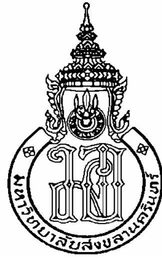

Maximized Uses of Phenolic Compounds in Retardation of Lipid Oxidation and Shelf-life Extension of Fish and Fish Products

Sajid Maqsood

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Science and Technology

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ABSTRACT

The role of phenolic compounds in prevention of lipid oxidation and fishy odour development in different systems was elucidated. Different phenolic compounds (catechin, caffeic acid, ferulic acid and tannic acid) at a level of 100 mg/kg had antioxidative activity differently. Among all the phenolic compounds tested, tannic acid exhibited the highest 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and ferric reducing antioxidant power (FRAP). Nevertheless, catechin showed the highest metal chelating activity ($P<0.05$), whereas caffeic acid had the highest lipoxygenase (LOX) inhibitory activity ($P<0.05$). Impact of different phenolic compounds at a level of 100 mg/l on lipid oxidation of menhaden oil-in-water emulsion and mackerel mince was investigated. Tannic acid showed the highest efficacy in retardation of lipid oxidation for both model systems as evidenced by the lower peroxide value (PV), conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) values. Tannic acid also lowered the release of non-haem iron in the mince.

When tannic acid at a level of 200 mg/kg was added to the striped catfish slices, which were further stored under MAP (60%N_2 / 35%CO_2 / 5%O_2) (M_2), the lowest PV and TBARS values with the coincidental lowest non- haem iron content were obtained. Myosin heavy chain of the control sample kept in air without tannic acid treatment was degraded by 17.85% after 15 days of storage as compared to fresh sample, whereas no change was noticeable in treated samples stored under MAP. Based on microbiological acceptability limit (10^7 cfu/g) and sensory test, the shelf-life

of control and treated samples stored under MAP was estimated to be 9 and 15 days, respectively. Therefore, tannic acid exhibited a synergistic effect with MAP on retarding lipid oxidation and microbial growth, thereby increasing the shelf-life of striped catfish slices during refrigerated storage.

Blood or haemoglobin retained in the fish muscle during post-mortem storage played an important role in acceleration of lipid oxidation as well as fishy odour development. Pro-oxidative activity of haemoglobins from different tropical fish (Asian seabass, tilapia and grouper) was influenced by pH (6, 6.5 and 7). Relative oxygenation of all haemoglobins decreased in the acidic conditions. Haemoglobin from seabass was more oxygenated and stable against autoxidation at both pH 6 and 7, compared to those from tilapia and grouper. Haemoglobin from tilapia and grouper was fully oxidised at pH 6 after 120 h. Haemoglobins accelerated lipid oxidation of washed Asian seabass mince more effectively at pH 6, compared with pH 6.5 and 7 as indicated by the higher PV and TBARS values. At the same pH values, haemoglobins from tilapia and grouper were more pro-oxidative than that from Asian seabass as evidenced by the higher PV and TBARS ($P<0.05$). Volatile lipid oxidation compounds were also formed at higher rate in the washed mince added with haemoglobin from tilapia or grouper with coincidental stronger fishy odour development, compared to the control and those added with haemoglobin from Asian seabass.

Tannic acid (200 and 400 mg/kg) was used to retard the lipid oxidation mediated by haemoglobin from tilapia in washed Asian seabass mince during 10 days of iced storage. Control samples (without tannic acid) had the highest PV within the first 2 days and possessed the greater amount of TBARS throughout the storage of 10 days ($P < 0.05$). With addition of tannic acid, the lipid oxidation of washed mince was retarded, especially when the higher level (400 mg/kg) was used, as indicated by lowered PV and TBARS values. The retarded formation of volatile lipid oxidation products and fishy odour intensity was also found in the samples added with 400 mg/kg tannic.

When ethanolic kiam wood extract (EKWE) (0.05 and 0.1%), containing tannic acid at a level of 545.57 mg/g was added to washed Asian seabass

mince containing haemoglobin and 5% menhaden oil, the decrease in PV and TBARS values were observed. Incorporation of 5% menhaden oil to the washed mince had no impact on the formation of PV and TBARS, compared with oil-free samples during the storage (P>0.05). Formation of volatile lipid oxidation compounds and fishy odour intensity was retarded in the sample containing menhaden oil and haemoglobin and treated with 0.1% EKWE, compared with those without the addition of EKWE after 10 day of storage in ice (P<0.05).

With the addition of tannic acid (0.02 and 0.04%) and EKWE (0.08%), formation of PV, TBARS and fishy odour in the fish emulsion sausages prepared from striped catfish was retarded effectively, compared to the control (P<0.05), especially when the tannic acid and EKWE was used at higher level. At the same level, EKWE showed the lower ability in retarding the lipid oxidation, in comparison with tannic acid. Both tannic acid and EKWE had no detrimental effect on the sensory attributes of sausages. However, EKWE added sample had lower L^* and higher a^* and ΔE^* values, compared to the control samples (P<0.05). After 20 days of storage, sample added with 0.04% tannic acid had higher hardness, gumminess and chewiness with more compact structure and no visible voids. Furthermore, the oil droplets with smaller size were dispersed more uniformly, compared with others (P<0.05). Thus, tannic acid or EKWE could serve as potential natural antioxidants in prevention of lipid oxidation and retardation of fishy odour development, which was mediated by haemoglobin in different fish and fish products.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Fish are considered to be of great nutritional importance. This is mainly due to their naturally high content of essential omega-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). Consumption of these fatty acids is believed to be beneficial to human health (Lee and Lip, 2003). Nevertheless, fish, especially fatty fish is susceptible to oxidation, which is associated with the rancidity and loss in nutritive value (Hsieh and Kinsella, 1989; Frankel, 1998a). Apart from high amounts of PUFAs, the presence of haem pigments and trace amounts of metallic ions makes the fish, especially dark flesh fatty fish, prone to lipid oxidation (Hsieh and Kinsella, 1989). Haemoglobin is also known as an important catalyst of lipid oxidation. Haemoglobin can be a source of activated oxygen due to haemoglobin autoxidation, and haem or iron can be released from the protein to promote lipid oxidation (Richards and Hultin, 2002). Lipid oxidation can be accelerated by reduction in pH and could be due to enhanced autoxidation of haemoglobin at reduced pH (Tsuruga *et al.,* 1998). Oxidation can also cause other detrimental effect such as discoloration, vitamin destruction and decomposition of essential fatty acids, leading to organoleptic failure and a decrease in nutritive value (Sherwin, 1978). To retard such a quality loss, synthetic antioxidants have been used to decrease lipid oxidation during the processing and storage of fish and fish products (Boyd *et al.*, 1993). However, the use of synthetic antioxidants has raised questions regarding food safety and toxicity (Chang *et al.,* 1977). The use of natural antioxidants is emerging as an effective methodology for controlling rancidity and limiting its deleterious consequences. Owing to the increasing demand for food devoid of synthetic additives, much effort has been paid to the discovery of new natural antioxidants such as phenolic compounds. In general, phenolic compounds play a role as antioxidants through different mechanisms of action, such as scavenging of free radicals (Antolovich *et al.,* 2002), quenching of

reactive oxygen species, inhibition of oxidative enzymes (Edenharder and Grunhage, 2003), chelating of transition metals or through interaction with bio-membranes (Liao and Yin, 2000). Therefore, these compounds have been considered as the promising candidates as potential protectors against food oxidation and biological aging of tissues.

Apart from using the antioxidants, appropraite packaging technology can be applied to retard lipid oxidation in fish. Modified atmosphere packaging (MAP) has been used to prolong the shelf-life of fish and fish products (Masniyom *et al.,* 2002). The removal and/or replacement of the atmosphere surrounding the product is required before sealing in vapor-barrier materials (McMillin *et al.,* 1999), thereby maintaining the quality of those products (Skibsted *et al.,* 1994). The modification process is achieved by lowering the amount of oxygen (O_2), moving it from 20% to 0%, in order to slow down the growth of aerobic microorganisms and retard the oxidation reactions. The removed oxygen can be replaced with nitrogen (N_2), commonly acknowledged as an inert gas, or carbon dioxide (CO_2), which can lower the pH or inhibit the growth of bacteria. MAP is widely used as supplement to ice or refrigeration storage to delay spoilage and extend the shelf-life of fresh fish and fish products (Reddy *et al.,* 1995; Pedrosa-Menabrito and Regenstein, 1998). Therefore, the use of selected phenolic compounds as natural extract in combination with MAP under the appropriate condition could be a promising means to extend the shelf-life of fish by lowering the rate of lipid oxidation, especailly mediated by haem protein released in fish, as well as the growth of spoilage microorganisms.

Additionally, phenolic compounds can be used as an potential antioxidants in high fat foods particularly those enriched with n-3 fatty acids, such as fish emulsion sausage, etc. Fish oil is actually the main dietary source of n-3 PUFA. An increase in consumption of n-3 PUFA has been reported to reduce the risk of coronary heart disease, decrease mild hypertension and prevent certain cardiac arrhythmias (Garg *et al.,* 2006). Therefore, phenolic compounds especially from natural sources like wood or bark of several trees could act as the antioxidant, which

could lower lipid oxidation. As a consequence, the desired quality with the nutritive value can be maintained during processing, handling and storage.

1.2. Review of Literature

1.2.1 Lipid oxidation

Lipid oxidation produces rancid odors and off-flavors, decreases shelf life and can alter texture and appearance of foods. Furthermore, it can decrease the nutritional quality and safety of foods. Lipid oxidation is one of the main factors limiting the quality and acceptability of muscle foods (Morrissey *et al.*, 1998). Oxidation of lipids is accentuated in the immediate post-slaughter period, during handling, processing, storage and cooking. This process leads to discoloration, drip losses, off-odor and off-flavor development, texture defects and the production of potentially toxic compounds (Morrissey *et al.*, 1998; Richards *et al.*, 2002).

1.2.2 Autoxidation

The oxidation of lipids occurs by a free radical chain reaction involving three processes: (1) initiation – the formation of free radicals; (2) propagation – the free radical chain reactions; and (3) termination – the formation of non-radical products (Frankel, 2005; Nawar, 1996) (Fig. 1). In the initiation step, a lipid free radical known as the alkyl radical (L•) is formed. Abstraction of a hydrogen atom from the central carbon of the *pentadiene structure* is found in most fatty acid acyl chains containing more than one double bond:

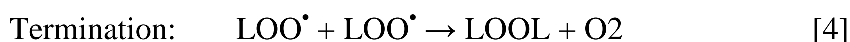
The alkyl radical contains an unpaired electron that reacts rapidly with the oxygen biradical to form peroxyl radicals (LOO•) [2] in the propagation step. The following hydrogen transfer reaction that occurs with unsaturated lipids to convert the peroxyl radical to a hydroperoxide (LOOH) happens slower than the previous step [3]. Termination is the last step of autoxidation. In the termination stage [4-6], peroxyl

radicals accumulate and react with each other forming non-radical products (Frankel, 2005; Erickson, 2002; Nawar, 1996; Ingold, 1962).

Initiation: $In^{\bullet} + LH \rightarrow InH + L^{\bullet}$ [1]

Propagation: $L^{\bullet} + O2 \rightarrow LOO^{\bullet}$ [2]

$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ [3]


Termination: $LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O2$ [4]

$L^{\bullet} + LOO^{\bullet} \rightarrow LOOL$ [5]

$L^{\bullet} + L^{\bullet} \rightarrow LL$ [6]

In general, high peroxide is not stable and can be decomposed to many products including acids, alcohols, aldehydes, ketones, etc. Those oxidation products could undergo polymerisation, and might induce protein denaturation, leading to the changes in functional properties (Shahidi and Naczk, 2004) (Fig. 1).

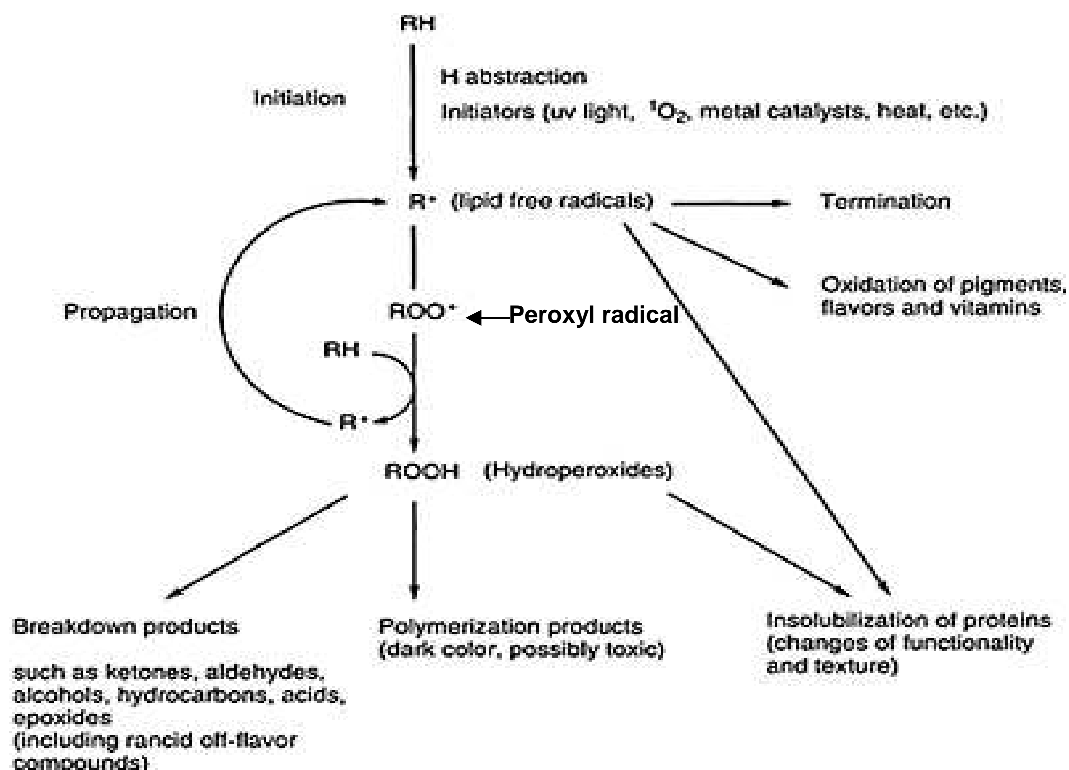


Figure 1. General scheme for autoxidation of lipids containing polyunsaturated fatty acids (RH) and their consequences.

