydroperoxides accelerated peroxidation of unsaturated fatty acids (Step-4 in Fig. 1.1). Also, degradation of hydroperoxides is considered as the beginning point of the formation of volatile compounds. After that, the hydroperoxide concentration reaches a level and then free radicals are generated by a bimolecular degradation mechanism (Step-5 in Fig 1.1). Reaction Step-5 is exothermic, while reaction step-4 is the endothermic by monomolecular decomposition of hydroperoxides, both of them are the branching reactions of the free radical chain. Before chain termination, a radical may form hundreds of hydroperoxide molecules at room temperature. Alkyl radicals in the presence of air can be transformed into peroxy radicals through the rapid radical chain reaction 1 (Step-1, Fig 1.1). Therefore, collision of two peroxy radicals causes chain termination (Step-8, Fig 1.1) (Karen, 2013).

Start: Formation of peroxy ($RO_2^{\cdot}$),

Alkoxy ($RO^{\cdot}$) or alkyl ($R^{\cdot}$) radicals

Chain propagation:

(1) $R^{\cdot} + O_2 \longrightarrow RO_2^{\cdot}$

(2) $RO_2^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$

(3) $RO^{\cdot} + RH \longrightarrow ROH + R^{\cdot}$

Chain Branching:

(4) $ROOH \longrightarrow RO^{\cdot} + OH^{\cdot}$

(5) $2ROOH \longrightarrow RO_2^{\cdot} + RO^{\cdot} + H_2O$

Chain Termination:

(6) $2R^{\cdot} \longrightarrow$

(7) $RO_2^{\cdot} + R^{\cdot} \longrightarrow$ } Stable Products

(8) $2RO_2^{\cdot} \longrightarrow$

Fig 1.1. Basic steps in the autooxidation of olefins.

(Source: Karen, 2013)

The photooxidation proceeds basically through two pathways - direct photooxidation or by a photosensitizer. Direct photooxidation is caused by free radicals produced by UV light and then proceeds by normal free radical chain reactions (Frankel, 1991). With metals or metal complexes, oxygen turns to be active and forms either free radicals or singlet oxygen, then initiates lipid oxidation (Galliard et al, 1975). Photooxidation by a photosensitizer can be divided into type I or type II reactions. These reactions are competitive at the same time. Type

I reactions follows a free radical mechanism, and the reactions are most efficient at low oxygen concentrations (He et al., 1998). In type II reactions, the sensitizer reacts with oxygen to form $^1O_2$. These reactions basically produce hydroperoxides (Spikes, 1968). Chain-breaking antioxidants cannot inhibit the two types of photosensitized reactions. Contrast to type I reactions, type II reactions are affected by $^1O_2$ quenchers (Stratton and Liebler, 1997).

Enzymatic browning is another type of lipid oxidation. It is a chemical process of generating brown pigments by the enzyme polyphenoloxidase (PPO) and lipoxidase. They are found in plants, catalyze the oxidation of unsaturated fatty acids such as linoleic, linolenic and arachidonic acids, generate the product of cis, trans-1,3 diolefin hydroperoxide in addition of molecular oxygen (Y. Jiang et al. 2004). Enzymatic browning leads to food quality loss, especially during post-harvest storage of fresh fruits, juices and some shellfish. It has been reported that enzymatic browning may be responsible for up to 50% of all losses during fruit and vegetables production (Zhou, 2014). In the presence of air, the enzyme catalyzes the conversion of phenolics into quinones. And insoluble polymers melanins with black color yield after further polymerization. These melanins prevent the spread of infection or bruising in plant tissues. Plants with relatively higher polyphenoloxidase levels show a comparably high resistance to climatic stress (Yu, 2010). An example of the formation of melanins from a simple polyphenol, tyrosine, is shown in the figure below:

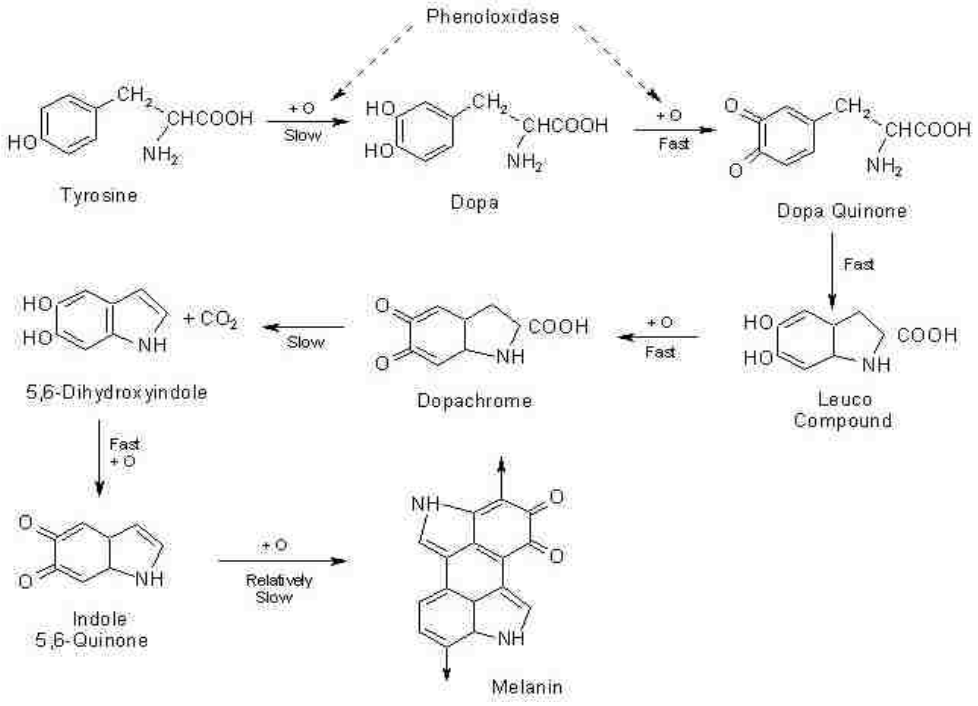


Fig 1.2 Formation of melanins from tyrosine

(Food and Agriculture Organization Database, 2009)

1.1.2 Negative effects of lipid oxidation on food and human health

At the nutritional level, the oxidation of fatty constituents is the major cause quality loss by nutritional and food safety deterioration. Quality attributes of food are affected by this process. Thus, aroma changes result from the formation of new volatile odorous compounds while food nutritive value and safety are impaired (Eriksson, 1982). Lipid oxidation of fish oil and other foods rich in fatty acids seriously affects their shelf-life and leads to nutritional value lose and consumer acceptability decline. Moreover, fatty acid oxidation products, such as the aldehydes, cause aging, mutagenesis, and carcinogenesis (George, 2007). Aldehydes, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), can be toxic by crosslinking to proteins or binding covalently to nucleic acids (Nair, Cooper, Vietti, and Turner, 1986). Polyunsaturated fatty acids with more than a single double bond are prone to

chemical and enzymatic oxidation, which are the same chemical processes involved in butter rancidity. In the fatty acid or fatty acyl chain, the hydrogen on the carbon atom between two adjacent double bonds (double bonds of fatty acids linked to bio-system are always separated by a single "–CH2–" group) is the weakest bond if the fatty acid is involved in esterification of a complex phospholipid. Activation of inflammatory cells can be initiated by lipid peroxidation that produces reactive oxidants. The reactive oxidants can abstract the weakly bound hydrogen via generation of chemically reactive oxygen species and produce enzymes like myeloperoxidase (Podrez, 2000). This free radicals reaction cannot be reduced by the normal pathways that control most of metabolic changes and results in oxidative attack on polyunsaturated fatty acids and phospholipids (Porter ,1995).

At the biological level, the lipid oxidations damage membranes, hormones and vitamins, which are essential for the normal cell activities. Considerable evidences indicate lipid oxidation participate in tissue injury and disease in the form of radicals, hydroperoxides, epoxides, and aldehydes (Frankel, 1992). It leads to certain pathological processes in living tissues, including aging, cancer, atherosclerosis and Alzheimer's disease. Moreover, it is considered that lipid oxidation is toxic compounds resulting in decomposition of membranes, inactivation of enzymes, and impairment of cell functions (Li, 2014). Oxidized lipids, such as oxygenated arachidonic acid products, the prostaglandins and leukotrienes, also exist diverse effects on the inflammatory and reparative responses of cells (Smyth, 2009). Therefore, antioxidants are considered as effective agents for preventing rancidity and limiting its deleterious consequences (Frankel, 1987).

1.2 Fish Oil

1.2.1 Background

Fish oil is a unique species compared to other commercial oils due to three characteristic features: the high unsaturation degree and carbon atoms number of the constituent fatty acids; the high content of the long chain omega-3 type polyunsaturated fatty acids (n-3 PUFAs); and the variety of fatty acids present in the triacylglycerols (Ackman and Lamothe, 1989). Although fish is a dietary source of omega-3 fatty acids, these fatty acids cannot be synthesized by fishes but obtained from algae or plankton (Falk, 1998). The species of fish caught specifically for fishmeal and fish oil mainly includes menhaden, anchovy, capelin and sardines (Table 1.1).

Table 1.1 Species of fish caught for fish oil and fishmeal production

Species	Country
Anchovy	Peru, Chile, South Africa, Namibia, Mexico, Morocco
Jack (Horse) Mackerel	Peru, Chile, China, Vanuatu
Capelin	Norway, Iceland, Russian Federation
Menhaden	USA: Atlantic and Gulf of Mexico
Blue Whiting	Norway, UK, Russian Federation, Ireland
Sand eel	Denmark, Norway, Faroe Islands
Norway Pout	Denmark, Norway, Faroe Islands
Sprat	Denmark, Russian Federations

(Source: Ibraheem, 2014)

The report from Food and Agriculture Organization (FAO) in 2010 showed 55.1 million tons of global aquaculture production and 90 million tons of global marine catches on the state of the world fisheries and aquaculture in 2009. Twenty seven million tons of global marine catches were severed for non-food purpose. About 20 million tons were applied to fishmeal and fish oil. The figures from FAO in past decades indicated a continuously growing trend in world aquaculture production and the amount of captured fish keep relatively stable for fishmeal and fish oil production (Fig 1.3). Fisheries and aquaculture researches become more significant and hence deserve considerable investigations for socio-economic and biological purposes (Fabrizio, 2013).

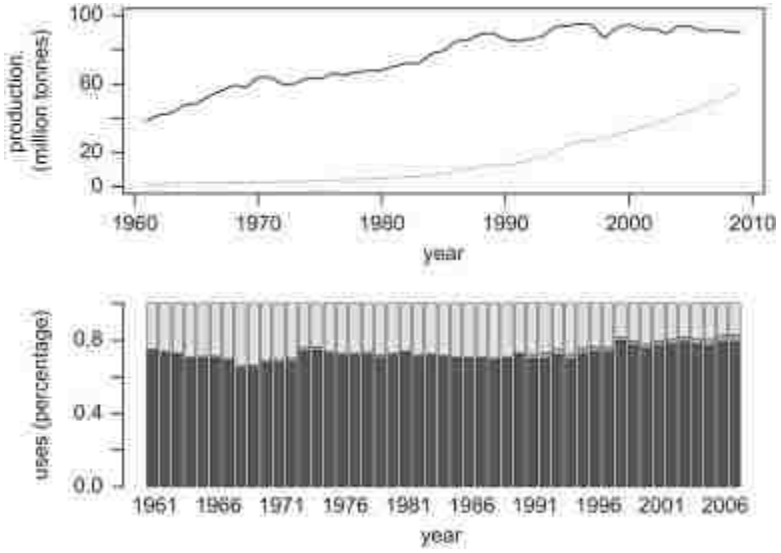


Fig 1.3 Production from aquaculture and fishery (chart above) and uses for food, other uses and feed (chart below)

(Source: Ibraheem et al., 2014)

Generally, fish oil can be recovered by different methods. Typical recovery methods aim to minimize oil decomposition and products denaturation. Wet reduction is the most common process applied in fish oil production (Tuominen, 2003). It enables a high volume recovery of

fish oil and can produce edible fish oil by subsequent refining steps. Other recovery methods include hydrolysis, silage production (also called autolysis), dry rendering and solvent extraction. In addition, the focus of these processes is typically to recover for edible, pharmaceutical or industrial application which must meet regulatory standards (Bimbo, 2013).

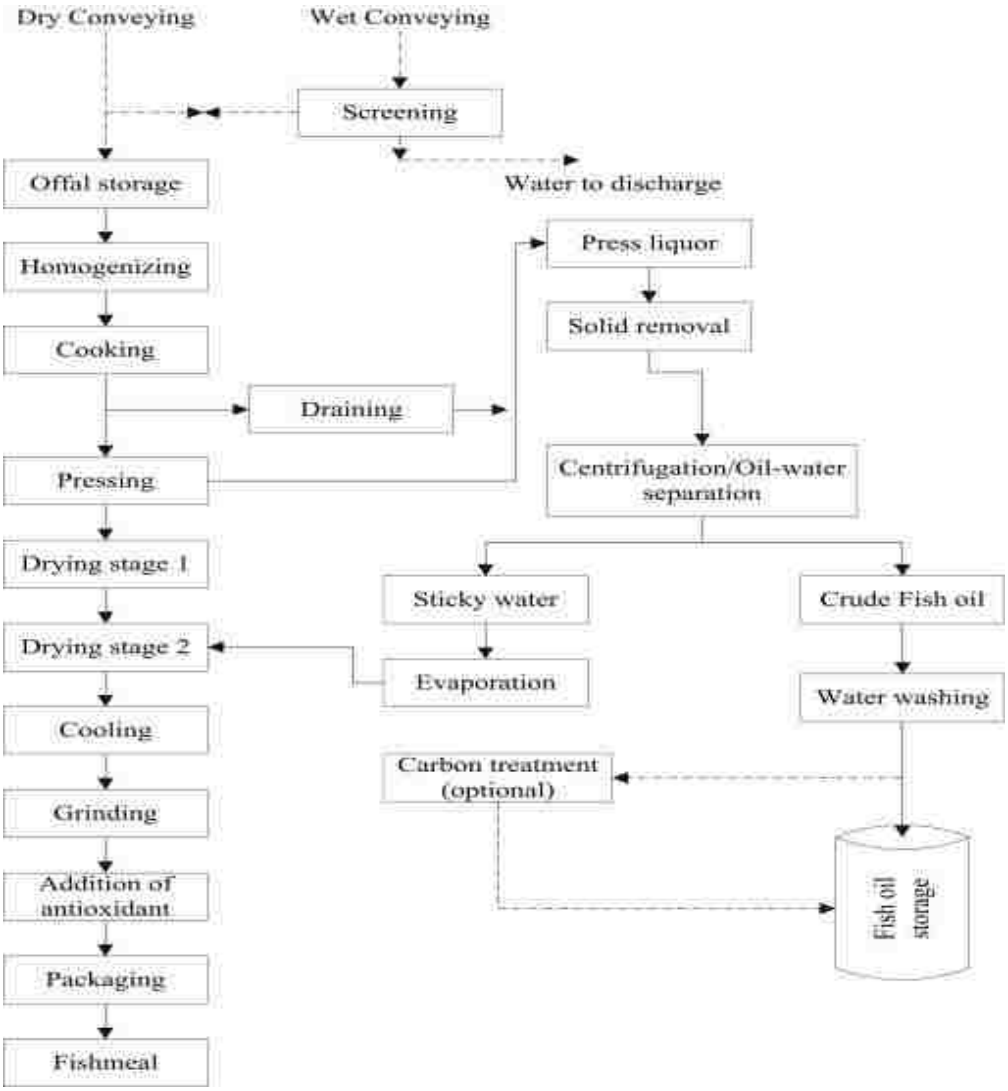


Fig 1.4 Basic stages of fishmeal and fish oil production at a large-scale fishmeal plant. (Source: Bimbo, 2013)

1.2.2 Unsaturated fatty acids and omega-3 polyunsaturated fatty acids

Unsaturated fatty acids have one or more allyl groups. Due to allyl groups, they are readily oxidized to hydroperoxides, such as oleic, linoleic and linolenic acids (Carolina, 2014). After a series of reaction, a large number of degradation compounds generate. Therefore, unsaturated acyl lipids are considered as unstable food constituents that prone to be rancid during usual food storage (Geiser, 1991). There is a table below showing common unsaturated fatty acids and their sources.

Table 1.2 Unsaturated fatty acids.

Common Name	Lipid Numbers	Structural Formula	Naturally Occurring in
α-Linolenic acid	C18:3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	Flaxseeds, chiaseeds, walnuts
Stearidonic acid	C18:4	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_4COOH$	Seed oils of hemp, blackcurrant, corn gromwell
Eicosapentaenoic acid	C20:5	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	cod liver, herring, mackerel, salmon, menhaden and sardine
Docosahexaenoic acid	C22:6	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$	maternal milk, fish oil
Linoleic acid	C18:2	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	Peanut oil, chicken fat, olive oil
γ-Linolenic acid	C18:3	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_4COOH$	borage oil, black currant oil, evening primrose oil and safflower oil

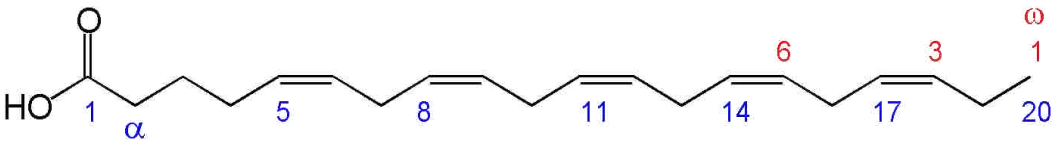
Table 1.2 continued

Dihomo-γ-linolenic acid	C20:3	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_6COOH$	only in trace amounts in animal products
Arachidonic acid	C20:4	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	meat, eggs, dairy
Docosatetraenoic acid	C22:4	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_5COOH$	-
Palmitoleic acid	C16:1	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$	Macadamia nuts
Vaccenic acid	C18:1	$CH_3(CH_2)_5CH=CH(CH_2)_9COOH$	dairy products such as milk, butter, and yogurt
Paullinic acid	C20:1	$CH_3(CH_2)_5CH=CH(CH_2)_{11}COOH$	guarana
Oleic acid	C18:1	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	olive oil, pecan oil, canola oil
Elaidic acid	C18:1	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	hydrogenated vegetable oil
Gondoic acid	C20:1	$CH_3(CH_2)_7CH=CH(CH_2)_9COOH$	jojoba oil
Erucic acid	C22:1	$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$	-
Nervonic acid	C24:1	$CH_3(CH_2)_7CH=CH(CH_2)_{13}COOH$	King Salmon, flaxseed, Sockeye salmon, sesame seed, macademia nuts
Mead acid	C20:3	$CH_3(CH_2)_7CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$	-

(Source: Guesnet, 2011; Beltran et al., 2004; Horrobin, 1990; Huang, 1996; Avato, 2003; Villarreal, 2007)

Fish oils contain long-chain polyunsaturated fatty acids, which are known as omega-3 fatty acids. “Polyunsaturated” indicates that more than one double bond existing between carbon atoms. “Omega-3” (also called ω-3 or n-3) refers to the location of the first double bond, which is considered as the "tail" of the carbon chain, in contrast, the carboxylic acid (-COOH) end, which is considered as the beginning of the chain, thus "alpha" (Moghadasian, 2008). Therefore, Omega-3 fatty acids contain the first double bond at the third position from the side of methyl carbon. Then another category of long-chain polyunsaturated fatty acids, omega-6 fatty acids (n-6), have the first double bond occurs at the sixth position in the similar way. In a variety of fatty acids of fish oils, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are two typical n-3 fatty acids rich in fatty fish (J. Moreillon et al. 2013). These two polyunsaturated acids have either 5 or 6 double bonds in a carbon chain of 20 or 22 carbon atoms, respectively (Vitek et al. 2012). Both of them exist a strong positive effect on human health (Elmira, 2012).

EPA (Eicosapentaenoic acid):



DHA (Docosahexaenoic acid)

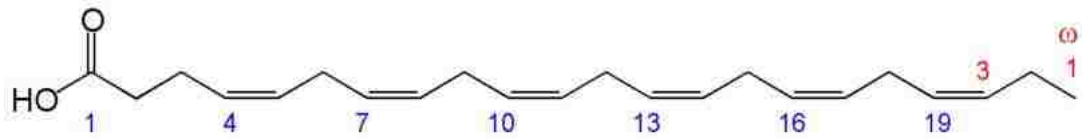


Fig 1.5 Chemical Structures of EPA and DHA

(Source: Vitek, 2012)

1.2.3 Health effects of PUFAs

The high content of long-chain polyunsaturated fatty acids (LC-PUFAs) in sea food products has high health value. Fish oil contain omega-3 fatty acids, especially long-chain docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3) (Kolakowska et al., 2006). Both of two fatty acids have evident health benefits. DHA is an essential source for brain and retina development both in fetus and infants (Kolanowski et al., 2007). Phospholipids containing DHA can be used to build structure of neuronal membranes (Green et al., 1999). Animal and human studies have shown that n-3 PUFA deficiency reduces DHA concentration in brain, and is associated with poorer development of visual acuity and neural development. Moreover, it will lead to a variety of central nervous system disorders, including Alzheimer's disease and psychiatric illnesses, such as schizophrenia and depression and mood disorders (Assisi, 2006). Additionally, recent epidemiological studies (Larrieu et al., 2004) reported that high consumption of fish shows inverse relationship with cognitive decline, and development of dementia or Alzheimer's disease. The beneficial effects of DHA are also associated with fatal cardiovascular disease (Brouwer, 2008). With intake of DHA, cardiovascular diseases (CVD) may be prevented (Kris et al., 2002) and the symptoms in rheumatoid arthritis are relieved. Low consumption of DHA nonpharmacologically protects human body from platelet-related cardiovascular events (Guillot et al., 2009). EPA may have function on both cardiovascular prevention and immunological health (Ryan et al., 2010). It was recently reported that in Japan EPA Lipid Intervention Study (JELIS) patients who were randomly consumed 1800 mg of EPA daily (with statin) had a 19% relative decrease in major coronary events compared with those only receiving the statin. EPA may

have benefits in preventing major coronary events (Kris et al., 2009). A study showed that EPA inhibit against the inflammatory symptoms of edema, erythema, and blood flow (Khan et al., 2005). Additionally, intervention studies suggested that cognitive function is improved after the consumption of EPA for 6 months to Alzheimer-type and cerebrovascular dementia patients (Otsuka and Ueki, 2001).

1.3 Antioxidants

1.3.1 Introduction

Antioxidants are compounds that prolong the shelf life of foods by protecting them against deterioration caused by oxidation, such as fat rancidity and color changes (Karovicova and Simko, 2000). Antioxidant technology plays an important role in utilization of fats and oils as raw materials in food processing and food markets. Thus, the proper and effective use of antioxidants is dependent on a basic understanding of the chemistry characteristics of fats, mechanism of oxidation, and function of an antioxidant in counteracting different type of deterioration (Struckey, 1972). By interfering with the formation of the free radicals that initiate and propagate oxidation, antioxidants for fats and oils can break the free radicals reaction chain. Antioxidants have to form stable and low-energy free radicals that will not further propagate the oxidation of fats and oils. From this point of view, the most convenient compounds are the phenolic compounds whose structure allows them to form low-energy radicals through stable resonance hybrids. At present, synthetic phenolic antioxidants are being used as food additives. Due to their possible toxicity, the majority of countries have regulations for controlling the use of antioxidants in food applications. These regulations identify specific approved antioxidants, establish permitted use levels and require labelling.

However, antioxidant permitted in one country may be prohibited in another country. Commonly used antioxidants in food processing are usually classified into two groups - natural antioxidants and synthetic antioxidants (Karovicova and Simko, 2000).

1.3.2 Synthetic antioxidants

Synthetic antioxidants are obtained through chemical synthesis and purification. The advantages of synthetic antioxidants are inexpensive cost, wide range of application and medium to high antioxidant activity. These pure compounds are added to withstand various treatments and conditions as well as to prolong shelf life of the foods (Jolana and Peter, 2000). Typical types of synthetic antioxidant are listed in Table 2 below. Today, almost all processed foods have synthetic antioxidants incorporated.

Table 1.3 Chemical structure and applications of the most important synthetic antioxidants.

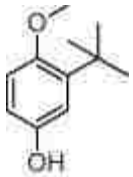
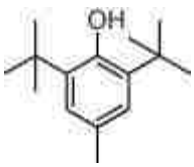
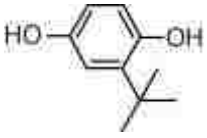
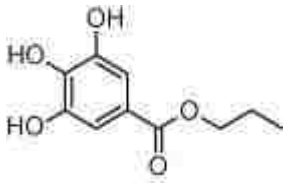
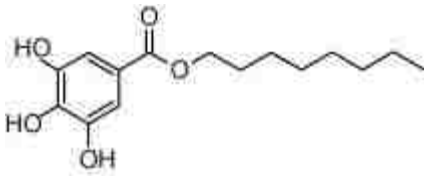
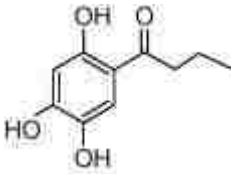
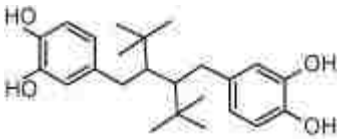
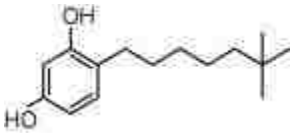
Compound name	Chemical Structure	Applications	Reference
BHA (butylated hydroxyanisole)		Food antioxidants	Branen (1975)
BHT (butylated hydroxytoluene)			Botterweck et al. (2000) Aguillar et al. (2011) Aguillar et al. (2012)
TBHQ (*tert*-butylhydroquinone)		Animal processed food antioxidant	Gharavi and Kadi (2005)

Table 1.3 continued

PG (propyl gallate)		Food antioxidants	Soares et al. (2003)
OG (octyl gallate)		Food and cosmetic antioxidant, antifungal properties	Kubo et al. (2001
2,4,5-Trihydroxy butyrophenone		Food antioxidant	Astill et al. (1959)
NDGA (nordihydroguaiaretic acid)		Food antioxidant	Evan and Gardner (1979)
4-Hexylresorcinol		Prevention of food browning	Chen et al. (2004)

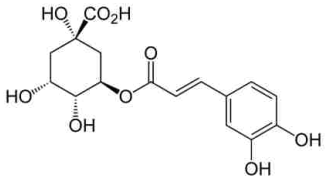
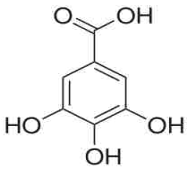
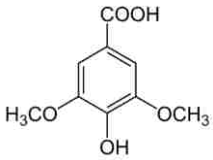
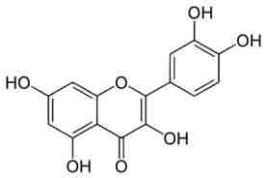
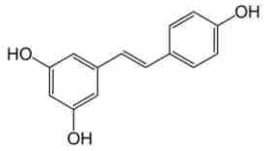
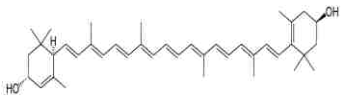
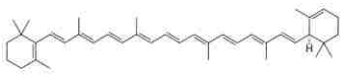
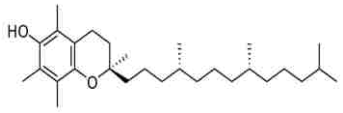
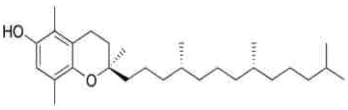
BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) are the most widely used chemical antioxidants. Between 2011 and 2012, the European food safety authority (EFSA) reevaluated all the available information on these two antioxidants, including those contradictory data that have been published. The EFSA established revised acceptable daily intakes (ADIs) of 0.25 mg/kg bw/day for BHT and 1.0 mg/kg bw/day for BHA and noted that adults and children were unlikely to exceed these intakes (EFSA, 2011 and EFSA, 2012).