Source: Shahidi and Naczk (2004).

1.2.3 Lipid oxidation induced by lipoxygenase (LOX)

LOXs (EC 1.13.11.12) are non-haem iron-containing dioxygenases that are ubiquitous in plants and animals (Gardner, 1995; Kuhn and Thiele, 1999; Shibata and Axelrod, 1995). The expression levels of different LOXs in tissues vary widely and can differ among the same organism with age, tissue and environmental factors. The oxidation processes initiated by these enzymes become deleterious under certain conditions. LOX are initiators of uncontrolled oxidation processes that are one of the main causes of food quality decay. LOXs catalyze the regio- and antio-selective dioxygenation of polyunsaturated fatty acids (PUFAs) containing a 1(*Z*), 4(*Z*)-pentadiene system to form hydroperoxide products (Nanda and Yadav, 2003). The most widely accepted reaction mechanism for the LOX-catalyzed peroxidation is radical-based, where the fatty acid is oxidized by the ferric iron within the active site of the enzyme to form a fatty acid radical and a ferrous iron. This radical intermediate is then attacked by dioxygen to form the fatty acid hydroperoxide. LOXs are responsible for the development of rancidity in fat-containing foods (Baysal and Demirdoven, 2007). Generation of off flavors, loss of nutritional value and loss of economical value of food products are the result of LOXs activity (Grechkin, 1998). Due to these adverse effects associated with the activity of these enzymes, a considerable research effort has therefore been devoted to the identification of appropriate inhibitors for these enzymes (Kim and Uyama, 2005; Rescigno *et al.,* 2002; Schneider and Bucar, 2005 a, b). Most common inhibitors currently known for LOX are polyphenolic compounds (Banerjee, 2006). Polyphenols in general are very powerful chain-breaking antioxidants and able to retard the adverse oxidation reactions initiated by LOX, but they usually exert their function by radical scavenging or reduction of the oxidation products rather than inhibiting the enzyme (Banerjee, 2006).

1.2.4 Lipid oxidation mediated by haemoglobin

1.2.4.1 Structure and function of haemoglobin

Haemoglobin is the major haem protein of red blood cells and is responsible for the transportation of oxygen to the tissues (Perutz, 1990). Haemoglobin consists of four polypeptide chains, two of α-chains and two of β chains. The four chains are held together by covalent attractions. Each α chain is in contact with β chain (Fig. 2). The conformation of α and β subunits differs only by addition of one helix in the β subunit. The haemoglobin from different fish species varies with respect to the difference in the amino acid sequences. Differences in the numbers and identity of amino acids have been reported in haemoglobin from different sources (Perutz, 1990; Jensen *et al.,* 1998).

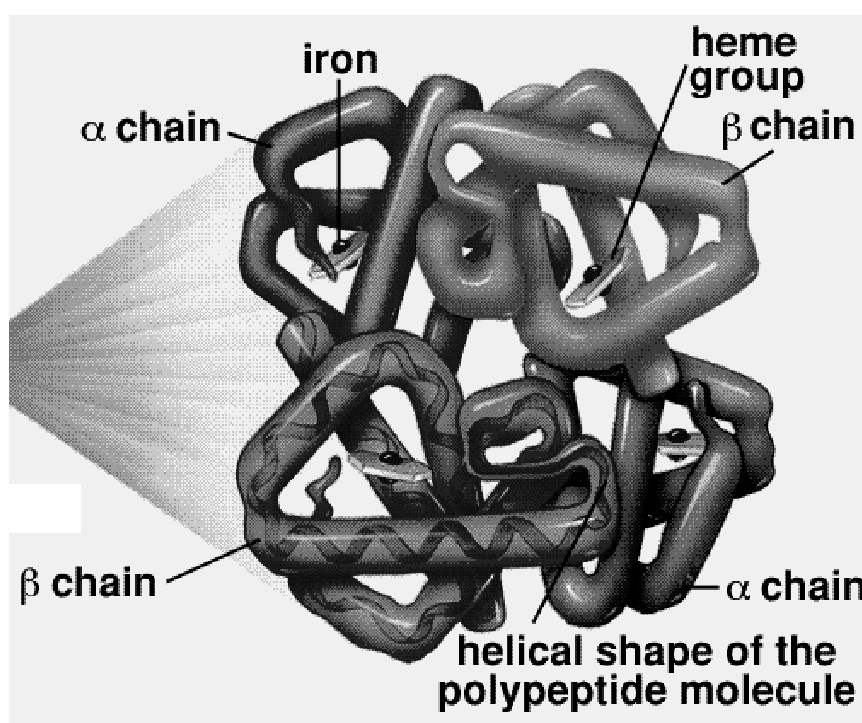


Figure 2. Haemoglobin structure displaying haem group with iron attached.

Source: Mader (1997).

Capacity of haemoglobin to bind with oxygen depends on the presence of non-polypeptide units namely a haem group. The haem buried in the hydrophobic pocket of the haemoglobin consists of an organic part and an iron atom (Dickerson and Geis, 1986). The organic part, protoporphyrin, is made up of four pyrrole rings. The four pyrroles are linked by methene bridge to form tetrapyrrole ring. Four methyl, two vinyl and two propionate side chains are attached to tetrapyrrole ring. The iron atom in the haem binds to the four nitrogen in the center of the protoporphyrin ring. The iron can form two additional bonds, one on either side of the haem plane. These binding sites are termed the fifth and sixth coordination positions. At the fifth coordination position, the iron is directly bonded to histidine of globin called proximal histidine while the sixth coordination position is the oxygen binding site. The iron atom can be in the ferrous (2+) or the ferric (3+) oxidation state and the corresponding forms of haemoglobin are called ferrohaemoglobin and ferrihaemoglobin, respectively. Ferrihaemoglobin is also called as methaemoglobin, where water molecule occupies the sixth coordination site. Only ferrohaemoglobin, the 2+ oxidation state, can bind oxygen. Therefore, most haemoglobin molecules are found either with no oxygen (deoxyhaemoglobin, unliganded Hb) or with four oxygens (oxyhaemoglobin, liganded Hb) (Fermi *et al.,* 1984).

Binding of oxygen in haemoglobin is cooperative. This means that binding at one haem facilitates bindings of oxygen at other haems on the same molecules (Jensen *et al.,* 1998). Oxygen binding property of haemoglobin is also affected by pH. In the physiological range, a lowering of pH decreases the oxygen affinity of haemoglobin. Increasing the concentration of CO_2 also lowers the oxygen affinity. These linkages between the binding of O_2 and concentration of H^+ and CO_2 are known as Bohr effect (Riggs, 1988). Haemoglobin of some fish expressed a large decrease in both oxygen affinity and cooperative at low pH. This characteristic of haemoglobin is known as the Roots effect (Brittain, 1987). Typically, Bohr effect expresses its role when blood pH drops from about 7.4 to 6.5 (Stryer, 1988a). A further decrease in blood pH is considered the Root effect (Manning *et al.,* 1998).

Many fish species have multiple haemoglobin components, which are characterized by significant differences in the amino acid sequences and in functional

properties (Brunori, 1975; Brunori *et al.,* 1975). This multiplicity is in contrast to human haemoglobin which has a single main haemoglobin. Based on the multiplicity, teleost fish fall into three major classes: class I, II and III (Weber *et al.,* 1976). The class I haemoglobin as in carp is an electrophoretically anodal haemoglobin that has Bohr, Root, phosphate and temperature effects (Jensen *et al.,* 1998). The class II haemoglobin as in trout and eel consist of both anodal component and cathodal haemoglobin that exhibits high oxygen affinity and small Bohr effect and low temperature sensitivities (Zolese *et al.,* 1999). The class III haemoglobin as in tuna is haemoglobin that is sensitive to pH but insensitive to temperature (Rossi-Fanelli, 1960).

1.2.4.2 Lipid oxidation mediated by haemoglobin

When tested in a washed cod muscle model system, a crude haemoglobin preparation was highly effective in catalyzing lipid oxidation (Richards and Hultin, 2002; Richards and Hultin, 2000). In fish, haemoglobin has been identified as one of the most potent pro-oxidants and can start oxidation according to several mechanisms (Richards and Hultin, 2002; Kristinsson and Hultin, 2004). In seafood processing, blood may not be removed prior to, for example, filleting and mincing. So, in postmortem fish, haemoglobin can react with the muscle lipids and accelerate lipid oxidation (Richards *et al.,* 1998), therefore, its rapid removal or inhibition is important to prevent the onset of lipid oxidation.

Haemoglobin can be a source of activated oxygen due to haemoglobin autoxidation, and haem or iron can be released from the protein to promote lipid oxidation. Plasma is recognized for its many components that inhibit lipid oxidation (Wayner *et al.,* 1987), but fish plasma is 1.2–3% lipid, much of it in the form of lipoproteins (McDonald and Milligan, 1992), which could be a source of oxidizable lipid. Haemoglobin can be present in many different forms (Everse and Hsia, 1997). In its reduced state, the iron can be bound to O_2, which is stabilized via hydrogen bonding by the nearby distal histidine, or it can be without the oxygen, such as at low pH (Hargrove *et al.,* 1997) or at low oxygen tension (Stryer, 1988b). The iron can oxidize to form the brown methaemoglobin under the right solution conditions. Both

the oxidized and the reduced forms can be pro-oxidative. Several different mechanisms of the pro-oxidative power of haemoglobin have been proposed (Everse and Hsia, 1997). Oxyhaemoglobin (Fe^{2+}-O_2) can autoxidize to methaemoglobin (Fe^{3+}) releasing its oxygen as a superoxide anion radical ($O_2^{\bullet-}$). This radical can further dismutate to hydrogen peroxide, which can activate the methaemoglobin to form hypervalent ferryl haemoglobin ($Fe^{4+}{=}O$). Although this species is only transient in nature and has a short half-life, it is capable of peroxidizing lipids and is thought to be the main species responsible for haemoglobin-induced lipid oxidation in muscle products (Alayash, 1999). This ferryl species has a protein radical form and exerts its action by abstracting an electron from the lipid substrate leaving a free radical substrate species, which can cause further oxidation. The superoxide released on autoxidation can also lead to the formation of species ($HOO^{\bullet}$ and $HO^{\bullet}$) that are pro-oxidative, $HO^{\bullet}$ more than $HOO^{\bullet}$. In a second mechanism, which is poorly understood, the protein may act as a pseudo-lipoxygenase (Everse and Hsia, 1997).

The autoxidation reaction is enhanced by a low pH, while it is reduced at an alkaline pH as interactions with distal histidine become stronger (Hargrove *et al.,* 1997; Shikama*,* 1998). Part of this enhancement of autoxidation at a low pH comes from the increased dissociation of the tetramer to dimers for mammalian haemoglobins (Tsuruga *et al.,* 1998) and possibly full dissociation of fish haemoglobin to monomers (Manning *et al.,* 1998). This high sensitivity of some fish haemoglobin components for protons is believed to be due to the presence of positive charges at the subunit interfaces in which protonation cause a dramatic shift in the spatial arrangement of the subunits (Mylvaganam *et al.,* 1996). Dissociation is also accomplished as the protein is diluted, e.g., on erythrocyte lysis (Hargrove *et al.,* 1997). The dissociated form is also more pro-oxidative and has an increased tendency to lose its haem (Benesch and Kwong, 1995). The presence of preformed lipid hydroperoxides and other oxidation products may also cause an increase in the autoxidation of haemoglobin (Lynch and Faustman, 2000; Everse and Hsia, 1997). Deoxygenation is furthermore favored at low pH, and this phenomenon may play an important role in oxidation as the pH is lowered (Richards and Hultin, 2000). Formation of the reactive $HOO^{\bullet}$ and very reactive $HO^{\bullet}$ from superoxide is favored as

the pH is lowered. The former can easily penetrate into and through lipid bilayers, while $HO^{\bullet}$ cannot due to its high reactivity. Both $HOO^{\bullet}$ and $HO^{\bullet}$ can participate in lipid oxidation (Hultin and Kelleher, 2000). The stability of the haem in the protein is also compromised as the pH is lowered likely due to opening of the haem crevice (Falcioni *et al.,* 1978), which may lead to increased exposure of the haem to fatty acids or its partition into membrane bilayers (Rao *et al.,* 1994; Atamna and Ginsburg, 1995).