TBHQ (tert-Butylhydroquinone) stabilizes and preserves the freshness, nutritive value, flavour and color of animal food products. In 2004 the EFSA published a scientific review entitled by the impact of this antioxidant on human health and stated that there was no scientific proof of its carcinogenicity despite previous data conflicting. Octyl gallate is considered as a safe food additive because it can be hydrolyzed into gallic acid and octanol, which are found in many plants and do not pose a threat to human health (Joung et al., 2004). NDGA (Nordihydroguaiaretic acid) is known to cause renal cystic disease in rodents despite being a food antioxidant (Evan and Gardner, 1979).

1.3.3 Natural antioxidants

In the recent years, natural antioxidants has increasingly been demanded due to the growing concern consumers about potential toxicological effects of synthetic antioxidants (Juntachote et al., 2006; Naveena et al., 2008; Gonzalez et al., 2008). Plants are the generous source to supply man with valuable bioactive substances (Tayel and Tras, 2012). Different plant products are being evaluated as natural antioxidants to preserve and improve the overall quality of meat and meat products. These natural antioxidants from plants, in the form of extracts, have been obtained from different sources such as fruits (grapes, pomegranate, date, kinnow), vegetables, (broccoli, potato, drumstick, pumpkin, curry, nettle), herbs and spices (tea, rosemary, oregano, cinnamon, sage, thyme, mint, ginger, clove) and investigated to decrease the lipid oxidation. There is a list showing several natural antioxidants found in common plants.

Table 1.4 Natural Antioxidants in Common Plants.

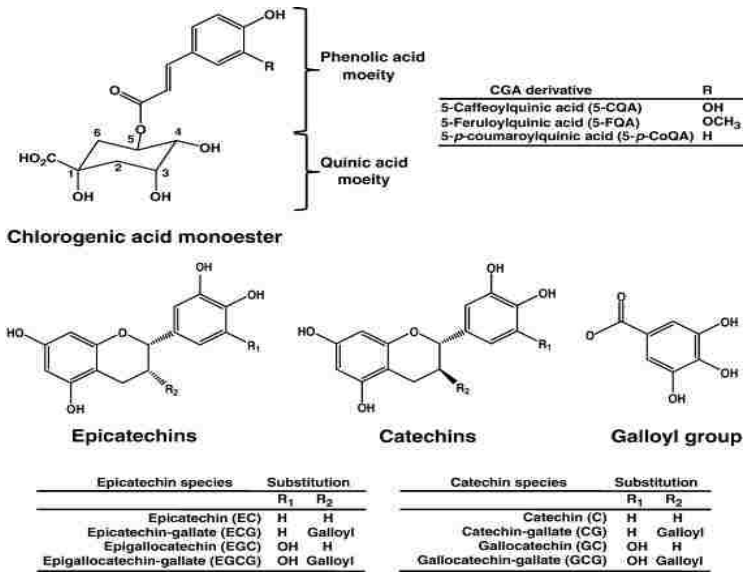
Classification	Compound name	Chemical Structure	Natural Sources
Phenolic acids and their esters	Chlorogenic acid		Peach, prunes (Cheng and Crisost, 1995)
	Gallic acid		Gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants (Reynolds and Wilson, 1991)
	Syringic acid		Palm oil, vinegar
Flavonoids	Quercetin		Broccoli, apples, plums, blueberry (USDA Database, 2011)
	Resveratrol		Red grapes, red wine (Baur and Sinclair, 2006)
Carotenoid terpenoids	Lutein		carrots, winter squash, tomatoes, green beans, cilantro, Swiss chard
	α-Carotene		
Tocopherol	α-tocopherol		Wheat germ oil, sunflower, and safflower oils (Reboul et al., 2006)
	β-tocopherol		

1.3.4 Antioxidant-rich drinks

Coffee, one of the most common beverages in world market, is rich in bioactive phytochemicals including methylxanthines, amino acids, phenolic acids and polyphenols (Fig. 1). Caffeine is the primary methylxantine in coffee, and is well known for its stimulatory and metabolic effects (Acheson et al., 1980). Additionally, the natural abundance of phenolic and polyphenolic constituents in coffee has drawn attention of researchers. It has been reported that their biological activities including: antioxidant activities (Frei and Higdon, 2003), stimulation of vasodilation (Lorenz et al., 2009), regulation of xenobiotic-metabolizing enzymes, modulation of glucose absorption and utilization (Arion et al., 1997). Most recent epidemiological studies indicated that proper daily consumption of coffee have contributed to positive effect on human health, particularly associated with its polyphenolic content - chlorogenic acids (Li et al., 2007). In fact, coffee intake may improve glucose metabolism and insulin sensitivity, therefore lead to reducing the risk of type 2 diabetes, coronary heart disease, ischemic stroke, depression, Alzheimer's and other central nervous system diseases (Huxley et al., 2009). Moreover, coffee consumption has shown inverse relation with the death caused by heart disease and respiratory disease and stroke (Freedman et al., 2011).

Green tea is an evergreen plant that grows primarily in temperate climate regions of Asia which mainly include China, India, Sri Lanka and Japan. Green tea extract is a great source of flavonoids with the status of food additive and showed potential benefits on human health as antiviral, antiallergic, anti-inflammatory, antitumor effects due to antioxidant activities (Lambert et al., 2010; Rietveld and Wiseman, 2003). The main compounds responsible for this antioxidant activity are gallic acid and eight major catechins: catechin, epicatechin,

catechin gallate, epicatechin gallate, gallocatechin, epigallocatechin, gallocatechin gallate, epigallocatechin gallate (Poon, 1998 and Zeeb et al., 2000). Green tea has been utilized in food with optimum concentrations to extend its shelf life and protect food without changing sensorial properties (Diana et al., 2008). In addition, it has been reported that green tea is a potential source of antioxidants to be added in plastic for protection during polymer processing/manufacturing and also incorporated in polymers for developing of antioxidant active packaging (Dicastillo et al., 2012). Additionally, it is also a widely consumed beverage and shows preventive effects against chronic diseases including cancer. Green tea polyphenols are thought to be responsible for cancer preventive effect and modulation of redox activity. Various laboratories data have suggested that green tea polyphenols act as direct antioxidants by scavenging reactive oxygen species or chelating transition metals (Carol et al., 2013).



CGA derivative	R
5-Caffeoylquinic acid (5-CQA)	OH
5-Feruloylquinic acid (5-FQA)	OCH_3
5-*p*-coumaroylquinic acid (5-*p*-CoQA)	H

Epicatechin species	Substitution	
	R_1	R_2
Epicatechin (EC)	H	H
Epicatechin-gallate (ECG)	H	Galloyl
Epigallocatechin (EGC)	OH	H
Epigallocatechin-gallate (EGCG)	OH	Galloyl

Catechin species	Substitution	
	R_1	R_2
Catechin (C)	H	H
Catechin-gallate (CG)	H	Galloyl
Gallocatechin (GC)	OH	H
Gallocatechin-gallate (GCG)	OH	Galloyl

Fig 1.6 Structure of chlorogenic acid and catechin derivatives abundant in coffee and tea respectively. (Source: Ferruzzi, 2010)

Wine is an alcoholic beverage that contains various polyphenols extracted from grapes during the vinification (Rastija and Šarić, 2009a). The polyphenolic compounds are found in a large quantity in red wines, including flavonoids, anthocyanins and tannins. They were believed to have antioxidant activity in protecting the body cells against oxidation. Moreover, polyphenolic compounds influence their astringency, bitterness and color in red wines. The viticulture practices, different enological techniques, the varieties, the harvesting year of grapes and the wineries are all influence factors of the polyphenolic composition of wines (Cliff, King, and Schlosser, 2007). Polyphenolic substances in wines are usually subdivided into two groups: flavonoids and nonflavonoids. Flavonoids in red wines contain anthocyanins, catechins, and flavonols. The main flavonols are myricetin, quercetin, kaempferol, syringetin and laricitrin (Mattivi et al., 2006). Anthocyanins are the main phenolic compounds responsible for the color of red wines and act as antioxidants (Rossetto et al., 2004). Non-flavonoid compounds in red wine are mainly phenolic acids, such as various benzoic and cinnamic acid derivatives. They have functional effects on defining the sensorial characteristics of wines, and present typically long-aged "oak wood" taste products. Phenolic acids are also largely associated with the astringency and bitterness in young wines (Monagas et al., 2005). Hydroxycinnamic acids and their tartaric esters are made up of the main class of non-flavonoid phenolics in red wines and involved in the browning reactions. They are also precursors of volatile phenolic compounds (Vrhovsek, 1998). Gallic acid is the main hydroxybenzoic acid in red wine, based on the hydrolysis of flavonoid gallate esters. Wines matured in oak generate high levels of hydroxybenzoic acid derivatives, such as ellagic acid. It is observed that a diet rich in polyphenolic compounds may positively influence health due

to properties of antioxidant. In general, wine is rich in antioxidant compounds which have a functional role on acting against free radicals (Renauld and Lorgeril, 1992). Also, polyphenolic compounds in wine are considered to be associated with the beneficial health effects, due to the protection on the cardiovascular system against the effect of the free radicals produced by human aerobic metabolism (Virgili and Contestabile, 2000; Halpern et al., 1998). Epidemiological evidence indicated that the moderate consumption of wines reduced the incidence of coronary heart disease (CHD), atherosclerosis and platelet aggregation (Tedesco et al., 2000).

CHAPTER 2
FREE RADICAL SCAVENGING ACTIVITY, PHENOLIC CONTENT AND ANTI-LIPID-OXIDATION OF DIFFERENT PHENOL ANTIOXIDANTS

2.1 Introduction

Lipid oxidation is initiated with active free radicals under oxidative stress (Carocho and Ferreira, 2013). The free radicals could be internally generated by normal metabolism of a biological system or by external factors such as smoking, pollution, and radiation. They result in the homeostasis of lipid metabolism and acceleration of lipid oxidation in the human body (Patil et al. 2010). Lipid oxidation has been long considered as a major problem in the storage of fatty food products. Oxidative changes can result in repugnant flavors, nutrition substances damage and toxic compounds generation (Kanner et al., 1992). Antioxidants are compounds that prolong the shelf life of foods by protecting them against deterioration caused by oxidation. They are usually classified into two groups - natural antioxidants and synthetic antioxidants. Natural phenolics are reported to be capable of scavenging the free radical and stabilizing normal lipid metabolism (Xu, 2012). The phenolics are a group of phytochemicals and biosynthesized as the secondary metabolites from all kinds of plants. For example, vitamin E as a group of tocopherols and tocotrienols, is lipophilic antioxidants and rich in cereals such as rice bran, nuts, or corns (Xu, 2012). Other common phenolics such as quercetin (QCT), chlorogenic acid (CA), gallic acid (GA) or syringic acid (SA) are widely found in a variety of fruits and vegetables including apples, peppers, and culinary herbs etc. (Xu, 2012). These phenol-based antioxidants are reported to be effective modulators of redox homeostasis under oxidative stress and reduce the risks of the diseases involved by lipid oxidation (Robards et al., 1999). Therefore, by retarding the lipid oxidation,

antioxidants play an important role on promoting health status and preventing the development of chronic diseases in human health. Compared with the natural antioxidants, synthetic phenol antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG) are well known as food preservers or antioxidants and have been widely used in food industry for extending the shelf-life of various food products (Chao et al., 2009). However, the performances of the synthetic and natural antioxidants in different antioxidant assays have not been fully explored and compared.