Lipid peroxidation catalyzed by trout haemoglobin was found to increase when the pH was lowered from neutrality to 6.0, and levels of deoxyhaemoglobin and methaemoglobin were higher at pH 6.0 than at pH >7 (Richards and Hultin, 2000). The affinities for oxygen of haemoglobins from different species of fish, poultry, and beef at pH values below or at neutrality may be different (Richards *et al.,* 2002). Factors affecting oxy/deoxygenation of haem proteins during post-mortem periods may be crucial to understanding lipid oxidation and fish rancidity development. The large pH shifts that occur during acid or alkali solubilization can have an impact on both the pro-oxidative properties of haemoglobin and the susceptibility of the muscle to lipid oxidation. It was shown that after treatment at pH 3, washed cod muscle became slightly more susceptible to lipid oxidation, while alkaline treatment slightly protected the muscle from trout haemoglobin-mediated lipid oxidation (Kristinsson and Hultin, 2004). The same authors demonstrated that exposure of trout haemoglobin to low pH increased its pro-oxidative properties. Richards and Hultin (2000) showed that there was rapid lipid oxidation of washed cod muscle at pH 3.5 catalyzed by trout haemoglobin, while there was a considerable lag phase and a slower rate of oxidation at pH 7.8. Pazos *et al.* (2005a) reported that in washed cod muscle a decrease in pH from 7.6–6.4 decreased the lag phase and increased the rate of lipid oxidation. A further decrease in pH to 3.5 decreased the lag phase and increased the rate further.

1.2.4.3 Retardation of haemoglobin mediated lipid oxidation by natural phenolic extracts

There has been a growing interest in the use of natural phenolic antioxidants to enhance the oxidative stability, maintain or improve the intrinsic quality and nutritional value of seafood products (Pazos *et al.,* 2005b). The antioxidant effects of various plant extracts as well as individual phenolic compounds have been evaluated and tested in different fish model systems. Green tea and tea catechins were reported as excellent antioxidants toward lipid oxidation in a cooked ground mackerel model system (He and Shahidi, 1997). The antioxidant effects of tea catechins were comparable to or even better than those of synthetic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroxyquinone (TBHQ) and the natural antioxidant α-tocopherol. A flavonol-enriched fraction produced from cranberry juice powder and white grape dietary fiber concentrate were also found to provide excellent protection against haemoglobin induced lipid oxidation in washed cod muscle model (Sanchez-Alonso *et al.,* 2007). A well-designed model system comprising washed fish muscle and added haemoglobin as oxidation catalyst has been widely employed for oxidation study of fish muscle (Decker *et al.,* 2005; Richards and Hultin, 2000). It has intact myofibrillar proteins and membranes which closely resembles the physical structure of fish muscle. Compared with other model systems such as bulk oil, emulsions or microsomes, the performance of candidate antioxidant in washed fish model can provide more accurate predictions of its antioxidant effectiveness in real fish muscle system. A number of potent antioxidant compounds have been isolated and identified from different types of seaweeds, including phlorotannins, sulphated polysaccharides, carotenoid pigments such as fucoxanthin and astaxanthin, sterols, catechins and mycosporine-like amino acids (MAAs) (Miyashita and Hosokawa, 2008). Algal polyphenols, especially phlorotannins (polyphloroglucinol phenolics) derived from brown algae have recently become a focus of intensive research due to their superior antioxidant properties. The brown algae (Phaeophyta) *Fucus vesiculosus* (Linnaeus) extract was tested towards haemoglobin-mediated lipid oxidation in washed cod muscle and cod protein isolate (Wang *et al.,* 2010). In both washed cod muscle and protein isolates, phlorotannin-

enriched ethyl acetate (EtOAc) fraction from *F. veisculosus* showed higher inhibitory effect than crude 80% ethanol (EtOH) extract (Wang *et al.,* 2010). The addition of oligomeric phlorotannin-rich subfraction (LH-2) from *F. veisculosus* separated by Sephadex LH-20 chromatography completely inhibited the initiation of lipid peroxidation in both systems throughout the entire study period (8 days). Its effectiveness at 300 mg/kg level was comparable to that of 100 mg/kg propyl gallate (PG), a highly effective synthetic antioxidant in muscle foods (Wang *et al.,* 2010).

Natural phenolic extract prepared from cranberry have been shown to retard the lipid oxidation mediated by haemoglobin (Lee *et al.,* 2006). Fractions from cranberry enriched in phenolic acids (Fraction 1), anthocyanins (Fraction 2), flavonols (Fractions 3 and 4) and proanthocyanidins (Fractions 5 and 6) were prepared from cranberry powder using Sephadex LH-20 chromatography and tested to inhibited the haemoglobin mediated lipid oxidation in washed cod muscle. Addition of cranberry fractions at a level of 74 mol quercetin equivalents per kg of washed cod muscle extended the induction time of thiobarbituric acid reactive substances (TBARS) formation (Lee *et al.,* 2006). The ability of the different cranberry fractions to scavenge DPPH radicals did not reflect their relative ability to inhibit lipid oxidation in the washed cod muscle system. Quercetin was tentatively identified as a component in cranberry that was especially effective in inhibiting haemoglobin mediated lipid oxidation (Lee *et al.,* 2006). The ability of flavonol and proanthocyanidin-enriched fractions to inhibit haemoglobin-mediated lipid oxidation in spite of efforts to wash away the added polyphenolics prior to haemoglobin addition indicated that these classes of polyphenolics had binding affinities for insoluble components of washed cod muscle. Isolated fractions from a crude cranberry extract were more effective at inhibiting haemoglobin-mediated lipid oxidation than the crude extract (Lee *et al.,* 2006). This demonstrates the potential utility of isolating certain classes of polyphenolics from a crude plant extract to increase efficacy (Lee *et al.,* 2006).

White grape dietary fiber concentrate (WGDF) was found to be effective against haemoglobin-mediated oxidation of washed cod mince, with and without 10% added herring oil during iced storage (Sanchez-Alonso *et al.,* 2007). The

addition of WGDF to the model system completely and significantly inhibited the haemoglobin-mediated development of rancid odor during the entire ice storage (10 days) (Sanchez-Alonso *et al.,* 2007). Controls fortified with 10% herring oil were oxidized at the same rate as oil-free controls and were also significantly stabilized by 2% WGDF. Adding 2-4% whole WGDF, or an ethanol extract to a lean or fatty minced fish product appeared to be a promising way of preventing rancidity during ice storage (Sanchez-Alonso *et al.,* 2007).

1.2.5 Factors affecting lipid oxidation

The extent of oxidation can be influenced by both intrinsic and extrinsic factors such as the concentration of prooxidants, endogenous ferrous iron, myoglobin, enzymes, pH, temperature, oxygen consumption reaction and the fatty acid composition of the meat (Castell *et al.,* 1965; Nawar, 1996; Slabyj and Hultin, 1984; Undeland *et al.,* 2003). Fish, in particular, contain higher levels of unsaturated lipids than those of mammals and birds. Thus, fish lipids undergo more rapid oxidation after capture, even at low temperature storage (Foegeding *et al.*, 1996; Pacheco-Aguilar *et al.,* 2000). Pacheco-Aguilar *et al.* (2000) reported that the shelf-life of oily Monterey sardine was limited by lipid oxidation, as shown by the increase of peroxide value (PV) during storage at 0ºC up to 15 days. Sohn *et al.* (2005) reported that the total lipid hydroperoxide content of Pacific saury (*Cololabis saira*), Japanese Spanish mackerel (*Scomberomorus niphonius*) and chub mackerel (*Scomber japonicus*) tended to increase in both dark and ordinary muscle throughout 4 days of iced storage.

The concentration of ferrous iron and its ability to activate the lipid oxidation reaction will be a key factor causing differences in lipid oxidation among species. In general, dark meats tend to have more reactive iron. Chaijan *et al.* (2004) reported that lipid and myoglobin contents were higher in dark muscle than in ordinary muscle of both sardine and mackerel. Other constituents of meat including enzymatic and non-enzymatic reducing systems can accelerate oxidation by converting iron from the inactive ferric form to the active ferrous state (Foegeding *et al.,* 1996). Changes of PV, conjugated diene and TBARS in sardine muscle indicated that lipid oxidation occurred throughout 15 days of iced storage. Apart from a plenty of unsaturated fatty

acids, haem protein as well as reactive iron in the muscle contributed to the accelerated oxidation (Chaijan *et al.,* 2006).

Like most chemical reactions, lipid oxidation rates increase with increasing temperature and time. Saeed and Howell (2002) reported that the rate of lipid oxidation in frozen Atlantic mackerel increased with increasing storage time and storage temperature. NaCl is also able to catalyze lipid oxidation in muscle tissue (Nambudiry, 1980). Alternatively, the Na^+ may replace iron from a cellular complex via an ion exchange reaction (Kanner and Kinsella, 1983). The displaced iron may then participate in the initiation of lipid oxidation. It is most likely that meat or meat products containing salt such as surimi and cured meat are susceptible to lipid oxidation (Chaijan, 2008).

The living cells possess several protection mechanisms directed against lipid oxidation products. Glutathione peroxidase reduces hydroperoxides in the cellular membranes to the corresponding hydroxy-compounds. This reaction demands supply of reduced glutathione and will therefore cease post mortem changes when the cell is depleted of that substance. The membranes also contain the phenolic compound, α-tocopherol (Vitamin E), which is considered as the most important natural antioxidant. Tocopherol can donate a hydrogen atom to the radicals $L^•$ or $LOO^•$ functioning as an antioxidant. It is generally assumed that the resulting tocopheryl radical reacts with ascorbic acid (Vitamin C) at the lipid/water interface, regenerating the tocopherol molecule.

1.2.6 Impact of lipid oxidation on rancidity

Fatty acid hydroperoxides produced during propagation do not have a direct adverse effect on the flavor and aroma of foods. However, lipid hydroperoxide decomposition produces alkoxyl radicals ($LO^•$), which in turn can cause the decomposition of the fatty acid. β-scission reactions occur after hydroperoxides decompose into alkoxyl radicals. These highly energetic alkoxyl radicals have the ability to abstract an electron from the carbon-carbon bond on either side of the oxygen radical in order to cleave the fatty acid chain.

Hydroperoxide is a primary oxidation product during fish storage which is readily decomposed to a variety of volatile compounds including aldehydes, ketones and alcohols (Frankel *et al.,* 1994). The β-scission reaction is important because it causes fatty acids to decompose into low molecular weight, volatile compounds (Decker, 2005; Chaiyasit *et al.,* 2007). The aldehydes and ketones produced from the β-scission reaction are the source of the characteristic rancid flavors and aromas in foods (Coleman and Williams, 2007). Human olfactory receptors usually have remarkably low organoleptic thresholds to most of these volatile compounds (Ke *et al.,* 1975; McGill *et al.,* 1977).

Rancid or fishy odor has been identified as a common off-flavor associated with fish flesh and directly related with the formation of the secondary lipid oxidation products (Ke *et al*., 1975; Sohn *et al.,* 2005). Varlet *et al.* (2006) reported that carbonyl compounds, such as heptanal or (*E,Z*)-2,6-nonadienal, show a high odorant intensity in salmon (*Salmo salar*), giving the flesh its typical fishy odor. The fishy volatiles identified in the boiled sardine were dimethyl sulfide, acetaldehyde, propionaldehyde, butyraldehyde, 2-ethylfuran, valeraldehyde, 2,3-pentanedione, hexanal and 1-penten-3-ol (Kasahara and Osawa, 1998). Rancid odor is often related to a significant number of volatile compounds that can be produced from oxidation of polyunsaturated fatty acids (PUFA). Aldehydes are the main volatile secondary oxidation products responsible for off-flavors and odors during storage and treatments of foods. Several volatiles have been associated with the characteristic odors and flavors of oxidized fish, described as rancid, painty, fishy and cod-liver like (Pearson *et al.,* 1977). Oxidation of unsaturated fatty acids in fish was related to the formation of *E*-2-pentenal, *E*-2-hexenal, *Z*-4-heptenal, (*E*,*E*)-2,4-heptadienal and 2,4,7-decatrienal (Frankel, 1998a). Other volatiles formed during oxidation of fish lipids are 1-penten-3-ol, 1-octen-3-ol, 1, 5-octadien-3-one and 2,6-nonadienal, some of them having high odor impact (Milo and Grosch, 1993).

Fish volatiles have been conventionally analyzed by gas chromatographic (GC) techniques. Simultaneous steam distillation with solvent extraction has been employed for determining volatiles in fish muscle, but is time- and solvent-consuming, which may result in the loss or degradation of some of the volatile

compounds (Prost *et al.,* 1998). Analysis of volatiles in fish and seafood has been widely performed by several headspace techniques (Girard and Nakai, 1994; Medina *et al.,* 1999; Frankel *et al.,* 1989; Alasalvar *et al.,* 2005). Both, dynamic headspace and purge-and-trap (DHS techniques) coupled with gas chromatography have been extensively used for the analysis of aroma compounds in fish muscle and provided better sensitivity and efficacy than static head space (Iglesias and Medina, 2008).

Solid-phase microextraction (SPME) is an alternative extraction technique developed by Pawliszyn (1997) in the late 90s that combines sampling and sample preparation in one step (Arthur and Pawliszyn, 1990). It is an economical method for sample preparation before gas chromatography and provides several advantages over other well established techniques for analyzing volatiles in foods in terms of sensitivity, selectivity and suitability for routine analysis (Wardencki *et al.,* 2004). This technique, used in the headspace mode (HS-SPME), is particularly suitable for the analysis of volatiles and has been used for the analysis of flavor and freshness in several foodstuffs including seafood. The method has been applied to determine the concentration of aliphatic amines (Li *et al.,* 2004), volatiles of yellowfin tuna (Edirisinghe *et al.,* 2007), smoked fish (Guillen and Errecalde, 2002), fish stored under controlled atmospheres and microencapsulated fish oils (Wierda *et al.,* 2006) . SPME-based techniques require careful optimization and selection of several parameters having significant influence in the analyses. Variables such as the type of fiber which determines the specificity of the extraction, the sample amount, the time and temperature of extraction, the salting-out effect or the desorption time of the fiber in the injector affect the pre-concentration efficiency. From the determination of volatiles in fish muscle by SPME-GCMS, 79 compounds were identified and 16 of them were selected (Table 1 and Fig. 3) as representatives of lipid oxidation in fish muscle (Iglesias and Medina, 2008). Volatiles in fish oil emulsion and products fortified with fish oil were detected by HS-SPME method showing a high sensitivity for detecting lipid oxidation (Iglesias *et al.,* 2007). Hexanal analysis showed an increment of 50% between the first and second day and *E*-2-hexenal showed an increment of the relation peak area/IS peak area of 81% (Iglesias *et al.,* 2007). The incubation of 0.5 g of fish oil emulsion at 60 °C during 30 min leads to the most

effective extraction of volatiles associated with lipid oxidation of fish oil emulsions. The HS-SPME method coupled with GC–MS allowed the qualitative and quantitative analysis of the volatiles derived from oxidation of real fish oil enriched foods such as milk and mayonnaise (Iglesias *et al.,* 2007).