Total phenolic content (TPC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays are the traditional assays of evaluating the antioxidant activity of food products. The two in vitro assays can evaluate implicit correlation with the health promoting antioxidant potential in most antioxidant studies. However, the unilateral evaluation by those single chemical-based spectrophotometric assays may not totally reflect the real antioxidant performance in a lipid-related environment, especially in human serum. In fact, the mechanism of lipid oxidation involved in a biological system is complicated. In this study, a fish oil emulsion model was applied to simulate the human serum and examine the actual anti-lipid-oxidation capability of the selected antioxidants and drinks. The results in this study could demonstrate the relationship of the performances obtained from different antioxidant assays. However, the conventional phenolic content, scavenging DPPH free radical assays may not be correlated to the actual antioxidant capability of protecting lipid oxidation in an emulsion model associated with the anti-lipid-oxidation activity in human serum or body. Therefore, the results could provide useful information for understanding the difference between

phenolic content, scavenging DPPH free radical activity, and real anti-lipid-oxidation or health promoting capability in an emulsion.

2.2 Method

2.2.1 Chemicals and materials

Hexane and methanol (HPLC grade) was from Fisher Chemicals (Fair Lawn, NJ, USA). Sodium bicarbonate was obtained from Mallinckrodt Co. (Paris, KY, USA). Synthetic antioxidant standards Trolox, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butylhydroquinone (TBHQ) and natural antioxidant standards, including catechin, resveratrol (RSV), α-tocopherol (α-T), quercetin (QCT), gallic acid (GA), syringic acid (SA) and chlorogenic acid (CA), as well as Folin-Ciocalteu reagent, 2,2-diphenyl-l-picrylhydrazyl (DPPH), Tween 20, menhaden fish oil, boron trichloride in methanol (BCl_3-methanol), heptadecanoic acid (C17:0), and eicosapentaenoic acid (EPA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 Determination of phenolic content and scavenging DPPH free radical activity

In this study, the antioxidant standards Trolox, BHA, BHT, TBHQ, PG, RSV, α-T, QCT, GA, CA, SA and catechin were prepared as the stock solutions of 1 mmol/L concentration, respectively. Then, they were diluted to 200 μmol/L as test solutions. Phenolic content was evaluated by the Folin-Ciocalteu method reported in the study of Jang and Xu with minor modification (Jang and Xu, 2009). Each test solution (100 μL) was mixed with 0.75 mL of ten-fold diluted Folin-Ciocalteu reagent and 0.75 mL of sodium bicarbonate (60 g/L). The

mixture was incubated at 25℃ for 90 min, and then its absorbance was measured by an UV-Visible spectrophotometer at 750 nm. Finally, the result was unified into μmol of catechin equivalent/L according to the standard curve of catechin.

Scavenging DPPH free radical activity was determined by the method described in the study of Yue and Xu (Yue and Xu, 2008). An aliquot of 0.2 mL of the test solution was mixed with 1.8 mL of DPPH solution (0.1 mmol/L) in a spectrophotometer cuvette for 30 min at 25℃ in the dark. The absorbance was measured at 0 and 30 min under wavelength 517 nm, respectively. Then, the difference of the absorbance was calculated and then converted to μmol of Trolox equivalent/L based on the standard curve of Trolox. Additionally, EC50 value was calculated via the scavenging DPPH free radical percentages of each antioxidant at a series of concentrations (1-1000 μmol/L).

Fig 2.1 UV Spectrophotometer

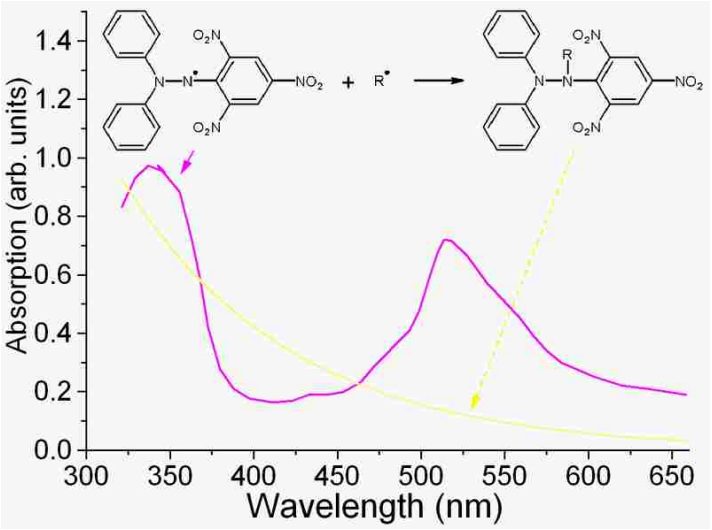


Fig 2.2 Absorbance wavelength of DPPH

(Source: Cowie and Valeria, 2008)

Scavenging DPPH free radical percentage (%) = $(1 - Abs_{sample}/Abs_{blank}) \times 100$

where Abs_{sample} was the absorbance difference at 0 and 30 min; Abs_{blank} was the absorbance difference at 0 and 30 min of the blank in which the same volume of methanol replaced the test solution. EC_{50} value of each individual antioxidant was calculated by semilog regression analysis in SAS program (SAS, 9.1.3, Cary, NY, US).

2.2.3 Determination of anti-lipid-oxidation capability in the fish oil emulsion

The fish oil emulsion is made up of 1 % of menhaden fish oil and 0.1 % of Tween 20 in phosphate buffer (pH 7.0) based on the procedure in the study of Zhang et al. (2013). After the emulsion was homogenized by a microfluidizer materials processor (M-110P, Microfluidics, Newton, MA, USA), 1 mL of each antioxidant stock solution (1 mmol/L) was added into 19 mL of the fish oil emulsion in a 40-mL vial. One milliliter of distilled water rather than the antioxidant solution was used as a blank control group. All the vials were incubated and continuously agitated by a multiple magnet stirrer in a 37℃ water bath

(Multistirrer, VELP Company, Italy) for 72 h. The determination of EPA in the fish oil emulsion was operated at the 0 and 72 h, respectively. Two milliliters of C17:0 (100 μg/mL) in hexane was added into 2 mL of the emulsion from each vial. After centrifugation and evaporation, the upper layer was collected. And the dried residue was reacted with 2 mL of BCl_3 reagent at 60 ℃ for 30 min. The EPA derivative was extracted by 1 mL of hexane and transferred to a GC vial after being cooled down in an ice bath for 10 min. The GC condition in EPA analysis was the same as that in the study of Zhang et al. (2013). The anti-lipid-oxidation capability was expressed by the percentage of retained EPA in the fish oil emulsion after 72 h incubation. It was calculated by the formula below:

$$\text{Anti-lipid-oxidation capability (\%)} = (C_t / C_0) \times 100$$

where C_0 was the level of EPA at 0 h; C_t was the level of EPA at 72 h in the same emulsion sample.

Figure 2.3 Fish oil emulsion mode with 37℃ water bath

2.2.4 Data analysis

The phenolic content, scavenging DPPH free radical activity, EC_{50} value, and anti-lipid-oxidation capability in fish oil emulsion were made in triplicates and expressed as

mean and standard deviation. The calculations were performed by Microsoft Excel (Redmond, WA, US). The significant difference between the mean values of two groups was analyzed by using one-way ANOVA at P value < 0.05(SAS, 9.1.3, Cary, NY, US).

2.3 Results

2.3.1 The phenolic contents, scavenging DPPH free radical activities, and EC_{50} values

QCT showed the greatest performance in the phenolic content assay by phenolic content of 401.75 ±10.44 μmol of catechin equiv/L, which was followed by CA (238.61 ±16.73), GA (234.98 ±16.91), PG (208.61 ±1.91), RSV (178.71 ±9.72), and SA (173.71 ±9.96) (Figure 2.4). BHT, α-T, TBHQ, BHA and Trolox had comparatively lower phenolic content (μmol of catechin equiv/L) in a range of 99.20 ±2.67 for BHT to 52.43 ±3.24 for Trolox. Except for α-T, the phenolic content of the naturally existing antioxidants were all above 170 μmol of catechin equiv/L, while it was below 100 μmol catechin equiv/L for the synthetic antioxidants except for PG (Figure 2.4).

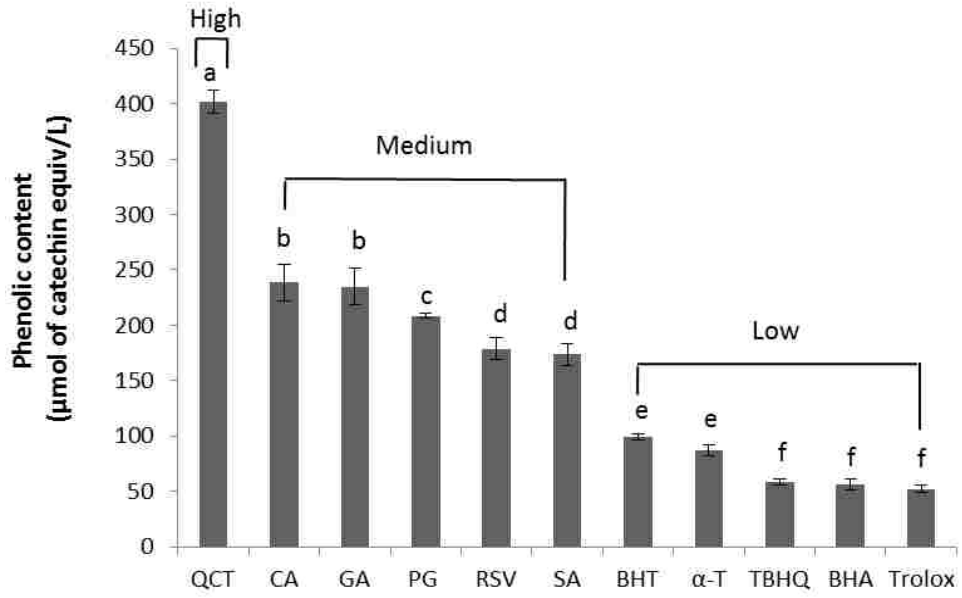


Figure 2.4 Phenolic contents of the eleven individual antioxidants

In the DPPH assay, the high performance group contained QCT (275.09 ± 1.20), PG (274.18 ± 5.24) and GA (270.55 ± 3.15 μmol of Trolox equiv/L) (Figure 2.5). The scavenging DPPH activities of CA, α-T and Trolox were at a similar level of 202.52 ± 0.45, 197.97 ± 4.67, and 197.06 ± 5.68 μmol of Trolox equiv/L, respectively (Figure 2). They were followed by TBHQ (149.64 ± 3.88) and SA (120.39 ± 8.26 μmol of Trolox equiv/L), while the activities of BHA, RSV and BHT were below 100 μmol of Trolox equiv/L (Figure 2.5).

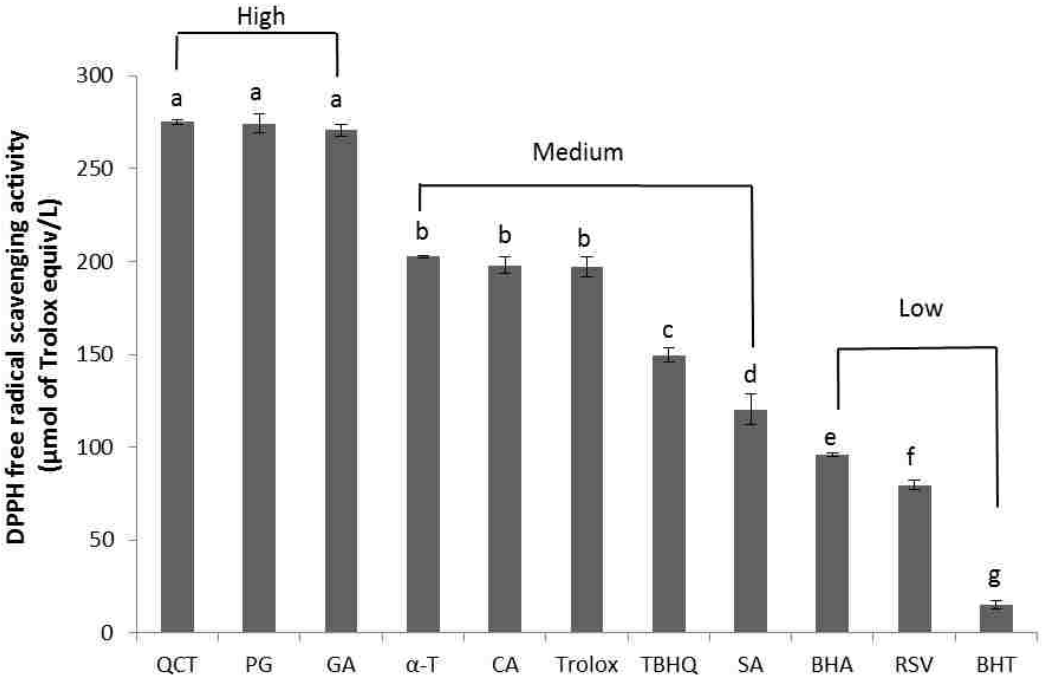


Figure 2.5 DPPH free radical scavenging activities of the eleven individual antioxidants

Also, the percentages of scavenging DPPH free radical activity of GA, PG, and QCT increased in a fast rate at the concentration lower than 100 μmol/L (Figure 2.6). They were followed by CA, α-T, Trolox, and TBHQ which had the maximum free radical scavenging percentages at a concentration of approximately 500 μmol/L (Figure 2.6).