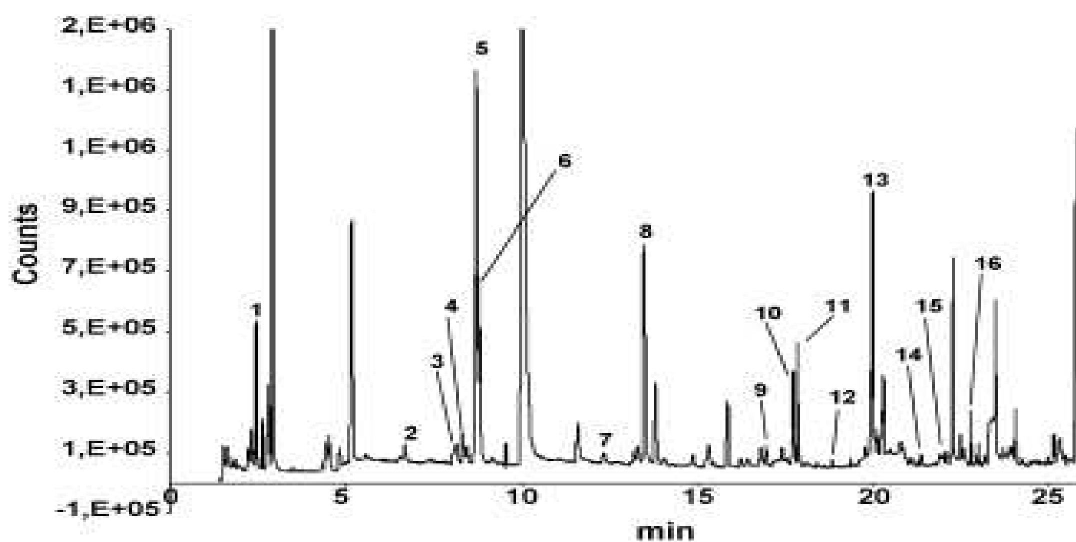


Figure 3. Chromatogram of oxidized Atlantic horse mackerel muscle. Peaks are identified as shown in Table 1.

Source: Iglesias and Medina (2008).

Table 1. Selected compounds and their SIM mass in oxidized Atlantic horse mackerel analyzed by HS-SPME

Number	Compound name	Retention time (min)	SIM mass
1	Propanal	2.51	29 + 57 + 58
2	2-Ethylfuran	6.75	53 + 81 + 96
3	1-Penten-3-one	8.30	27 + 55 + 84
4	Pentanal	8.40	29 + 41 + 44 + 57 + 58
5	1-Penten-3-ol	8.83	57 + 86
6	2,3-Pentanedione	8.85	43 + 100
7	*E*-2-Pentenal	12.34	55 + 83 + 84
8	Hexanal	13.46	44 + 56 + 72 + 82
9	*E*-2-Hexenal	17.04	41 + 55 + 69 + 83 + 98
10	Heptanal	17.78	44 + 55 + 70
11	*Z*-4-Heptenal	17.92	41 + 55 + 68
12	2-Pentylfuran	18.94	81 + 138
13	1-Octen-3-ol	20.04	52 + 72
14	(*E*,*E*)-2,4-Heptadienal	21.05	81 + 110
15	(*E*,*Z*)-2,4-Heptadienal	21.43	81 + 110
16	3,5-Octadien-2-one	22.80	81 + 95 + 124

Sources: Iglesias and Medina (2008).

1.2.7 Lipid oxidation in muscle foods

Lipids are the most variable components in meats and there are differences between species, muscles types and age of animals. They can be categorized by their location in the muscle (Foegeding *et al.,* 1996). In adipose tissue, stored triglycerides are arranged in large globules within the cell and they vary greatly in amount and composition within species and as a function of diet. In muscle tissue, muscle lipids are integral parts of various cellular structures, including the cell membrane, mitochondria and microsomes. These intracellular fats include most of the phospholipids of the tissue and have partial association with proteins (Erickson, 2002). In fatty fish muscle, fat occurs as extra-cellular droplets in the muscle tissue. In red muscle, distinct fat droplets exist within the cells, whereas in white muscle, the fat is well dispersed outside the muscle cells (Foegeding *et al.,* 1996). One of the major causes of quality deterioration in muscle foods is lipid oxidation (Erickson, 2002). The level of lipid present does not determine the oxidative susceptibility of the muscle. The type of lipid and its reactivity determines the occurrence of lipid oxidation in muscle foods (Frankel, 2005). Lean beef muscle consists of about 2-4% triacylglycerols and 0.8-1% phospholipids containing 44% polyunsaturated fatty acids. *In vitro* studies of individual lipid classes indicate their oxidative stability of a food system. Free fatty acids oxidize faster than triacylglycerols, while the reactivity of membrane lipids is greater than that esterified triacylglycerols (Erickson, 2002). Fish muscle is more susceptible to lipid oxidation than beef because of their higher polyunsaturated phospholipid fraction (Frankel, 2005). Fish muscle is noticeably different than mammalian or avian muscles because they contain a larger percentage of unsaturated fatty acids making them the most unstable towards lipid oxidation (Erickson, 2002; Foegeding *et al.,* 1996).

The presence of antioxidants and/or catalysts and food processing operations can alter the oxidative stability of muscle foods (Lee *et al.,* 1997). Antioxidants are the most important defense mechanism for lipid oxidation in muscle foods. They inhibit oxidation by reducing the rate of oxidation or by reducing the maximal level of oxidation (Erickson, 2002). In muscle foods, many oxidative reactions occur in the aqueous environment, hence, water-soluble antioxidants should

be highly effective (Decker, 1998b). Free radical scavengers have shown to be effective inhibitors of meat flavor deterioration (Wu and Brewer, 1994). Prooxidants in muscle foods include transition metal ions, singlet oxygen generation systems and enzymic initiation systems (Erickson, 2002). Biological oxidation is due almost exclusively to metal ion-promoted reactions (Kanner, 1992). All food products are subjected to numerous processing treatments prior to storage. During processing, an opportunity to alter patterns of oxidation arises. Mincing of muscle tissues disrupts cellular integrity and exposes more of the lipids to prooxidants. It will also dilute the antioxidant concentration, increase the exposure of tissue to oxygen, and increase surface area, accelerating oxidation process (Erickson, 2002). Heating modifies lipid oxidation by dislodging iron from haem compounds, disrupting cellular integrity, breaking down preexisting hydroperoxides and inactivating enzymes associated with lipid oxidation (Erickson, 2002; Watts, 1962). Iron is essential for all living things because it is necessary for oxygen transport, respiration and the activity of many enzymes (Decker and Hultin, 1992). Muscle foods contain the prominent amounts of iron, a known pro-oxidant. Lipid oxidation can be initiated by the presence of soluble low molecular weight iron, which produces hydroxyl radicals from hydrogen peroxide (Decker and Hultin, 1992; Wu and Brewer, 1994).

During fish processing and storage, quality may decline as a result of several reactions affecting both protein and lipid fractions and decreasing the nutritional and sensory properties of the product. Indeed, due to their high level of unsaturated long chain polyunsaturated fatty acids, fatty fish products are very susceptible to oxidation. Lipid oxidation in fish starts immediately after the harvest and during the processing, making the fish smell rancid. Thiobarbituric acid reactive substances (TBARS) was higher in the minced fillets than in the mince because less subcutaneous fat and dark muscle were removed during hand-mincing, indicating that the settings of the skinning-deboning machine can strongly influence the final quality of the product (Eymard *et al.,* 2005). The total lipid hydroperoxide content and TBARS of the yellowtail (*Seriola quinqueradiata*) dark muscle were higher than those of the ordinary muscle during 2 days of iced storage. Those changes were accompanied with the increasing intensity of fishy, spoiled and rancid off-odor as well

as increasing metmyoglobin formations which results in discoloration. However, no correlation was found between the content of total lipid hydroperoxide and the odor intensities in ordinary muscle (Sohn *et al.*, 2005). Antioxidant is capable of delaying, retarding or preventing the development of rancidity or other flavor deterioration due to oxidation (Gordon, 1990). The rate of PV increase was significantly higher in the herring-fillets stored for 6 and 9 day than in fillets stored for 0 and 3 days on ice (Undeland and Lingnert, 1999). It is therefore reasonable to assume that critical changes increasing the susceptibility toward lipid oxidation took place in the fillets between 3 and 6 days on ice (Undeland and Lingnert, 1999). Kolakowska (1981) observed that mince produced from 2-day Baltic herring had a faster increase in oxidation products during subsequent frozen storage than herring stored for a period longer or shorter than 2 days. It cannot be excluded that compounds arising from protein changes or lipid hydrolysis had reached levels at propagation stage of oxidation at which they might possess an inhibiting effect (Undeland and Lingnert, 1999). The rate of degradation process of this kind leads to protein alteration in particular, however, it has a large impact on lipid oxidation dynamics, both positively and negatively. It has been suggested that the production of protein radicals is crucial to the initiation of lipid oxidation (Srinivasan and Hultin, 1995) and that proteolysis brings lipids and catalysts, which are normally separated, in close contact with each other (Harris and Tall, 1989). Both ice storage and frozen storage significantly promoted all of the oxidation products measured. However, the larger effect arose from storage on ice, which stresses the importance of limiting the holding on ice of herring prior to frozen storage (Undeland and Lingnert, 1999). None of the oxidation products were significantly affected by the interaction between ice and frozen storage. The development of rancid odour further supports a minimisation of the ice storage period. Rancidity in herring fillets could be detected sensorially after 2.5 days on ice (Undeland and Lingnert, 1999). The development of lipid oxidation during storage seemed to depend on the composition of the mince matrix and its initial oxidative status (Eymard *et al.,* 2009). The compounds resulting from lipid oxidation can modify proteins by inducing cross linking, resulting in modifications of amino acids of nutritional interest and a decrease in protein functionality (solubility, hydrophobicity) due to protein denaturation (Pokorny *et al.,* 1976).

1.2.8 Antioxidants

Antioxidants are substances, synthetic or naturally occurring, that can delay the onset or slow the rate of oxidation of autoxidizable materials. Antioxidants are regarded as compounds capable of delaying, retarding or preventing autoxidation processes. According to the USDA Code of Federal Regulations, “antioxidants are substances used to preserve food by retarding deterioration, rancidity or discoloration due to oxidation” (Dziezak, 1986). Synergists are substances that enhance the activity of antioxidants without possessing antioxidant activity of their own (Nawar, 1996). It has been suggested that an ideal food-grade antioxidant should be safe, not impart color, odor or flavor, be effective at low concentrations, be easy to incorporate, survive after processing, and be stable in the finished product (carry through) as well as available at a low cost (Coppen, 1983).

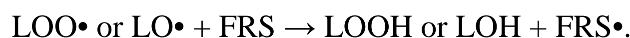
The activity of antioxidants is strongly influenced by numerous factors. Thus, compounds that are effective antioxidants in one system may be unsuitable in other systems. Some factors influencing antioxidant activity are the nature of the lipid substrate, the hydrophilic-lipophilic balance of the antioxidant, physical and chemical environments and interfacial interactions (Chang *et al.,* 2003; Porter, 1993). Antioxidants act at different levels in the oxidative sequence involving lipid molecules. They may decrease oxygen concentration, intercept singlet oxygen, prevent first-chain initiation by scavenging initial radicals such as hydroxyl radicals, bind metal ion catalysts, decompose primary products of oxidation to non-radical species and break chains to prevent continued hydrogen abstraction from substrates (Shahidi, 2000, 2002).

According to their mechanism of action, antioxidants can be classified as primary or secondary antioxidants. Primary antioxidants are chain breaking antioxidants and can inhibit lipid oxidation by interfering at the propagation or initiation phase or in β-scission reactions by accepting free radicals to form stable free radicals. Secondary antioxidants are considered preventative antioxidants, such as chelators, oxygen scavengers and singlet oxygen quenchers. These antioxidants decrease the rate of oxidation through numerous mechanisms; however, they do not

convert free radicals into more stable products (Chaiyasit *et al.,* 2007; Frankel, 2005). Antioxidants can inhibit lipid oxidation by numerous mechanisms.

1.2.8.1 Free radical scavengers and chain breaking antioxidants

Free radical scavengers and chain breaking antioxidants have the ability to slow or inhibit oxidation by interfering with either chain initiation and/or propagation. The following reaction demonstrates the ability of free radical scavengers (FRS) to interact with either peroxyl (LOO•) or alkoxyl (LO•) radicals (Decker, 2005; Frankel, 2005):



LOO• or LO• + FRS → LOOH or LOH + FRS•.