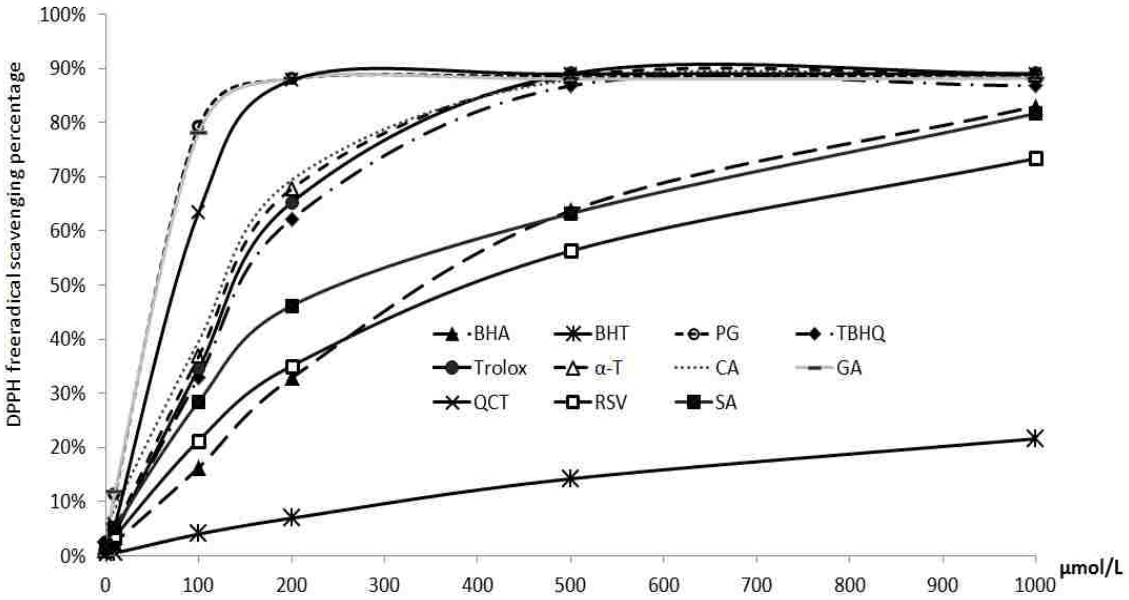
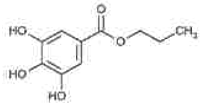
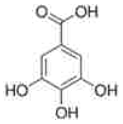
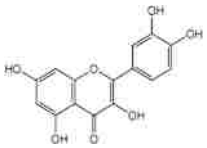
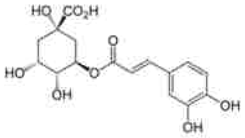
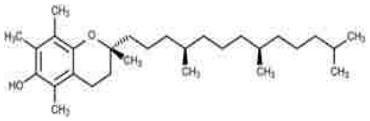
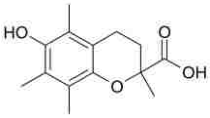
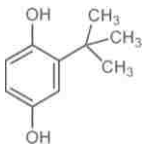
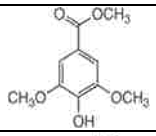
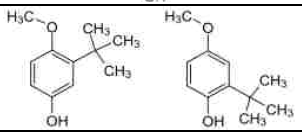
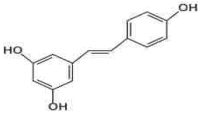
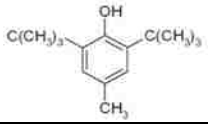


Figure 2.6 The changes of DPPH free radical scavenging percentages with different concentrations of the eleven individual antioxidants

Since DPPH free radical scavenging is a dynamic process, a portfolio of concentrations were used to evaluate the DPPH free radical scavenging capability. And EC50 value was used to reflect the capability. Basically, EC_{50} is defined as the capability of an antioxidant and necessary concentration to reach 50% of scavenging DPPH free radical activity (Chen, Bertin et al. 2013). In other word, the small EC50 value means great free radical scavenging capability while large EC50 value means relatively low scavenging capability. Therefore, the order of EC_{50} of scavenging DPPH free radical from low to high was PG, GA, QCT, CA, α-T, Trolox, TBHQ, SA, BHA, RSV and BHT (Table 2.1). PG and GA had smaller EC50 value than other antioxidants, which means they have greater capability than others. BHT had not reached EC_{50} even at a level of 1000 µmol/L, so it has a very low scavenging DPPH free radical capability compared to other antioxidants.

Table 2.1 Chemical structures and EC_{50} values of the twelve individual antioxidants in DPPH assay

Name	**Chemical structure**	**EC_{50} (μmol/L)**
PG		35.48 ± 2.78
GA		36.31 ± 1.97
QCT		54.95 ± 2.67
CA		114.82 ± 5.10
α-T		120.23 ± 8.14
Trolox		128.28 ± 6.33
TBHQ		151.36 ± 7.54
SA		257.04 ± 2.28
BHA		316.22 ± 15.76
RSV		354.81 ± 21.35
BHT		>1000

2.3.2 Anti-lipid-oxidation capabilities in the fish oil emulsion

Generally, the unsaturated fatty acids in fish oil, especially EPA and DHA, are vulnerable and easily oxidized at a similar rate under oxidative stress (Zhang et al., 2013). Thus, the retention rate of EPA in the emulsion could reflect the oxidation status of fish oil at some extent. The higher retention rate of EPA indicates the greater anti-lipid-oxidation capability of the emulsion. It was found in this study that the emulsion mixed with BHA, PG and α-T were top three groups with the highest retention rate of EPA or anti-lipid-oxidation capabilities which were 66.78 ± 3.07, 66.16 ± 1.55 and 62.81 ± 2.33 %, respectively. They were followed by TBHQ (53.36 ± 2.06 %), QCT (50.23 ± 8.26 %), RSV (49.43 ± 6.59 %), Trolox (36.45 ± 3.91 %) and SA (27.72 ± 3.07 %) (Figure 2.7). However, the emulsions mixed with BHT, GA or CA showed very low anti-lipid oxidation capability and retained less than 15 % of EPA (Figure 2.7).

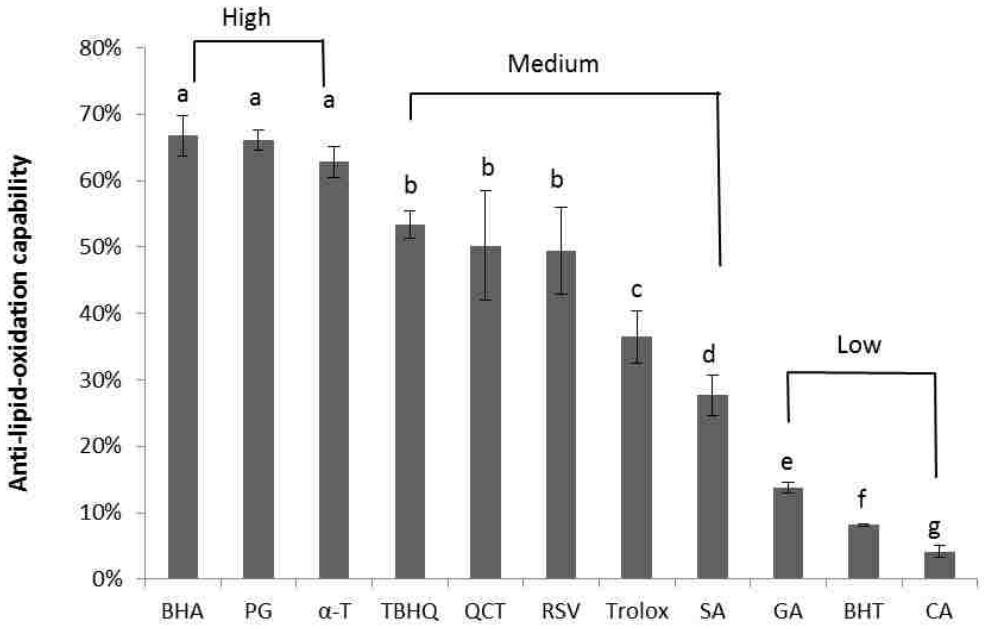


Figure 2.7 Anti-lipid-oxidation capabilities of the eleven individual antioxidants

2.4 Discussion

The phenolic content assay is based on the degree of phenolics in reducing Folin-Ciocalteu reagent (phosphotungstic and phosphomolybdic acids mixture) to produce blue color oxides of tungstene and molybdene under the alkaline condition (Azlim Almey, et al., 2010). The reducing degree mainly depends on the reducing power from the hydroxyl group linked with benzene ring in the antioxidant molecular structure. For example, QCT contains five of the hydroxyl groups in positions 3, 3′, 4′, 5, 7 respectively, and a carbonyl group in fourth position (Table 2.1). Thus, QCT molecule has stronger activity in reducing Folin-Ciocalteu reagent (Hollman, van Trijp et al. 1997). In addition, the multiple hydroxyl groups of CA, GA, PG and RSV were associated with their relatively high performance in the phenolic content assay (Table 2.1, Figure 2.4). Since the reaction media of phenolic content assay is aqueous, the lipophilic antioxidant, such as α-T may have limited access to the Folin-Ciocalteu reagent and then carry out the reduction, although there are multiple hydroxyl groups on its structure. Similarly, the activity of scavenging free radicals also depends on the degree of hydroxylation of phenol since the active hydroxyl group could contribute hydrogen to annihilate free radicals. In the DPPH assay, the annihilation causes the color change of the DPPH solution which is further measured by a spectrophotometer (Cieśla, Kryszeń et al. 2012). With the highest phenolic content, QCT also dominated in the scavenging free radical activity of DPPH solution among the eleven selected individual antioxidants. Additionally, PG and GA with the multiple active hydroxyl groups exhibited relatively high activity in scavenging DPPH free radicals (Figure 2.5). However, the anti-radical ability of each antioxidant was not linearly correlated to their concentrations (Figure 2.6). For example, the activity of PG

or GA increased faster than QCT with the increasing concentration below 200 μmol/L, but there was no significant difference after their concentrations were over 200 μmol/L (Figure 2.6). Although the activity of BHA has increased at a lower rate than SA or RSV at concentrations below 200 μmol/L, it exceeded them after the concentration was higher than 300 and 500 μmol/L, respectively. The orders of EC_{50} values GA < α-T < BHT and QCT < CA in this study were also reported by Mishra, Ojha et al. (2012) and Chen et al. (2013), respectively (Table 2.1).

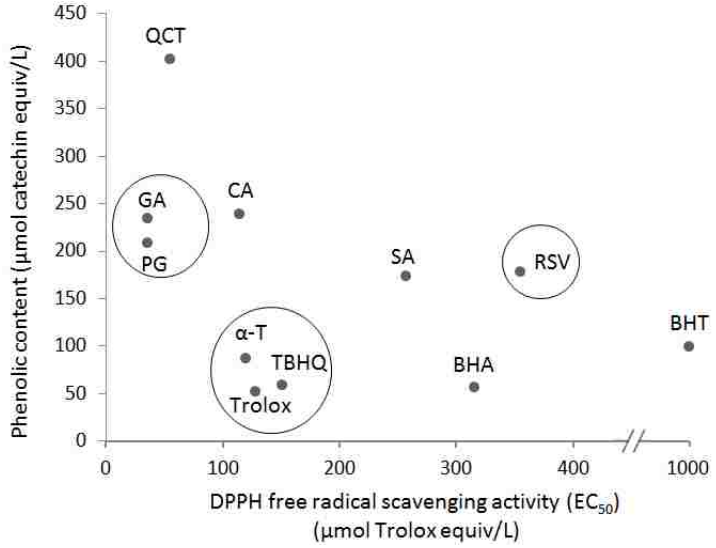
Although both phenolic content and DPPH were commonly used assays, the antioxidant potential revealed from the two methods could not build up necessarily positive correlation to each other. Since DPPH free radicals annihilation is a dynamic procedure varied by different concentrations, a specific concentration cannot really reflect the actual antiradical capability of a certain antioxidant. Thus, EC_{50} value of DPPH scavenging activity of each individual antioxidant was used to compare with their respective phenolic content and anti-lipid-oxidation capability. In this study, QCT has highest phenolic content assay and could also effectively scavenge DPPH free radical (Figure 2.8a). The DPPH scavenging free radical capabilities of PG and GA was slightly higher than QCT, however, the phenolic content of PG or GA was approximately 50 % lower than QCT (Figure 2.8a). Furthermore, although the DPPH annihilation ability was close in Trolox, α-T or CA, the phenolic content of CA was about four times higher than Trolox and α-T. On the other hand, there was no significant difference between their phenolic contents, but TBHQ had a better performance in scavenging DPPH free radical than BHA. Although it was reported that phenolic content was not highly correlated with DPPH radical scavenging ability, the cause of poor correlation

was the different responses of various antioxidants profile in different plant species or the color interference in the phenolic content and DPPH assays (Liu, Ardo et al. 2007). In this study, the interference of sample impurities or the various performance of phenolic complex in the reaction was excluded since individual antioxidant standard was used. Thus, the possible causes of the lack of evident linear relationship of phenolic content and DPPH free radical scavenging activity were the different mechanisms, steric accessibility or ionic strength of the reagents and test samples involved in the two assays (Apak et al., 2013).

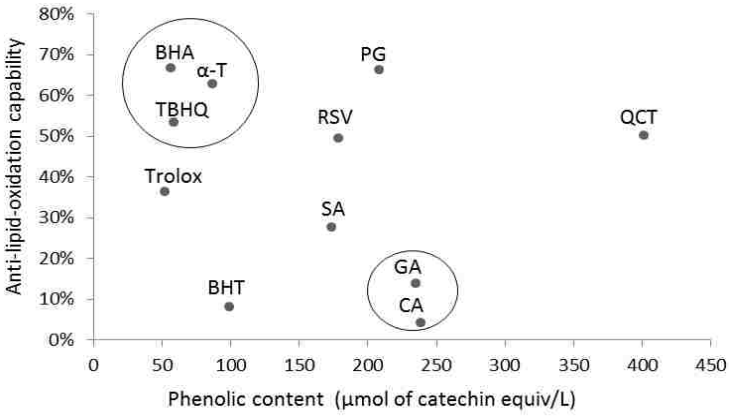
Compared with the selected natural antioxidants, most synthetic antioxidants had the lower phenolic contents but more effective in inhibition of lipid oxidation. For example, QCT with the highest phenolic content subjected to BHA which has the low phenolic content in inhibiting lipid oxidation in the fish oil emulsion (Figure 2.8b). Also, phenolic content of BHA, Trolox and TBHQ were all below 60 μmol of catechin equiv/L, but their anti-lipid-oxidation capability were approximately five to ten times higher than GA and CA which phenolic contents were above 230 μmol of catechin equiv/L (Figure 2.8b). Similarly, the anti-lipid-oxidation capability of BHT was approximately eight times higher than that of α-T, though there was no significant difference of phenolic content between these two antioxidants (Figure 2.8b). The great difference of the performance of α-T was due to the amphiphilic system of fish oil emulsion, so α-T was able to align itself at the oil-water interface and efficiently stabilize lipid oxidation in the fish oil emulsion despite its low phenolic content (Maqsood and Benjakul 2010). Therefore, the phenolic content of each antioxidant was not in a necessary correlation with its corresponding anti-lipid-oxidation capability. In Figure 2.8c, PG had the best antioxidant performance in DPPH assay and fish

oil emulsion, while BHT was not effective in both evaluation assays. However, not all the individual antioxidants had an ideal correlation between DPPH annihilation ability and anti-lipid-oxidation capability. For example, one remarkable contrast is GA which played the top role in DPPH assay, but the anti-lipid-oxidation capability was lower than most selected individual standards (Figure 2.8c). Although CA had similar DPPH free radical scavenging activity with Trolox and α-T, its anti-lipid-oxidation capability was significantly lower than the other two groups (Figure 2.8c). Additionally, PG and BHA were in the group of high anti-lipid-oxidation capability, while, both QCT and RSV were classified as the group with medium anti-lipid-oxidation capability (Figure 2.7). However, the effectiveness of scavenging DPPH free radical in BHA was approximately ten times lower than that of PG and seven times lower in RSV than QCT. Therefore, the poor correlation between DPPH free radical annihilation ability and anti-lipid-oxidation capability in the fish oil emulsion may be the reason that the free radicals commonly used in conventional evaluation assays were synthesized stable free radicals, while, natural reactive oxygen species involved in the lipid oxidation would result in more complicated mechanism of antioxidant performance (López-Alarcón and Denicola, 2013). Both radical scavenging activity and reducing power are only based on unilateral mechanism for assessing the antioxidant capability of test samples. They cannot be used to predict the overall antioxidant potential since the substrates in biological system are faced with external oxygen pressure and also the natural free radicals involved in lipids were not taken into consideration (Chaiyasit, Elias et al. 2007). Few evaluation studies for individual natural or synthetic antioxidants have been carried out in lipid medium, while they only focused on the effect of the antioxidants on the oxidative

stability of pure oil such as walnut, flaxseed, or rice bran oil with the determination of oxidative stability index or the peroxide value (Mart ńez, 2013; Bera, 2006; Jennings and Akoh, 2009). In this study, a fish oil emulsion model consisting of complex lipid substrate was much similar to the real biological systems such as human blood or serum and reflected relatively comprehensive antioxidant activity of diverse antioxidants.



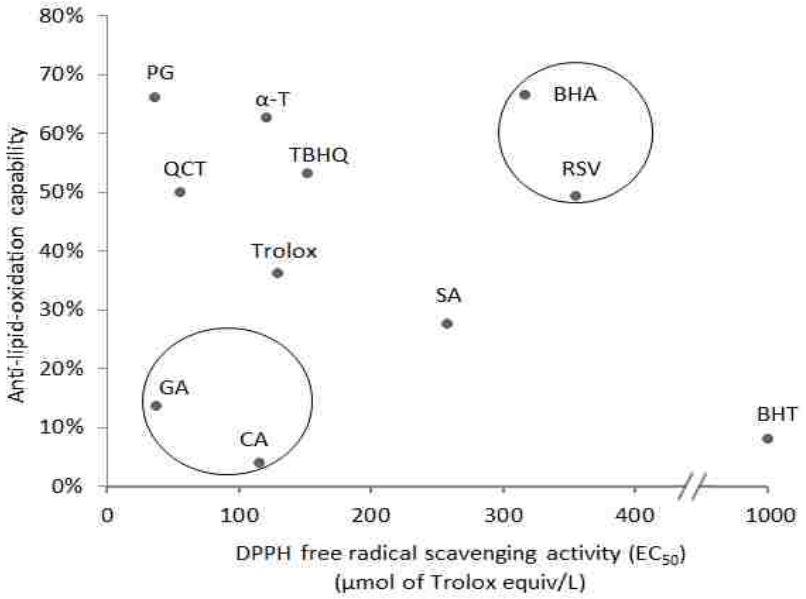
(a)



(b)

Figure 2.8. The correlation of TPC with DPPH free radical scavenging activity (a),and the correlation of anti-lipid-oxidation capability with TPC (b) or DPPH free radical scavenging activity (c)

Figure 2.8. (Continued)



(c)

2.5 Conclusion

In this study, eleven individual synthetic and natural antioxidants were evaluated in their phenolic content, DPPH free radical scavenging activity, for exploring the correlation between these factors and anti-lipid-oxidation capability in fish oil emulsion. As a result, most selected natural antioxidants showed relatively higher antioxidant activity in total phenolic content and DPPH assays. However, synthetic antioxidants had better performance in preventing lipid oxidation in the fish oil emulsion. Moreover, the result indicated that high phenolic content or DPPH free radical scavenging activity was not necessarily correlated with the great antioxidation capability in inhibiting lipid oxidation. Because the former two methods were based on simple chemical reactions, they cannot reflect the complex interaction in the real biological lipid system. But the composition and interaction of fish oil emulsion was relatively similar to the lipid environment in cell membrane or human serum so it can be used to assess the antioxidant promoting status of human bodies. Therefore, this

study provided not only useful information of total phenolic content and DPPH free radical scavenging activity of the selected eleven phenol-based antioxidants, but also the correlation among the antioxidant activity exhibited in traditional phenolic content or DPPH methods and the developed fish oil emulsion model.

CHAPTER 3
TOTAL PHENOLIC CONTENT, DPPH FREE RADICAL SCAVENGING ACTIVITY, AND ANTI-LIPID-OXIDATION CAPABILITY BY USING FOUR BEVERAGES

3.1 Introduction

Total phenolic content (TPC) and scavenging artificial free radicals activity assays are two traditional assays to evaluate the antioxidant activity of food products. The results obtained from the two *in vitro* assays indicated implicit correlatiton with the health promoting antioxidant potential in most antioxidant studies. However, their results may not truly correspond to the actual capability of anti-lipid-oxidation and health status promotion because the chemical reactions involved in the assays are totally irrelevant to the lipid oxidation naturally occurring in human body. The lipids in the blood and cell membrane were considered as most vulnerable under oxidative stresses (Boots, Haenen, and Bast, 2008; Serdar, Aslan, Dirican, Sarandöl, Yeşilbursa, and Serdar, 2006). After they are oxidized, enormous lipid oxidation products are generated to initiate cell inflammatory and development of cardiovascular and other chronic diseases (Lobo, Patil, Phatak, and Chandra, 2010). A number of epidemiological studies have confirmed that the increase of antioxidants in blood or serum significantly inhibits the harmful lipid oxidation and reduces the risk of developing those chronic diseases (Rautenbach and Venter, 2010). Therefore, lipid should be considered as the important substrate or subject to be directly tracked in performing a health-promoting antioxidant potential evaluation. In this study, a fish oil emulsion model was developed to mimic the human serum environment and used to evaluate the anti-lipid oxidation capability. Compared with the traditional assays, the antioxidant capability obtained from the oil emulsion

may more closely reflect the real activity in preventing the serum lipid oxidation and reducing inflammatory oxidation products in the body.

Consumptions of fruits, vegetables, cereals, and grains are important for promoting the health status and antioxidant level in the body. The health promoting function of the beverages made from natural antioxidant-rich plants and fruits have also been widely recognized (Beard and Ryan, 2011). Tea, brewed from the leaves of plant *Camellia sinensis*, is well known for the anti-lipidemic, anti-inflammatory and anti-thrombogenic activities (Castelnuovo, Giuseppe, Iacoviello, and Gaetano, 2012). A variety of phenolics in coffee could also effectively counteract against chronic diseases (Higdon and Frei, 2006). The presence of anthocyanins and hydroxycinnamate derivatives in grape wines was reported to contribute to the serum cholesterol lowering, neuroprotective and anti-aging activities (Mar, Mateos, Garc á, Puertas, and Villar, 2012). They are antioxidant-rich beverages and could increase the antioxidant level of body without intake of excessive carbohydrates. Several previous studies reported the correlations of TPC, DPPH free radical scavenging activity, and reducing power by using either pure antioxidant standards or a single type of sample (Orak, 2007; Zhang and Wang, 2009; Sulaiman et al., 2011). However, the approach of this study was to use the four different beverages as antioxidant sources to assess the correlations of the results of the two traditional assays and real anti-lipid-oxidation capability in an emulsion. Also, the diversities and concentrations of phenolics in the four beverages were measured by using an HPLC method and compared with their TPCs. In general, the relationships of the results obtained from the traditional antioxidant activity assays and real anti-lipid-oxidation capability in an emulsion were reviewed. Compared with the TPC and DPPH assays, the emulsion

model could be a more specific and economical *in vitro* method of evaluating the health promoting antioxidant activity.

3.2 Materials and methods

3.2.1 Chemicals and materials

HPLC grade methanol, acetonitrile, acetic acid, and 1-butanol were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). Sodium bicarbonate was obtained from Mallinckrodt Co. (Paris, KY, USA). Tween 20, menhaden fish oil, DPPH, Folin-Ciocalteau reagent, boron trichloride in methanol (BCl_3-methanol), heptadecanoic acid (C17:0), EPA, Trolox, TMP, TBA, myricetin, catechin, quercetin, gallic, ellagic, syringic, protocatechuic, chlorogenic, caffeic, *p*-coumaric and ferulic acid standards were obtained by Sigma-Aldrich (St. Louis, MO, USA). The green tea powder, ground coffee, Merlot red wine, and Chardonnay white wine used in this study were obtained from a local grocery store.

3.2.2 Beverage preparation and phenolics determination by HPLC method

Green ea and coffee were prepared by brewing 1 g of the tea powder and ground coffee with 200 mL of distilled water in a coffee maker (Mr. Coffee, Jarden Corporation, Cleveland, OH, USA), respectively. Then the beverages were cooled down to room temperature before use. The tea, coffee, or wines was filtered by 0.45 μm microporous film and transferred to an HPLC vial. The HPLC method for quantifying phenolics and anthocyanins was the same as the study of Xu (2012). The levels of each phenolic and total anthocyanins were expressed as mg/L of beverage.

3.2.3 TPC and DPPH free radical scavenging activity assays

TPC Ten-fold diluted Folin-Ciocalteau (0.75 mL) was added in 0.1 mL of ten-fold diluted tea, coffee, red wine, or white wine. Then, 0.75 mL of sodium bicarbonate (60 g/L) was mixed into the reaction solution and incubated at 25℃ for 90 min. The absorbance was measured by a UV-visible SpectraMax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 750 nm. The results were converted to mmol of Trolox equivalent/L of beverage by using the calibration curve of Trolox standard.

DPPH The DPPH method was based on the procedure described in the study of Yue and Xu with modification (Yue and Xu, 2008). An aliquot of 0.2 mL of ten-fold diluted each beverage was reacted with 1.8 mL of DPPH solution (0.1 mmol/L) in dark for 30 min at room temperature. The difference of the absorbance at 0 and 30 min was used to calculate DPPH free radical scavenging activity by using the calibration curve of Trolox standard and expressed as mmol of Trolox equivalent/L of beverage.

The DPPH free radical scavenging percentages of the four beverages with different TPCs were md. Each beverage was diluted to TPC at 1, 10, 100, 200, 500, or 1000 μmol of Trolox equivalent/L based on the results from the TPC assay. A group of Trolox solutions were also prepared based on the six concentrations. An aliquot of 0.2 mL of each solution was used in the DPPH free radical scavenging assay as described above. The percentage of scavenging DPPH free radicals of each diluted sample was calculated by the formula below:

Percentage of scavenging DPPH free radicals (%) = $(1 - Abs_{sample} / Abs_{blank}) \times 100$

where Abs_{sample} was the difference of the absorbance at 0 and 30 min of the diluted sample or Trolox; Abs_{blank} was the difference of the absorbance at 0 and 30 min of the blank in which the same volume of distilled water replaced the sample or Trolox. EC_{50} value of each beverage in scavenging DPPH free radicals was obtained through semilog regression analysis in SAS program (SAS, 9.1.3, Cary, NY, US).