Peroxyl radicals are found in the greatest concentration of all radicals in a system and have lower energy than other radicals. Therefore, they preferentially react with the low energy hydrogens of the free radical scavenger rather than the unsaturated fatty acid, resulting in the formation of a free radical scavenger radical (FRS•). The resulting low energy FRS• will be less likely to catalyze the oxidation of unsaturated fatty acids. The inactiviation of the FRS• occurs during a termination reaction with another FRS• or lipid radical (Decker, 2005; Buettner; 1993; Frankel, 2005). Free radical scavengers can be physically classified into two groups: 1. hydrophilic (water loving/polar) and 2. lipophillic (oil loving/non-polar). The difference in the behavior of these two types of FRS in food systems is referred to as the *antioxidant polar paradox*. The premise of this theory is based on the observation that, in emulsified oils, non-polar FRS are more effective than polar FRS, while polar FRS are more effective than non-polar FRS in bulk oils (Frankel, 2005; Chaiyasit *et al.,* 2007; Porter, 1993; Decker, 1998b). The key to this phenomenon is the ability of the FRS to concentrate where lipid oxidation is most prevalent. Polar FRS concentrate at oil-air or oil-water interfaces in bulk oils, where the majority of oxidation occurs due to high concentrations of oxygen and prooxidants. In emulsions, non-polar FRS accumulates in the lipid phase and at the oil-water interface where interactions between hydroperoxides at the droplet surface and prooxidants in the aqueous phase occur (Decker, 1998b; Chaiyasit *et al.,* 2007; Decker, 2005).

To be used in food applications, synthetic FRS must be sufficiently active enough to be used at low concentrations (below 0.02%) and are not toxic. They must also be stable to processing and cooking conditions. Compared to natural FRS, synthetic FRS are more effective, can be used at lower concentrations, are less expensive and can be prepared with consistent quality without an effect on flavor, color and aroma of the food product (Frankel, 2005; Pokorny, 2007). However, synthetic FRS are "label unfriendly" additives (Chaiyasit *et al.,* 2007). Some of the most commonly used synthetic FRS in food systems are propyl gallate, butylated hydroxyl toluene (BHT), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) (Decker, 2005; Frankel, 2007). In the past couple of decades; use of natural FRS has increased due to worries about the possible hazardous effects of synthetic FRS and also current trends against the use of regulated/artificial food additives. The benefits of using natural FRS include GRAS (generally recognized as safe) status, allowance to use higher concentrations and worldwide acceptance. The negative side of natural FRS includes wide variation in concentration of active components due to source and extraction methods and undesirable effects on flavor, color and aroma of foods (Frankel, 2005; Pokorny, 2007).

Potentially active compounds from natural sources such as fruit, herbs, roots, bark and leaves have been extensively studied, since there is much interest on their FRS activity in relation to human health. Natural compounds that possess FRS activity are polyphenols, such as flavonoids, bioflavonoids, isoflavones, and tannins, as well as some vitamins including vitamin A, C and E. The role of these compounds is to interrupt the free radical chain reaction involved in oxidation. Polyphenols have strong FRS properties which can help protect cells against adverse effects of reactive oxygen species, free radicals and pro-oxidative metal ions (Dufresne and Farnworth, 2001; Aviram *et al.,* 2002). Of the tea catechins, green tea extracts have been found to have higher phenol content and greater chain-breaking activity than black tea extracts (Manzocco *et al.,* 1998). Carotenoids, found in fruits and vegetables, are another major group of natural compounds which have FRS properties. Lycopene has a high FRS potential due to its capacity to inactivate free radicals in lipid phases (Sies *et al.,*

1992; Ribeiro *et al.,* 2003) and by interfering with reactions of damaging oxidizing agents and free radicals (Ribeiro *et al.,* 2003; Henry *et al.,* 1998).

1.2.8.2 Metal inactivators and chelators

Metal inactivators and chelators are compounds that can inhibit lipid oxidation by mechanisms that do not involve the deactivation of free radical chains. These metal inactivators decrease the ability of metal ions to promote initiation reactions and the decomposition of hydroperoxides into secondary aldehydes (Frankel, 2005, Pokorny, 2007). Chelators inhibit metal-catalyzed reactions by: prevention of metal redox cycling, formation of insoluble metal complexes, steric hindrance of metal-lipid interactions or oxidation intermediates (e.g. hydroperoxides) and/or occupation of all metal coordination sites (Decker, 1998a). Most chelators accumulate in the aqueous phase of foods, however, in order to inactivate lipid-soluble metals, some chelators must also partition into the lipid phase (Decker, 2005). Conversely, under certain conditions, some chelators can increase metal solubility or alter the redox potential of metals, thus increasing oxidative reactions (Decker, 1998a). Ethylenediamine tetraacetic acid (EDTA), one of the most effective metal chelators, along with citric, tartaric and phosphoric acids are compounds which can deactivate metals by forming stable coordination complexes with pro-oxidant metals, thus effectively inhibiting both metal-catalyzed initiation and decomposition of hydroperoxides (Frankel, 2005). However, the antioxidative and/or pro-oxidative properties of metal chelators are often concentration dependant. It has been found that, when present at an EDTA: iron ratio of > 1, EDTA will perform as a strong metal chelator, in contrast, at an EDTA: iron ratio of ≤ 1, EDTA can behave as a prooxidant (Mahoney and Graf, 1986).

1.2.8.3 Singlet oxygen quenchers

The use of quenching agents is an effective way to reduce singlet oxygen oxidation. Quenching agents may decrease singlet oxygen promoted oxidation by quenching the excited triplet sensitizer or singlet oxygen by chemical or physical means. Chemical quenching involves the reaction of singlet oxygen with the quenching agent to produce stable products. Physical quenching returns singlet oxygen to triplet oxygen without the consumption of oxygen and any chemical changes of quenching agent (Min and Boff, 2002; Frankel, 2005). Natural food components such as carotenoids, tocopherols and ascorbic acid have been found to be effective quenching agents (Min and Boff, 2002). Carotenoids can chemically quench singlet oxygen when the singlet oxygen attacks the double bonds of the carotenoid, resulting in carotenoid breakdown products such as aldehydes and ketones. Physical quenching does not lead to breakdown products. During physical quenching, there is a transfer of energy from the singlet oxygen to the carotenoid, producing an excited state carotenoid and ground state triplet oxygen. The energy from the excited carotenoid is dissipated by vibrational and rotational interactions with the surrounding solvent to return it to the ground state (Decker, 2005). Lycopene has been found to be one of the most efficient singlet oxygen quenchers of the biological carotenoids (Di Mascio *et al.,* 1989).

1.2.8.4 Lipoxygenase inhibitors

The use of inhibitors can be another means to lower or prevent the enzymatic oxidation induced by lipoxygenase. The green tea extract showed inhibitory effects on both LOX-catalyzed and haemoglobin-catalyzed oxidation of arachidonic acid and linoleic acid (Liu and Bonnie, 2004). Similar effect of green tea extract was found in LOX from tilapia and grey mullet. Catechin mixtures prepared from tea effectively prevented the prooxidant activity of lipoxygenase (LOX) in fish skin extract (Mohri *et al.,* 1999). The typical application of green tea polyphenols (GTPs) significantly inhibited epidermal cyclooxygenase (COX) and LOX (E.C. 1.13.11.12), which are used as markers of skin tumor promotion (Huang *et al.,* 2005). The gill LOX pretreated with green tea extract (GTE) showed a 90.2% inhibition of tilipia gill

LOX and a 76.7% inhibition of grey mullet gill LOX (Liu and Bonnie, 2004). GTE can inhibit both the LOX-catalyzed dioxygenation and the autoxidation of the polyunsaturated fatty acids (PUFA) (Liu and Bonnie, 2004). Green tea polyphenols have potent free radical quenching and antioxidant activities (Wiseman *et al.,* 1997) and have structural features that may specifically interfere with the arachidonic acid cascade, including the LOX pathway (Hussain *et al.,* 2005). Green tea polyphenols are very potent inhibitors of mackerel LOX, with EGCG (epigallocatechin gallate) as the most effective inhibitor (IC_{50} 0.13 nM) followed by ECG (epicatechin gallate) (IC50 0.8 nM), EC (epicatechin) (IC_{50} 6.0 nM), EGC (epigallocatechin) (IC50 9.0 nM) and Ct (catechin) (IC50 22.4 nM) (Banerjee, 2006). Both animal and plant LOXs are inhibited by phenolic compounds, mainly phenolic acids and their related esters and flavonoids, which are naturally occurring antioxidants (Kohyama *et al.,* 1997). Therefore, antioxidants such as flavonoids which act as free radical quenchers (Zhou *et al.,* 2005) may act as LOX inhibitors. In addition, it has been proposed that the inhibitory effects of antioxidants depend on the physico-chemical state of the substrate and the type of LOX (Noguchi *et al.,* 2002).

1.2.8.5 Multiple antioxidant functions

Antioxidant compounds may reinforce each other in multi-component systems by cooperative effects known as “synergism”. Synergism imparts more protection against lipid oxidation than the sum of the activities of the compounds when used separately (Coleman and Williams, 2007). In addition, the use of synergistic antioxidant mixtures can allow for a reduction in the concentration of each antioxidant (Abdalla and Roozen, 1999). If both initiation and propagation are suppressed, successful synergistic inhibition can be achieved. A commonly used combination of synergistic compounds in foods is pairing of metal inactivators with chain breaking antioxidants (Nawar, 1996; Frankel, 2005). An example of synergism between two compounds is the combined antioxidative effect of ascorbic acid and butylated hydroxy toluene (BHT). Ascorbic acid has the capability to chelate metals, therefore limiting their ability to initiate lipid oxidation. BHT, a phenol and a chain breaking antioxidant, has been shown to be much more effective at retarding lipid oxidation in the presence of ascorbic acid (Coleman and Williams, 2007). Recently,

the synergistic antioxidant effect between rosemary extract and BHT was studied and it was found that a comparable antioxidant activity of rosemary, BHT and α-tocopherol was considerably higher than BHT (Romano *et al.,* 2006). This indicated the positive interaction between rosemary extract and BHT on increasing the total antioxidative activity. Rosemary methanolic extract enhances the antiradical efficiency of BHT through synergistic interactions (Romano *et al.,* 2006). Positive interactions of the rosemary methanolic extract with ascorbic acid and α-tocopherol on antioxidative efficiency was also reported by Romano *et al.* (2006). Several studies have shown that plant polyphenols have a synergistic effect with other antioxidants present in plant material (Graversen *et al.,* 2008). Lettuce extract had a clear antioxidative effect as evidenced by a lag phase for formation of conjugated dienes and α-tocopherol and especially quercetin acted synergistically in prolongation of the lag phase both following initiation in the lipid phase and in the aqueous phase (Altunkaya *et al.,* 2009). Antioxidants localised at or near the interface of the liposomes such as quercetin and α-tocopherol acted synergistically with lettuce extract as an antioxidant, while the hydrophilic antioxidant ascorbic acid showed no synergism (Altunkaya *et al.,* 2009). Synergistic interactions with respect to antioxidant activity and biological functions can also be found between flavonoids such as soy and green tea (Bertipaglia de Santana *et al.,* 2008).

1.2.8.6 Phenolic antioxidant from natural sources and their antioxidative activity

Different phenolic compounds with antioxidative activity from various natural sources have been reported. Flavonoids are the most widely studied class of polyphenols with respect to their antioxidant and biological activities. They have powerful antioxidant activities *in vitro*, being able to scavenge a wide range of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and chlorine species, such as superoxide, hydroxyl and peroxyl radicals, and peroxynitrous acid and hypochlorous acid (Hernandez *et al.,* 2009).

Green tea is the most abundant source of tea polyphenols, mostly in the form of gallic acid and its catechin derivatives. The major green tea catechins are epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC). In general, approximately 30% of the dry weight of green tea is catechin (Ho *et al.*, 2009). EGCG, ECG, EGC, theaflavin digallate (TF-2), theaflavin monogallate (TF-1), and theaflavin (TF) showed higher DPPH radical- and superoxide-scavenging abilities than carnosol, carnosic acid, and BHT (Chen and Ho, 2007).

A wide range of plant polyphenols or plant extracts have been evaluated for their antioxidant properties in unsaturated marine oils, meat and fish model systems (He and Shahidi, 1997). Flavonoid components obtained from grape by-products were effective in retarding lipid oxidation in different systems containing fish lipids, including bulk oil, oil-in-water emulsion and frozen fish muscle (Pazos *et al.,* 2005b). Grape polyphenols are also reported to have potential in preventing lipid oxidation in various food systems. Cranberries are a good source of phenolic compounds including flavonols, anthocyanins and proanthocyanidins (Chen *et al.,* 2001). The chloroform extract of cranberries at low levels (0.01, 0.05, 0.1 and 0.2%) was highly inhibitory towards lipid oxidation in mechanically separated turkey (MST) (Kathirvel *et al.,* 2009). Grape antioxidant dietary fibre (GADF) containing high amounts of dietary fibre and phenolics antioxidants such as phenolics acids, anthocyanidins, proanthocyanidins, catechins and other flavonoids, was successfully used as an ingredient in minced fish (Sanchez-Alonso *et al.,* 2007). Polyphenols derived from brown seaweeds are also potent ferrous ion chelators (Chew *et al.,* 2008) and their metal chelating potency depends upon their unique phenolic structure and the number and location of the hydroxyl groups present in the molecules (Santoso *et al.,* 2004). Medicinal plants from Leguminosae family are high in polyphenol contents (Chew *et al.,* 2009). Rutin was found to be abundant in *C. pulcherrima* leaves. A positive correlation was found between TPC and various antioxidant activities assessed in *in vitro* systems, indicating that polyphenols could be the major contributors to free radical scavenging and reducing ability of the extracts (Chew *et al.,* 2009). A high lipid peroxidation inhibition activity of extracts suggested that their

antioxidants from medicinal plants belonging to Leguminosae family could be utilised as alternative food ingredients and preservatives. Different antioxidative compounds have been identfied in the various natural products as shown in Table 2.