3.2.4 Preparation of fish oil emulsion and determination of anti-lipid-oxidation capability

Preparation of fish oil emulsion The fish oil emulsion were made up of 1 % of menhaden fish oil and 0.1 % of Tween 20 in phosphate buffer (pH 7.0) was homogenized by a microfluidizer materials processor (M-110P, Microfluidics, Newton, MA, USA). One milliliter of tea, coffee, red wine or white wine was added in 20 mL of the homogenized fish oil emulsion in a 40 mL test vial. For blank sample, 1 mL of distilled water was used instead of the beverage solution. Then, each vial was incubated in a 37℃ water bath and agitated by a multiple magnetic stirrer (Multistirrer, VELP Company, Italy) for 72 h.

Retention rate of EPA in fish oil emulsion The oxidation rates of EPA, DHA, and other unsaturated fatty acids in the emulsion were considered to have a positive correlation with each other (Zhang, Shen, Prinyawiwatkul, King, and Xu, 2013). Thus, the concentration of EPA in each vial was measured at 0 and 72 h respectively and used to represent the oxidation status in each emulsion following the GC method described in the study of Zhang et al. (2013). The anti-lipid oxidation capability of each beverage in the emulsion was expressed by the retention rate of EPA which was calculated by the formula below:

$$\text{Retention rate of EPA (\%)} = (C_t / C_0) \times 100$$

where C_0 was the concentration of EPA at 0 h; C_t was the concentration of EPA at 72 h in the same emulsion.

TBA Lipid oxidation in the fish oil emulsion was also determined by the method listed in the Current Protocols in Food Analytical Chemistry (2001). One milliliter of each emulsion was collected at 72 h and diluted by 19 mL of distilled water. An aliquot of 0.2 mL of the diluted emulsion was transferred to a test tube and added with 1.8 ml of TBA (0.2% in 1-butanol). After being heated at 95℃ for 35 min, the tube was then cooled down in an ice bath to terminate the reaction. Then, the absorbance of the reacted solution was measured at 532 nm using a UV-Vis spectrophotometer. The production of MDA was calculated based on the standard curve of TMP and expressed as mmol of MDA/L of the fish oil emulsion.

3.2.5 Data analysis

The phenolic concentration, TPC, DPPH free radical scavenging activity, and EC_{50} value of each sample as well as the production of MDA and retention rate of EPA in fish oil emulsion were measured in triplicates and expressed as mean and standard deviation. All the calculations were completed by Microsoft Excel (Redmond, WA, US). Also, one-way ANOVA was used to evaluate significant differences between the samples at $P < 0.05$ (SAS, 9.1.3, Cary, NY, US).

3.3 Results and Discussion

3.3.1 Diversities and concentrations of phenolics in the tea, coffee, red wine and white wine and their total phenolic contents

The diversities and concentrations of major phenolics in the tea, coffee, red wine and white wine samples are listed in Table 1. The red wine showed the greatest diversity and highest overall concentration of phenolics, including eleven different major phenolics and a group of anthocyanins. The level of total anthocyanins reached up to 140.46 ± 6.91 mg/L, while other phenolics such as catechin, gallic acid, protocatechuic acid, and chlorogenic acid were also at high levels of 55.08 ± 2.81, 46.76 ± 1.10, 15.67 ± 0.63, and 11.83 ± 0.27 mg/L, respectively (Table 3.1). Moreover, anthocyanins, catechin and gallic acid were also reported as the major phenolics in red wine at levels of 59.15 - 149.14 mg/L for catechin and 32.48 - 63.24 mg/L for gallic acid in previous studies (Porgal & Büyüktuncel, 2012; García-Falcón, Pérez-Lamela, Martínez-Carballo, & Simal-Gándara, 2007; Granato, Katayama, & Castro, 2011). The phenolics in white wine was mainly from white grape flesh, which was different from red wine which was fermented with red grape. Thus, white wine contained relatively low level of phenolics. As shown in Table 1, gallic acid was the dominant phenolic in the white wine sample (11.00 ± 0.08 mg/mL) and then followed by chlorogenic, caffeic, syringic, *p*-coumaric and ferulic acids. These phenolics were also found in other studies (Fernández-Pachón, Villaño, Troncoso, & García-Parrilla, 2006; Kallithraka, Salacha, & Tzourou, 2009). Both of them were reported to have a number of health benefits, especially in the prevention of coronary heart diseases, compared with other alcoholic beverages (Ray, Maulik, Cordis, Bertelli, Bertelli, & Das, 1999). The abundant phenolics in the tea sample were gallic acid (36.12 ±1.43 mg/L) and catechin (34.53 ± 1.71 mg/L) which were associated

with the anti-aging and anti-cancer properties, inhibition of plaque formation, and regulation of lipid metabolism by reducing triglycerides and total cholesterol oxidation (Morita et al., 2009). Three major phenolics, chlorogenic acid, protocatechuic acid, and caffeic acid were found in the coffee sample at levels of 57.20 ± 3.14, 10.90 ± 0.85, and 8.75 ± 0.56 mg/L, respectively. It was reported that chlorogenic acid in coffee could be effectively absorbed (up to 33% of the oral dose) in humans and responsible for anti-inflammatory and the prevention of cardiovascular, diabetes type II, or Alzheimer's disease (Farah et al., 2008). The overall concentrations of phenolics in the tea and coffee samples were at a close level and 85.59 and 76.85 mg/L, respectively, which were approximately three times greater than that of the white wine sample.

Table 3.1 Major phenolics and their concentrations in the four beverages

Phenolics (mg/L)	Tea	Coffee	Red wine	White wine
Gallic acid	36.12 ± 1.43		46.76 ± 1.10	11.00 ± 0.08
Protocatechuic acid		10.90 ± 0.85	15.67 ± 0.63	
Chlorogenic acid	1.66 ± 0.00	57.20 ± 3.14	11.83 ± 0.27	7.33 ± 0.02
Catechin	34.53 ± 1.71		55.08 ± 2.81	
Caffeic acid	10.57 ± 0.30	8.75 ± 0.56	6.78 ± 0.20	4.43 ± 0.01
Syringic acid			6.91 ± 0.07	2.66 ± 0.00
Ellagic acid			2.67 ± 0.05	
p-Coumaric acid	2.05 ± 0.00		0.94 ± 0.00	0.61 ± 0.00
Ferulic acid	0.66 ± 0.00		2.72 ± 0.01	0.54 ± 0.00
Myricetin			7.26 ± 0.38	

Table 3.1 continued

Quercetin Total anthocyanins			8.45 ± 0.52 140.46 ± 6.91	
Overall Concentration	85.59	76.85	305.53	26.57

From the results of TPC assay, the red wine sample showed the highest level of 37.16 ± 3.77 mmol of Trolox equivalent/L, while the other three beverages were all below 7 mmol of Trolox equivalent/L (Figure 1) since the red wine had a remarkable amount of anthocyanins as well as the highest overall concentration of phenolics. It was reported that the fruits (like guava, red grapes, and plums) rich in anthocyanins correspondingly had higher TPC than other less anthocyanins fruits (Fu et al., 2011). While the TPC of the red wine was approximately seven times higher than the tea, its overall concentration of phenolics was only four times higher than that tea sample. The TPC of the white wine was similar to the tea and higher than the coffee, even though it had the lowest overall concentrations of phenolics (Table 3.1). It was considered that the TPC results of the red and white wines were not only contributed by their phenolics but also some non-phenolic compounds. For example, The relatively high level of reducing sugars in the wines may also react with Folin-Ciocalteu reagent and result in their TPC results overestimated (Košmerl, Bertalanič, Maraš, Kodžulović, Šućur, & Abramovič, 2013). Therefore, the TPC assay may not be applicable to determine the sample with a certain level of reducing sugars. Compared with the wines, the TPC results of the tea and coffee may closely reflect the levels of phenolics in the beverages as their sugar content had very low level.

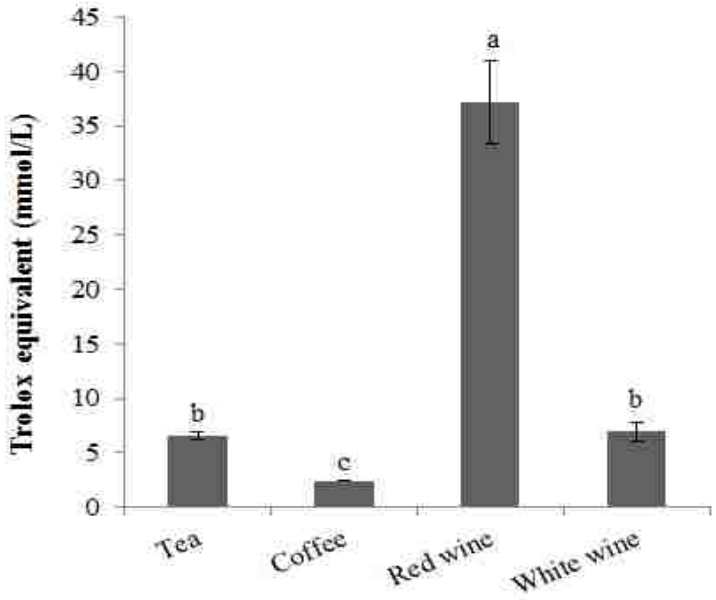


Figure 3.1 Total phenolic contents of the tea, coffee, red wine and white wine samples

3.3.2 DPPH free radical scavenging activities of the tea, coffee, red wine, and white wine

The order of DPPH free radical scavenging activities (mmol of Trolox equivalent/L) of the four beverages from high to low was red wine (17.76 ± 0.30) > tea (3.97 ± 0.12) > white wine (2.26 ± 0.07) > coffee (1.45 ± 0.09) (Figure 3.2). Similar to the difference between the overall concentrations of phenolics of the two samples, the red wine showed approximately four times higher DPPH free radical scavenging activity than the tea. Compared with the coffee and white wine samples, the tea sample exhibited greater free radical scavenging activity in the DPPH assay as it was rich in catechin and gallic acid which consist of multiple antioxidant hydroxyl groups (Table 3.1). Coffee had the poorest performance in the TPC or DPPH assay, even though the overall concentration of phenolics of the coffee was much higher than the white wine and similar to the tea. It was recognized that the types of phenolics in the coffee were not as effective as those in the tea in scavenging DPPH free radicals.

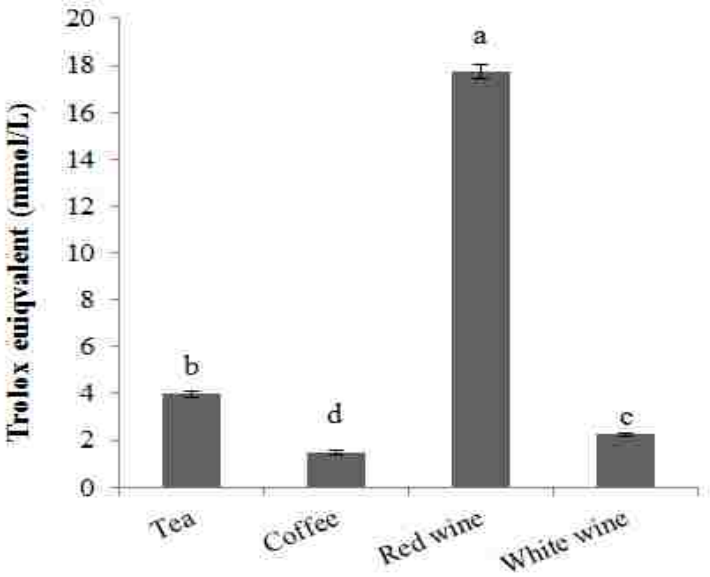


Figure 3.2 DPPH free radical scavenging activities of the tea, coffee, red wine and white wine samples

The EC_{50} obtained from the DPPH assay was usually referred to a required concentration to achieve a 50% of DPPH free radical scavenging activity (Chen, Bertin, & Froldi, 2013). In this study, the EC_{50} values of the four beverages were calculated based on the free radical scavenging activities of a series of the diluted beverages with different TPC values. All the beverages had a dose-dependent curve at the low TPC range, and then reached a plateau shape at relatively high TPC (Figure 3.3). The order of EC_{50} (μmol/L) value of the four beverages from low to high was red wine (18.70 ± 1.12) > tea (36.31 ± 2.25) > Trolox (151.36 ± 6.19) > white wine (190.55 ± 7.38) > coffee (331.13 ± 19.96). Since the lower EC_{50} value the higher effectiveness in scavenging DPPH free radicals, the red wine or tea sample had greater DPPH free radical scavenging activity than Trolox. However, a high level of TPC was required for the white wine or coffee sample to achieve the same scavenging activity, which means the efficiency of the scavenging DPPH free radical could vary from sample to sample at the same TPC value. Furthermore, although there was no significant difference between the

TPC results of the tea and white wine, the DPPH free radical scavenging activity of the tea was approximately three times higher than the white wine. Although the white wine had the lowest overall concentration of phenolics among the four beverages, it was more effective in scavenging DPPH free radical activity and had greater TPC than the coffee. Also, the overall concentrations of phenolics of the tea and coffee were at a similar level; however, the tea sample had greater performance in both of the TPC and DPPH assays. Thus, the TPC and DPPH free radial scavenging activity of a complicated sample may not be corresponded with each other. In general, the DPPH assay predicts quenching free radical or H-donor activity of the test sample, while the TPC assay is used to estimate reducing power of the sample through reacting Folin-Ciocalteu reagent to produce blue colored oxides (Sasikumar, Adithya, & Shamna, 2012). Theoretically, the TPC is positively correlated with the DPPH free radical scavenging activity. However, the types of phenolics, reducing sugars, and non-phenolics in a test sample may cause the correlation more complicate.

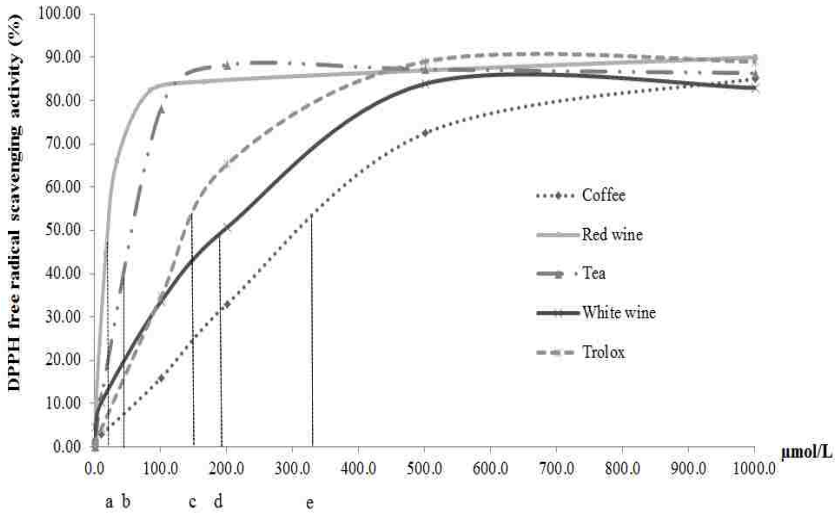


Figure 3.3 Changes of DPPH free radical scavenging activities of the four beverages diluted at different total phenolic contents and Trolox

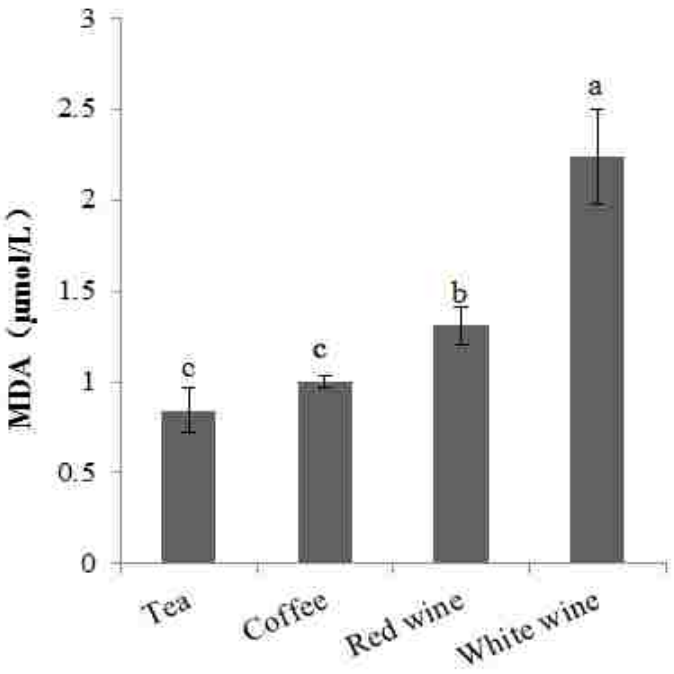
a. red wine (EC_{50}, 18.70 µmol/L) b. tea (EC_{50}, 36.31 µmol/L) c. Trolox (EC_{50}, 151.36 µmol/L) d. white wine (EC_{50}, 190.55 µmol/L) e. coffee (EC_{50}, 331.13 µmol/L)

3.3.3 Lipid oxidation in fish oil emulsions mixed with the coffee, tea, red wine and white wine

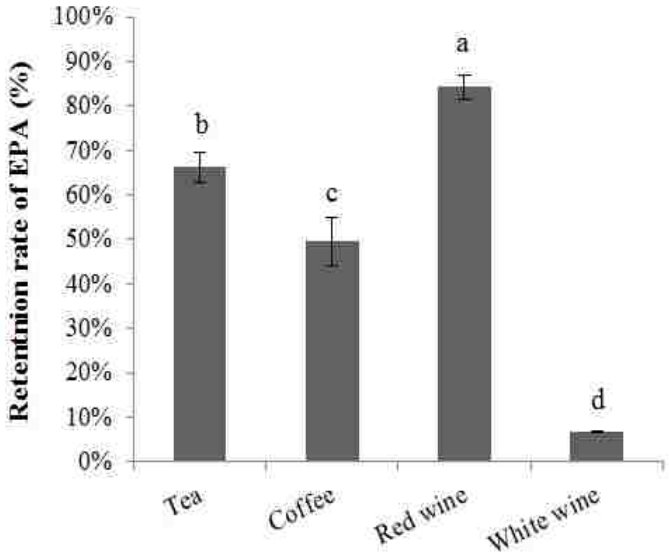
After 72 h incubation, the lipid oxidation product MDA and retained EPA in the fish oil emulsions were measured by using the TBA and GC methods, respectively. The result showed the emulsion mixed with the white wine had the highest amount of MDA (0.22 ± 0.03 mmol/L) and retained the lowest level of EPA (6.72 ± 0.14 %) among the four emusions (Figure 3.4a). The retention rate of EPA in the emulsion with the red wine was at the highest level of 84.25 ± 2.74 %, while the rate was 66.15 ± 3.40 and 49.49 ± 5.42 % in the emulsions mixed with the tea and coffee, respectively (Figure 3.4b). However, the level of MDA in the emulsion with red wine was 0.13 ± 0.01 mmol/L and signficantly higher than the emulsion with tea or coffee. It was considered that the synergistic effect of multiple phenolic acids and anthocyanins in red wine could significantly inhibit linoleic acid oxidation in a potassium phosphate buffer (Orak, 2007). There was no significant difference between the MDA levels of the emulsions with the tea and coffee (Figure 3.4b). Although the TPC result of the white wine was similar level as the tea and higher than the coffee, the performance of the white wine in inhibiting lipid oxidation in the fish oil emulsion was much poorer than the tea or coffee. It was suggested that the TPC value may not be correlated with the capablity of protecting lipid under oxidative stress.

Because MDA is the secondary lipid oxidation product, its production is associated with the degree of lipid oxidation. Therefore, the higher retention rate of EPA in the emulsion means the less MDA produced. However, this tendency was not observed in all the four emulsions (Figure 3.4). For example, the emulsion with white wine had the lowest EPA retention rate and highest MDA level, but the emulsion with red wine had the highest EPA retention rate

and did not exhibit the lowest MDA level among the four emulsions. In addition, the emulsion with tea retained 25% more EPA than the emulsion with coffee, while the MDA levels in the two emulsions were not significantly different. In fact, the MDA level measured by using TBA method is interfered by many factors involved in the sample, such as sugars, proteins etc. (Almandós, Giannini, Ciarlo, & Boeri, 1986). Similar to the TPC assays, the sugars in the red or white wine may react in TBA assay and cause the results overestimated. Therefore, directly monitoring the change of a vulnerable lipid substrate as this study could be more specific and reliable than the TBA method in either evaluation of the lipid oxidation status or determination of the antioxidant activity of a complicated sample.



(a)



(b)

Figure 3.4 The MDA levels (a) and retention rate of EPA (b) in the fish oil emulsions mixed with tea, coffee, red wine, and white wine after 72h incubation

3.3.4 Relationships of anti-lipid-oxidation capabilities, TPC, and DPPH free radical scavenging activities of the coffee, tea, red wine and white wine

The antioxidant activities obtained from the fish oil emulsion model and DPPH assay and TPC of the four beverages were summarized in Figure 3.5. The EPA retention rate of the emulsion with coffee (49.49 ± 5.42%) was approximately seven times greater than the emulsion mixed with white wine (6.72 ± 0.14%). However, the DPPH free radical scavenging activity of the coffee (1.45 ± 0.09 mmol of Trolox equivalent/L) was significantly lower than the white wine (2.26 ± 0.07 mmol of Trolox equivalent/L). In addition, the EPA retention in the emulsion with tea was approximately ten times higher than the emulsion with white wine, although the tea and white wine showed a similar level of TPC. The reasons may be that other non-phenolic compounds in the white wine could also react with the designed reagents in TPC and DPPH assays and then caused the results overrated. Also, the free radicals DPPH• or ABTS•+ usually used in traditional assays were totally different from natural reactive

oxygen species involved lipid oxidation (López-Alarcón & Denicola, 2013). Therefore, the results obtained from the TPC and DPPH free radical scavenging assays may not reflect the anti-lipid oxidation capability or anti-inflammatory in a biological emulsion. Also, the different chemical structures of antioxidants in the four beverages may also differently contribute to the overall anti-lipid-oxidation capablity, as they would affect their accessibility to the targeted lipid when antioxidation occurred (Balogh, Hegedűsa, & Stefanovits-Bányaia, 2010). The fish oil emulsion model introduced in this study simulated the lipids in human serum environment. The capability of preventing the vulnerable EPA substrate from oxidation in the emulsion could closely reflect the corresponding antioxidant capability of the sample in stabilizing the serum lipid, reducing inflammatory lipid oxidation products, and promoting the health status of body.

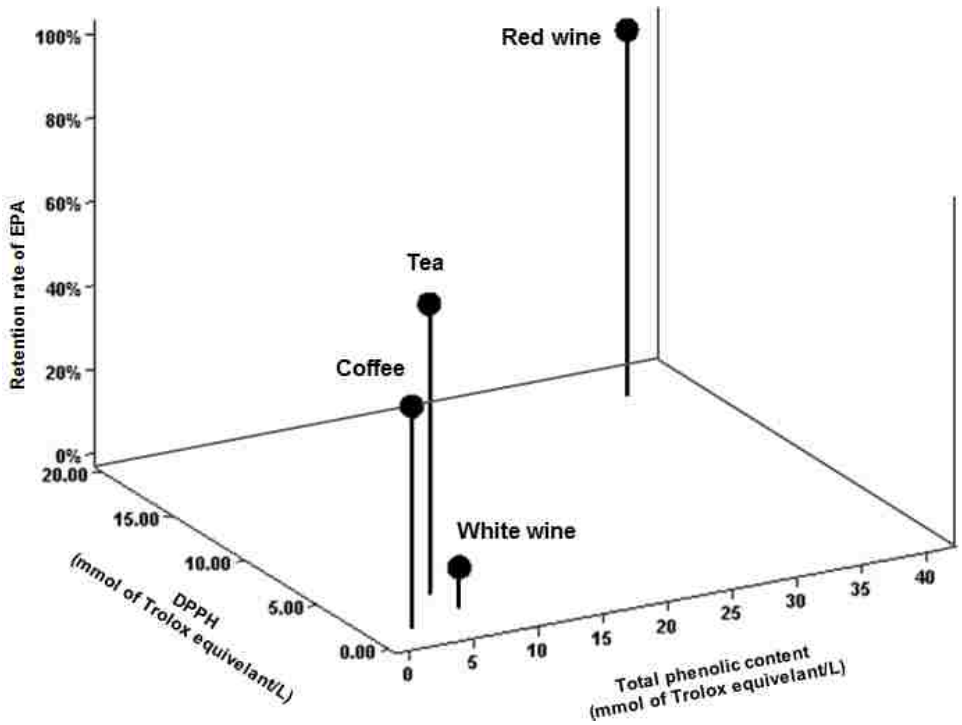


Figure 3.5 Relationships of the DPPH free radical scavenging activities, total phenolic contents, and capabilities of inhibiting lipid oxidation of the four beverages

3.4 Conclusion

Generally, the mechanisms of lipid oxidation and anti-lipid-oxidation are very complicated. The linear corrlections of the TPC, DPPH free radical scavenging activity, and real anti-lipid-oxidation were not observed in this study. Compared with the TPC and DPPH assays, the fish oil emulsion model was more similar to the human serum to evaluate the antioxidant activity of a test sample in inhibiting lipid oxidation. The results would closely correspond to the capability of the sample in promoting the antioxidation status in serum and providing the health promoting function.

CHAPTER 4
SUMMARY

In this study, eleven individual synthetic and natural antioxidants, four common drinks are evaluated in their phenolic content, DPPH free radical scavenging activity, for exploring the correlation between these factors and anti-lipid-oxidation capability in fish oil emulsion. As a result, most selected natural antioxidants showed relatively higher antioxidant activity in total phenolic content and DPPH assays. However, synthetic antioxidants had better performance in preventing lipid oxidation in the fish oil emulsion. Moreover, the result indicated that high phenolic content or DPPH free radical scavenging activity was not necessarily correlated with the great antioxidation capability in inhibiting lipid oxidation. Because the former two methods were based on simple chemical reactions, they cannot reflect the complex interaction in the real biological lipid system. But the composition and interaction of fish oil emulsion was relatively similar to the lipid environment in cell membrane or human serum so it can be used to assess the antioxidant promoting status of human bodies. Therefore, this study provided not only useful information of total phenolic content and DPPH free radical scavenging activity of the selected eleven phenol-based antioxidants and four drinks, but also the correlation among the antioxidant activity exhibited in traditional phenolic content or DPPH methods and the developed fish oil emulsion model.

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