Table 2. Phenolic antioxidant from various natural sources.

Sources	Antioxidative compounds	References
1. Fruits		
Berries	Flavanols, hydroxycinammic acids, hydroxybenzoic acids and anthocyanins	Hakkinen *et al.,* 1998, Belitz and Grosch, 1999, Wang and Lin, 2000, Yanishlieva-Maslarova and Heinonen, 2001, and Manach *et al.,* 2004.
North European berries	Trans-resveratrol, cinnamic acid, ferulic acid, p-coumaric acid, quercetin and morin	Ehala *et al.,* 2005.
Cherries	Hydroxycinnamicacids and anthocyanins	Belitz and Grosch, 1999, Yanishlieva-Maslarova and Heinonen, 2001, and Manach *et al.,* 2004.
Black grapes	Anthocyanins and flavonols	Belitz and Grosch, 1999, Yanishlieva-Maslarova and Heinonen, 2001, and Manach *et al.,* 2004.
Citrus fruits	Flavanones, flavonols and phenolic acids	Yanishlieva-Maslarova and Heinonen, 2001, Beecher, 2003, and Manach *et al.,* 2004.
Plums, prunes, apples, pears, kiwi	Hydroxycinnamic acids and catechins	Belitz and Grosch, 1999, Yanishlieva-Maslarova and Heinonen, 2001, and Manach *et al.,* 2004.
Chestnut (flower, skin leaf and fruit)	Phenolic acids and flavonoids	Barreira *et al.,* 2008.
2. Vegetables		
Aubergin	Anthocyanins and hydroxycinnamic acids	Manach *et al.,* 2004.
Chicory, artichoke	Hydroxycinnamic acids	Manach *et al.,* 2004.
Parsley	Flavones	Manach *et al.,* 2004 and Beecher, 2003.
Rhubarb	Anthocyanins	Manach *et al.,* 2004.
Sweet potato leaves	Flavonols, flavones,	Chu *et al.,* 2000.
Yellow onion, curly	Flavonols	Manach *et al.,* 2004.
Parsley	Flavones	Manach *et al.,* 2004.
Spinach	Flavonoids and *p*-coumaric acid	Bergman *et al.,* 2001.
3. Flours		
Oats, wheat, rice	Caffeic and ferulic acids	Yanishlieva-Maslarova and Heinonen, 2001 and Manach *et al.,* 2004.
4. Tea		
Black, green	Flava-3-ols and flavonols	Manach *et al.,* 2004 and Beecher, 2003.
5. Drinks		
Orange juice	Flavanols	Manach *et al.,* 2004.
Coffee	Hydroxycinnamic acids	Manach *et al.,* 2004.
Chocolate	Flavanols	Beecher, 2003 and Manach *et al.,* 2004.

Table 2 continues….

Sources	Antioxidative compounds	References
6. Herbs and spices		
Rosemary	Carnosic acid and carnosol,	Yanishlieva-Maslarova and Heinonen, 2001.
	Rosmarinic acid rosmanol	Ibanez *et al.,* 2003.
Sage	Carnosol, Carnosic acid, lateolin, rosmanul and rosmarinic acid	Yanishlieva-Maslarova and Heinonen, 2001.
	Rosmarinic acid	Zheng and Wang, 2001.
Oregano	Rosmarinic acid, phenolic acids and flavonoids	Yanishlieva-Maslarova and Heinonen, 2001. Exarchou *et al.,* 2002 and Belhattab *et al.,* 2004.
Thyme	Thymol and carvacrol,	Yanishlieva-Maslarova and Heinonen, 2001.
	Flavonoids and lubeolin	Exarchou *et al.,* 2002.
Summer savory	Rosmarinic, carnosol, carvacrol and flavonoids	Yanishlieva-Maslarova and Heinonen, 2001.
Ginger	Gingerd and related compounds	Yanishlieva-Maslarova and Heinonen, 2001.
7. Medicinal plants from Leguminosae family		.
Leaves and flowers of *Bauhinia kockiana*, *Caesalpinia pulcherrima* and *Cassia surattensis*	Flavones, flavonols, Rutin and Chlorogenic acid	Chew *et al.,* 2009
8. Beans		
Beans	Flavanols	Manach *et al.,* 2004.
9. Seaweeds and Algae		
Red and brown seaweed	Gallic acid, catechin, epicatechin, epicatechingallate, catechingallate, epigallocatechin and epigallocatechin gallate	Rodríguez-Bernaldo de Quirós *et al.,* 2010.
Brown alga; *Stypocaulon scoparium*	Gallic acid, catechin, epicatechin, rutin, *p*-coumaric acid, myricetin, quercetin and protocatechuic, vanillic, caffeic, ferulic, chlorogenic, syringic and gentisic acids.	Lopez *et al.,* 2010.
Seagrasses (*Zostera noltii* and *Zostera marina*)	Rosmarinic acid	Achamlale *et al.,* 2009.

Most of the natural plants (fruits and vegetables) containing varying amounts of phenolic compounds have been determined for antioxidative activities by *in vitro* assays. Total antioxidant capacity of various plant and beverages, assessed as Trolox – a water soluble vitamin E analogue – equivalent antioxidant capacity (mmol Trolox per kg of food or l of beverage) is shown in Table 3 (Ovaskainen *et al.,* 2008).

Table 3. Total antioxidant capacity of various plants (fruits and vegetables) in mmol Trolox/kg or l.

Food/beverage	mmol Trolox/kg or l	Food/beverage	mmol Trolox/kg or l
Spinach	8.5	Cola	0.1
Pepper	7.6–8.4	Black tea	3.6
Broccoli	3.0	Green tea	6.0
Carrot	0.4	Coffee (espresso)	36.5
Potato	0.8	Coffee (soluble)	32.5
Tomato	1.7	Beer (lager)	1.0
Blackberry	20.2	Cognac	1.3
Grape	2.5–3.9	Whiskey	1.7
Olive	10.4–14.7	Rum	<0.1
Pineapple	9.9	White wine	1.6–1.9
Strawberry	10.9–11.3	Rosè wine	1.5–2.4
Orange juice	3.0	Red wine	9.0–12.1

Source: Ovaskainen *et al.,* (2008).

1.2.9 Phenolic compounds as antioxidants

Natural antioxidants from dietary sources include phenolic and polyphenolic compounds. The mechanism by which these antioxidants exert their effects may vary, depending on the compositional characteristics of the food. Furthermore, the beneficial health effects of consuming plant foods have been ascribed, in part, to the presence of phenolics, which are associated with counteracting

the risk of cardiovascular diseases, cancer and cataract as well as a number of other degenerative diseases. This is achieved by preventing lipid oxidation, protein cross linking and DNA mutation and, at later stages, tissue damage (Shahidi and Naczk, 2004). Although, phenolic compounds and some of their derivatives are very efficient in preventing autoxidation, only a few phenolic compounds are currently allowed as food antioxidants. The major considerations for acceptability of such antioxidants are their activity and potential toxicity and/or carcinogenicity (Shahidi and Naczk, 2004). The approved phenolic antioxidants have been extensively studied, but the toxicology of their degradation products still is not clear. The role of polyphenolic compounds in preventing the generation of ROS and various radicals during lipid oxidation has been elucidated in Figure 4.

Some commercially produced plant phenolic compounds have recently been considered as antioxidants. Phenolic compounds such as tea catechins, grape procyanidins, rosemary extracts and olive oil hydroxytyrosol have been found to retard lipid oxidation in fish muscle-based food products (Pazos *et al.*, 2006; Tang *et al.*, 2001). Tang *et al.* (2001) reported that antioxidant potential of tea catechins added to mackerel and whiting mince was two-fold greater than that of α-tocopherol at the same concentration. Inhibition of haemoglobin-and iron-promoted oxidation in fish microsomes by phenolic compounds have been studied by Pazos *et al.* (2006). Isolated grape procyanidins expressed the highest antioxidative activity followed by propyl gallate, grape phenolic extract and olive oil hydroxytyrosol, respectively. Lee *et al.* (2006) reported that oligomeric polyphenols (e.g., proanthocyanidins) from cranberry extracts were least effective to inhibit haemoglobin-mediated lipid oxidation in washed fish muscle compared to the other classes of polyphenolics (e.g., phenolic acids, flavonols and anthocyanins). The reducing capacity or ability for donating electrons and the chelating properties may also contribute to the antioxidant activity of phenolic compounds (Pazos *et al.*, 2006). Even though many natural and synthetic compounds have antioxidant properties, only a few of them have been accepted as “generally recognized as safe” (GRAS) substances for use in food products by international bodies such as the Joint FAO/WHO Expert Committee for Food Additives (Hallagan and Hall, 1995).

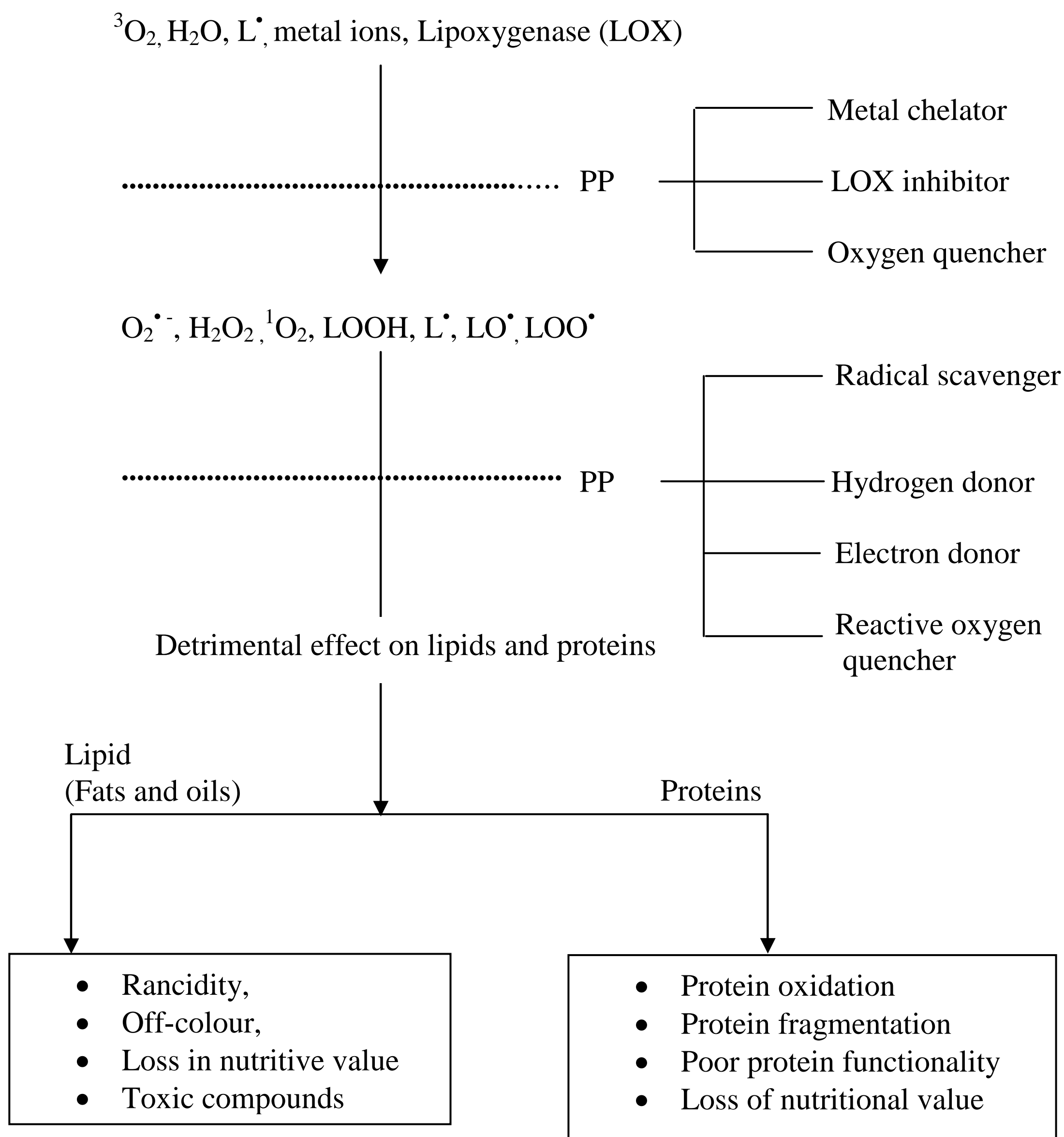


Figure 4. Elucidation of detrimental effects of lipid and protein oxidation and preventive role of polyphenols (PP).

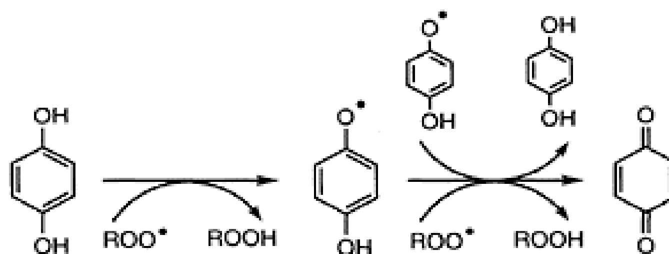
Source: Shahidi and Naczk (2004).

1.2.9.1 Antioxidative action of phenolic compounds

Phenolic antioxidants (AH) interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals. The reaction of antioxidant and lipid

radical is exothermic in nature. The activation energy increases with increasing A-H and R-H bond dissociation energy. Therefore, the efficiency of the antioxidants increases with decreasing A-H bond strength. The resulting phenoxy radical must not initiate a new free radical reaction or be subjected to rapid oxidation by a chain reaction. In this regard, phenolic antioxidants are excellent hydrogen or electron donors. In addition, their radical intermediates are relatively stable due to resonance delocalization and lack of suitable sites for attack by molecular oxygen (Sherwin, 1978; Nawar, 1996).

The phenoxy radical formed by reaction of a phenol with a lipid radical is stabilized by delocalization of unpaired electrons around the aromatic ring as indicated by the valence bond isomers.



However, phenol is inactive as an antioxidant. Substitution of the hydrogen atoms in the ortho and para positions with alkyl groups increases the electron density of the OH moiety by an inductive effect and thus enhances its reactivity toward lipid radicals. Substitution at the para position with an ethyl or *n*-butyl group rather than a methyl group improves the activity of the phenolic antioxidant; however, the presence of chain or branched alkyl groups in this position decreases the antioxidant activity (Gordon, 1990). Because these substituents increase the steric hindrance in the region of the radicals, they further reduce the rate of possible propagation reactions that may occur (Gordon, 1990).

The introduction of a second hydroxy group at the ortho or para position of the hydroxyl group of a phenol increases its antioxidant activity. The effectiveness of a 1,2- dihydroxybenzene derivative is increased by the stabilization of the phenoxy radical through intra-molecular hydrogen bond. Thus, catechol and

hydroquinone are more effective in their peroxynitrite scavenging activity than phenol (Heignen *et al.,* 2001). Similarly, flavonols containing a catechol moiety (3′- and 4′-OH) in ring B (rutin and monohydroxyethyl rutinoside) or an AC-ring with three OH groups (3-, 5-, and 7-OH) are potent scavengers. The 3-OH group is found to be the active center; its activity is influenced by electron-donating groups at the 5- and 7-positions (galangin, kaempferol, and trihydroxyethyl quercetin). In another study, Heim *et al.* (2002) found that multiple hydroxyl groups conferred substantial antioxidant, chelating, and, in some cases, pro-oxidant activity to the molecule. Methoxy groups introduce unfavorable steric effects, but the presence of double bond and carbonyl functionality in the C ring increases the activity by affording a more stable flavonoid radical through conjugation and electron delocalization. Finally, the antioxidant activity of hydroxyflavones is influenced by pH. The antioxidant potential increases, as determined by the TEAC assay, upon deprotonation of the hydroxyl group. This indicates that the mechanism of action of flavonoids is variable and, although abstraction of the hydrogen atom is involved for under protonated species, electron (not hydrogen) atom donation is involved in the deprotonated species (Lemanska *et al.,* 2001). Furthermore, the hydroxyl radical scavenging activity of phenolics involves multiple mechanisms, including hydroxyl bond strength, electron donating ability, enthalpy of single electron transfer and spin distribution of the phenoxy radical after hydrogen abstraction (Cheng *et al.,* 2002).

The effect of antioxidant concentration on autoxidation depends on many factors, including the structure of the antioxidant, oxidation conditions, and nature of the sample oxidized. Some phenolic antioxidants lose their activity at high concentrations and behave as pro-oxidants (Gordon, 1990). Antioxidant activity by donation of a hydrogen atom is unlikely to be limited to phenols. Endo *et al.* (1985) suggested that the antioxidant effect of chlorophyll in the dark occurs by the same mechanism as phenolic antioxidants. Electrostatic charge of oil droplets in emulsion and the pH have a major effect on the physical location and effectiveness of antioxidants and pro-oxidants in emulsions. Droplets stabilized with an anionic emulsifier are capable of accelerating lipid oxidation due to the electrostatic attraction that occurs between the negatively charged oil-water interface and the positively

charged metal ions present (Frankel, 2005; Mei *et al.,* 1999). The effectiveness of antioxidants can be influenced by the attractive/repulsive electrostatic interactions between charged antioxidants, which will affect their location and activity within the system. Electrostatic forces can also have an effect on charged pro-oxidants, in turn, affecting the location and activity of transition metals in the system. In addition, pH can alter the location of ionic antioxidants by altering their charge and solubility and, in addition, can alter the ionic interactions between prooxidants and other components (Mei *et al.,* 1999). Lipophillic antioxidants concentrate in the oil droplet, where lipid oxidation takes place, and are more effective in inhibiting oxidation than hydrophilic antioxidants which partition into the water phase (Decker, 1998b; Huang *et al.,* 1997). Emulsifiers can create a protective barrier around lipid droplets, deterring the penetration and diffusion of metals and radicals, which initiate lipid oxidation, into the lipid. The addition of metal chelators can decrease metal reactivity by binding metals and physically removing them from the lipid core and/or droplet interface, thus inhibiting the decomposition of lipid hydroperoxides, in turn retarding lipid oxidation (Decker *et al.,* 2002).

1.2.9.2 Preventive effect of different phenolic compounds on lipid oxidation in different systems

During the past decade, plant-derived substances have attracted a great deal of attention mainly for their ability to favor food preservation (Lugasi *et al.,* 2007). Several studies have been focused on the positive role of plant polyphenols as free radical scavengers and to control microbial growth and extend the shelf life of various food systems. Besides their antimicrobial and antioxidant activities, polyphenols may inhibit some enzymes that intensify food alteration (Banerjee, 2006). Antioxidant action is dependent on the ability of phenolics to scavenge free radicals and/or to chelate metals (Galati *et al.,* 2006). Fe binds to polyphenols via the ortho dihydroxy (catechol) or trihydroxy-benzene (galloyl) group. Tannins can also produce complexes with Fe^{+2} [$(Fe^{+2})_n$-tannic acid] (Lopes *et al.,* 1999). Thus, availability of Fe, which acts as a pro-oxidant in the lipid containing food can be reduced (Lopes *et al.,* 1999). Flavonoids are reported to act as a potential antioxidants in food lipid systems (Wanasundara and Shahidi, 1994). The effectiveness of flavonoids in

retarding lipid oxidation is related to their free-radical scavenging activity (Jovanovic *et al.,* 1994) or metal-chelating activity (Ramanathan and Das, 1993). Three structural groups are important determinants for radical-scavenging activity: the *ortho*-dihydroxy structure in the B-ring, as well as the 2, 3 double bond in conjugation with a 4-oxo function in the C-ring (Bors *et al.,* 1990). Flavonoids form binding complexes with metal ions by the 3- or 5-hydroxyl and 4-ketosubstituents or hydroxyl groups in *ortho* position in B-ring. All flavonoids (myricetin, quercetin, kaemferol, rutin and catechin) acted as antioxidants on oxidation of methyl linoleate. Quercetin and myricetin inhibited hydroperoxide formation better than α-tocopherol in methyl linoleate system. Catechin and rutin are more effective antioxidants in low density lipoprotein (LDL) oxidation than α-tocopherol (Teissedre *et al.,* 1996). In refined palm oil, myricetin, quercetin and kaemferol are more effective, but rutin and catechin were weaker than α-tocopherol. The antioxidant activity of flavonoids is generally governed by their chemical structures (Pekkarinen *et al.,* 1999). The antioxidant activity of flavonols increased as the number of phenolic hydroxyl groups in the B-ring increased. The positive relationship between increased hydroxylation and increased antioxidant activity of flavonols was reported in different lipid systems, such as bulk oils (Shahidi and Wanasundara, 1995; Wanasundara and Shahidi, 1994) and liposome system (Teissedre *et al.,* 1996).

Crude oils contain natural antioxidants making them more stable towards oxidation than refined oils where some of the natural antioxidants have been removed during the refining process (Johnson, 2002). The most preferable way to reduce lipid oxidation in refined bulk oil is the addition of antioxidants (Naz *et al.,* 2005), which has been intensively studied. As discussed previously, the "*antioxidant polar paradox*" is based on the theory that in oils with a low surface-to-volume ratio (bulk vegetable oils) polar antioxidants are more effective than non-polar antioxidants because they have the ability to concentrate at locations where oxidative reactions are the greatest (Chaiyasit *et al.,* 2007). In contrast, in oils with high surface-to-volume ratios (emulsified oils), non-polar antioxidants are strongly favored (Frankel, 2005; Frankel *et al.,* 1994; Porter, 1993; Huang *et al.,* 1997). Phenolic antioxidants are more effective in extending the induction period when added to oil that has not been

deteriorated to any great extent. However, they are ineffective in retarding decomposition of already deteriorated lipids (Mabrouk and Dugan, 1961). Thus, antioxidants should be added to foodstuffs as early as possible to achieve maximum protection against oxidation (Coppen, 1983).

A wide range of plant polyphenolics have been evaluated and tested for their antioxidant properties in unsaturated marine oils, meat and fish muscle model systems (He and Shahidi, 1997; Wanasundara and Shahidi, 1998). Phenolic compounds like caffeic acid, ferulic acid, tannic acid, etc. have proved to be efficient antioxidants in different fish model systems. Caffeic acid was demonstrated to be more active antioxidant than ferulic and *o*-coumaric acid for preventing lipid oxidation of frozen minced horse mackerel white muscle during storages at −10 and −18 °C (Medina *et al.,* 2009). The antioxidant effectiveness of two different families of phenolic compounds, hydroxycinnamic acids and catechins in chilled minced horse mackerel muscle was evaluated (Medina *et al.,* 2007). Among hydroxycinnamic acids, 10 ppm caffeic acid was highly effective in inhibiting lipid oxidation in fish muscle. Its antioxidant efficacy was similar to that of propyl gallate. Among all phenolic compounds tested, catechin showed the highest antioxidant activity. The efficacy in prevention of lipid oxidation in fish muscle using both caffeic acid and catechin (10 and 100 ppm) increased with increasing concentration (Medina *et al.,* 2007). The capacity of hydroxycinnamic acids for donating electrons appears to play an important role in retarding the development of rancidity in fish muscle. However, the ability of hydroxycinnamic acids for chelating metal ions and their distribution between oil and aqueous phases were not correlated with their inhibitory activities against oxidation (Medina *et al.,* 2007). Caffeic acid showed high antioxidant efficiency for preserving fish muscle against oxidation and could significantly inhibit the off-flavour formation and production of peroxides and TBARS (Medina *et al.,* 2007). The overall order of antioxidant efficiency of hydroxycinnamic acids was caffeic acid > ferulic acid = chlorogenic acid = *o*-coumaric acid. Thus, polyphenolic compound can effective retard lipid oxidation in fish and fish based products.

Grape seed extract has been evaluated for its antioxidative effect and has been reported to improve the oxidative stability of cooked beef (Ahn *et al.,* 2002),

turkey patties and cooled stored turkey meat (Lau and King, 2003; Mielnick *et al.,* 2006). Phenolics antioxidants such as phenolics acids, anthocyanidins, proanthocyanidins, catechins and other flavonoids were found in grape antioxidant dietary fiber (GADF) (Saura-Calixto, 1998). Recently, GADF had been successfully used as an ingredient in minced fish (Sanchez-Alonso *et al.,* 2007) because GADF exhibited relatively high antioxidant activity (Goni and Serrano, 2005) and it may be considered as antioxidant dietary fiber (Saura-Calixto, 1998). The extractable polyphenols from the grape seed contained 21% total phenolic compounds, which consisted of catechins (46.8%), benzoic acids (16%), flavonols (14%) and anthocyanidins (16.2%) (Perez-Jimenez *et al.,* 2008). When GADF was added, the chicken hamburger meat resisted to the lipid oxidation and showed lower values of TBARS during the storage (Sayago-Ayerdi *et al.,* 2009). The mechanism of the protective effect of GADF on lipid oxidation is mainly due to the presence of a number of oligomer procyanidins in GADF, such as catechin and epicatechin (Yilmaz and Toledo, 2004). Grape polyphenols have shown effectiveness in delaying lipid oxidation in minced fatty fish muscle during frozen storage (Pazos *et al.,* 2005b) and ability to preserve endogenous antioxidant systems (like vitamin E) in fish muscle (Pazos *et al.,* 2005c). In addition, Sanchez-Alonso *et al.* (2007) reported that the high levels of inhibition of lipid oxidation in minced horse mackerel during frozen storage was achieved when red grape antioxidant DF was added. Lipid oxidation in the different systems containing fish lipids including bulk oils, oil-in-water emulsions and frozen fish muscle was retarded by the flavonoid components obtained from grape by-products. The inhibition of oxidation in mackerel muscle was also achieved by natural grape components (Pazos *et al.,* 2005b). Less conjugated hydroperoxides, dienes and trienes were formed from PUFAs in samples with added white grape dietary fiber (WGDF) (2% and 4%) than in the control sample over a period of 6 months at –20 °C (Sanchez-Alonso *et al.,* 2008). Grape polyphenols could therefore be a potential source of antioxidants.

Cranberries are a good source of phenolic compounds including flavonols, anthocyanins and proanthocyanidins (Chen *et al.,* 2001; Foo *et al.,* 2000). The chloroform extract of cranberries was highly inhibitory on lipid oxidation in

mechanically separated turkey (MST) at low levels. Quercetin was largely responsible for the inhibitory potency of the chloroform extract. The amphiphilic nature of quercetin should allow some of the quercetin to partition more effectively into the membrane phospholipids, compared to less amphiphilic components in the extract. Apart from quercetin and glycosylated quercetin, proanthoanthocyanidins (PA) in cranberry fraction could be found. Cranberry PA has been shown to inhibit Cu^{2+} induced oxidation in low density lipoproteins (LDL) (Porter *et al.,* 2001). The PA were shown to bind to the lipoprotein which may explain the extended lag time prior to Cu^{2+}-induced LDL oxidation. Phenolic binding can physically orient the compound for efficient free radical scavenging or block sites on LDL, where copper needs to bind. When compared to other flavonoids, quercetin has been shown to prolong the lag time before the initiation of low density lipoprotein (LDL) oxidation effectively (Safari and Sheikh, 2003). Quercetin was able to inhibit lipid oxidation in marine oils rich in PUFA (Wanasundara and Shahidi, 1998). Heat-induced cholesterol oxidation can also be reduced by incorporation of 0.002% (w/w) quercetin (Chien *et al.,* 2006).

Grateloupia filicina is used in Korea and Japan as a food source and its antioxidant potential provides another benefit for its use in different food formulations. Seaweeds have different bioactive compounds, which are known to possess antioxidative activity in different food systems. Addition of the algal extracts to the reaction medium clearly slowed the rate of peroxide formation. *Grateloupia filicina* extracts effectively scavenged various reactive oxygen species and inhibited peroxidation of linoleic acid (Athukorala *et al.,* 2003). The algal extract was able to protect the oil from oxidation as reflected by lower TBARS formation (Athukorala *et al.,* 2003). Yan (1996) reported that *Sargassum kjellmanianurn* contained phlorotannin (brown algal phenols) which could prevent oxidation of fish oil. Algal genus Cysfoseira is a rich source of tetraprenyltoluquinols, which are α-tocopherol like compounds (Ruberto *et al.,* 2001). In addition, seaweeds contained vitamins and vitamin precursors, including a-tocopherol (Jensen, 1969), ß-carotene, niacin, thiamin, and ascorbic acid (Jensen, 1972).

The quince extract, mainly consisted of procyanidin B dimer (50.8%) and hydroxycinnamic acids (36.62%) was found to quench 2,2-diphenyl-1-

picrylhydrazyl radicals by nearly 59.3% (Fattouch *et al.,* 2008). As deduced from the lower peroxide value of the fillet fat fraction and the inhibition of thiobarbituric acid reactive substances formation, the quince extract (8.9 ± 0.4 mg phenolics/ml) was found to prevent fish oil from oxidative deterioration compared to control samples (Fattouch *et al.,* 2008). Medina *et al.* (2007) evaluated the reducing power of a range of pure polyphenols and reported that their donating electrons capacity seems to play the most significant role in retarding the development of rancidity in fish muscle.

Tannins and tannin derivatives such as gallic acid and ellagic acid are commonly found in higher herbaceous and woody plants (Labieniec and Gabryelak, 2006). Tannic acid is a water-soluble polyphenol containing sugar esters, mainly glucose, and phenol carboxylic acids, such as gallic acid, hexahydroxydiphenic acid, or its stable dilactone ellagic acid (Labieniec and Gabryelak, 2006; Rodriguez *et al.,* 2008). Hydrolyzable tannins such as tannic acid and epigallocatechin gallate have been reported to have natural antioxidant, antimicrobial, and antiviral activities (Akiyama *et al.,* 2001). Tannic acid has been well-described as antimutagenic and antioxidant compound. The antioxidant activity of tannic acid has been previously attributed to its capacity to form a complex with iron ions, interfering with the Fenton reaction (Lopes *et al.,* 1999). Lopes *et al.* (1999) found that tannic acid is able to reduce Fe(III) to Fe(II) (by means of the Fe(II) indicator ferrozine). As an antioxidant compounds, tannic acid was also shown to prevent lipid oxidation and radical-mediated DNA cleavage by scavenging oxygen and oxygen-derived radicals (Khan *et al.,* 2000). Tannic acid is able to prevent $OH^{\bullet}$ generation and 2-deoxyribose oxidative damage induced by Fenton reagents by forming a Fe (II) complex (Lopes *et al.,* 1999). The antioxidant action of tannic acid was similar to those of deferoxamine mesylate (DFO), EDTA, pyridoxal isonicotinoyl hydrazone (PIH), and ellagic acid**.** The mechanism of tannic acid as antioxidant is due to Cu (II) chelation, forming a copper–tannic acid complex that is less active in the participation of oxyradical formation (Andrade Jr *et al.,* 2005). Lopes *et al.* (1999) also reported an $OH^{\bullet}$ trapping activity of the iron complex with tannic acid. Sanchez-Moreno *et al.* (1999) reported that tannic acid showed much stronger activity in the inhibition of lipid oxidation than BHA, quercetin, DL-α-tocopherol, and caffeic acid. Tannic acid dramatically increased the

induction time of soybean oil oxidation. Khan *et al.* (2000) reported that the antioxidant property appears to be related to the number of hydroxyls on the tannic acid, while gallic acid and its structural analogues were found to be non-inhibitory or partially inhibitory on hydroxyl radical and singlet oxygen mediated cleavage of plasmid DNA.

Tannins are polyphenolic compounds commonly occurring in the barks, woods and fruits of many kinds of plants. Extraction of tannins from the bark of different trees was carried out (Yazaki and Collins, 1994). Kiam (*Cotylelobium lanceotatum craih*) trees are very common in the southern part of Thailand. Pieces of wood from the kiam tree have been traditionally submerged in sugar palm sap to prevent or retard microbial fermentation (Chanthachum and Beuchat, 1997). Balange and Benjakul (2009) reported that tannic acid (456.3 mg/kg) was found as the major component of the kiam wood extract. Thus, kiam wood extract can be used as an natural source of tannic acid for preventing lipid oxidation in the fish and fishery products.

1.2.9.3 Lipid oxidation in emulsion sausages

Emulsion sausage has been popular due to its acceptable taste and high nutritional value. Emulsion sausages, such as frankfurter, are widely consumed in both Western and Asian countries. A product is typically made of beef, or beef and pork, or chicken and has the fat content of 25–30%. Fish mince and surimi have recently been used as a raw material for emulsion sausage production, particularly in Asian countries (Konno, 2005). Fortification of 3-n PUFA to the fish sausage could be an alternative means to improve its fat quality and to increase n-3 PUFA consumption among the consumers. However, one of the main problems in food supplemented with marine fish oil is their high susceptibility to oxidation. Although lipid hydroperoxide are tasteless and odourless, the secondary oxidation products are responsible for the changes in aroma and flavour (Frankel, 1991). Oxidative deterioration is also related to the adverse changes in texture, appearance, and nutritional value (Min and Boff, 2002). Due to high susceptibility towards oxidation of fish oil, the enriched food products should be stored not over long periods of time, unless under special storage

conditions and in a special type of packing, especially avoiding an exposure to air. Otherwise the desirable n-3 PUFA may easily transform into toxic peroxides and other by-products of lipid oxidation could promote quality deterioration (Kolanowski and Laufenberg, 2006). The introduction of natural functional ingredients such as botanicals, plant extracts, seaweeds and whey proteins with biological activity into processed meat products is receiving abundant attention (Calvo *et al.,* 2008). Plant-derived ingredients possessing antioxidant and antimicrobial properties have the advantage of being readily accepted by consumers, as they are considered natural. Natural ingredients such as lutein, sesamol, ellagic acid, tannins, and leaf extract are natural active compounds exhibiting a variety of biological activities, including potent antioxidant effects (Ezdihar *et al.,* 2006; Bouaziz *et al.,* 2008) and antimicrobial properties (Micol *et al.,* 2005).

Phenolic compounds from natural sources have been recently applied in the sausages to prevent the lipid oxidation during refrigerated storage. Addition of sesamol, ellagic acid and olive leaf extract lowered lipid oxidation in raw and cooked pork sausages packed in air and under modified atmospheric packaging (80% O_2 : 20% CO_2 and 70% N_2 : 30% CO_2) during refrigerated storage (Hayes *et al.,* 2011). Those phenolic compounds displayed the antioxidative activity in the following order: sesamol 250 μg/g > ellagic acid 300 μg/g > olive leaf extracts 200 μg/g > lutein 200 μg/g for both raw and cooked pork sausages (Hayes *et al.,* 2011). Hayes *et al.* (2011) also reported that addition of lutein, sesamol, ellagic acid and olive leaf extract had no detrimental effect on pH, cooking losses, TVCs, tenderness, juiciness, texture or flavour. Therefore, lutein, sesamol, ellagic acid and olive leaf extract were effective as natural functional ingredients in suppressing lipid oxidation and have the potential to be incorporated into functional raw and cooked pork sausages (Hayes *et al.,* 2011).

Herbs and spices are traditionally used as food ingredients because of their antioxidant properties. Carnosol, rosmarinic and carnosic acids in rosemary (*Rosmarinus officinalis* L.) and oregano leaves (*Origanum vulgare* L.) have been screened for their preventive effect on the oxidation and colour of model pork batters

(Hernandez-Hernandez *et al.,* 2009). Rosemary extracts showed higher antioxidant activity possibly due to the presence of high concentrations of carnosic acid, carnosol and unidentified active compounds. However, ethanol oregano extracts containing high concentrations of phenols, mainly rosmarinic acid, efficiently prevented colour deterioration. The antioxidant effect of the studied extracts depends, not only on the concentration of phenol compounds (rosmarinic acid, carnosol and carnosic acid), but also on the extraction method and solvent used (Hernandez-Hernandez *et al.,* 2009).

Green tea catechins (GTC) and green coffee antioxidant (GCA) demonstrated the preventive effective on lipid oxidation of fish oil containing pork sausages (raw and cooked) during refrigerated storage under air and under MAP (80% O_2 : 20% CO_2 and 70% N_2 : 30% CO_2) (Valencia *et al.,* 2008). The inclusion of GTC (200 mg/kg) in fish oil containing sausages (FGTC 200) significantly reduced the extent of lipid oxidation after 7 days of storage. Stabilisation of fish oil against oxidation with GTC was previously reported by O'Sullivan *et al.* (2005). Jo *et al.* (2003) also reported that green tea extract reduced lipid oxidation in raw and cooked pork patties.

Natural pigments such as norbixin (NOR), lycopene (LYC), zeaxanthin (ZEA), β-carotene (CAR) or dextrose (used as a control (CON) were used to replace sodium erythorbate (NaEry), a synthetic compound applied as an antioxidant in sausage formulations (Mercadante *et al.,* 2010). All pigments used in the sausage formulations were able to maintain the oxidative stability of the sausages (MDA equivalents <0.38 mg/kg). Zeaxanthin and norbixin were the most efficient antioxidants of those tested. This antioxidant effect might be associated with the intermediate polarities of these two compounds, which would allow them to concentrate in the membrane lipids or emulsion interface, where lipid oxidation most likely takes place (Mercadante *et al.,* 2010).

Orange dietary fibre (ODF) (1%) and oregano essential oil (0.02%) (OEO) were comparatively studied for their antioxidative activity in bologna sausages

packed under vacuum, air and modified atmosphere (80% N_2 and 20% CO_2) during refrigerated storage (Viuda-Martos *et al.,* 2010). Samples treated with ODF + OEO stored in vacuum packaging showed the lowest TBARS values. ODF + OEO samples stored in vacuum packaging showed the lowest aerobic and lactic acid bacteria count (Viuda-Martos *et al.,* 2010). At day 6, the lowest oxidation values were found in the ODF + OEO samples packed in vacuum and MAP samples. The agents responsible for the antioxidant activity in both orange fiber and oregano essential oil are the bioactive compounds, mainly, polyphenols (Viuda-Martos *et al.,* 2010). The effect of the polyphenols contained in citrus fiber on lipid oxidation in both meat and fish-based products has been investigated (Sanchez-Zapata *et al.,* 2009; Viuda-Martos *et al.,* 2008).

1.2.10 Modified atmospheric packaging (MAP)

Modified atmosphere packaging (MAP) is the removal and/or replacement of the atmosphere surrounding the product before sealing in vapor-barrier materials (McMillin, 2008). MAP can be vacuum packaging (VP), which removes most of the air before the product is enclosed in barrier materials, or forms of gas replacement, where air is removed by vacuum or flushing and replaced with another gas mixture before packaging sealing in barrier materials. MAP has become increasingly popular as a method of food preservation. Shelf-life of the fish and fish products has been successfully extended by MAP (Hintlian and Hotchkiss, 1986). The shelf-life increased as a result of lag phase extension of several aerobic spoilage bacteria (Pastoriza *et al.,* 1996). The rapid growth of MAP technology for preservation of fish products is due to a number of interrelated factors such as (1) development on new polymeric high-barrier packaging materials; (2) extended market area for product with fresh characteristics; (3) consumer concerns about preservation additives in such products; and (4) favorable consumer perception of MAP technology (Ashie *et al.,* 1996). It is well known that a modified atmosphere (MA) with high CO_2 concentrations increases shelf-life, compared with storage under air (Reddy *et al.,* 1995; Pastoriza *et al.,* 1998). Modified atmospheres are commonly composed by high percentages of CO_2 and N_2, and low levels of O_2. High CO_2 concentrations coupled with low O_2 levels inhibit the growth of many spoilage bacteria; however, the presence

of O_2 has been demonstrated to slow the reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA), delaying the formation of off-odors (Boskou and Debevere, 1997). The MAP protects products against deteriorative effects (which may include discoloration, off-flavor and off-odor development, nutrient loss, texture changes, pathogenicity, and other measurable factors (Skibsted *et al.,* 1994; Yam *et al.,* 2005).

MAP has been used with various types of products, where the mixture of gases in the package depends on the type of product, packaging materials and storage temperature. Meat and fish need very low gas permeability films, so for non-respiring products (meat, fish, cheese etc.), high barrier films are used. The shelf-life increased as a result of lag phase extension of several aerobic spoilage bacteria and retardation of enzymatic spoilage (Ashie *et al.,* 1996). Shelf-life of fish under CO_2 atmosphere storage could be extended (Debevere and Boskon, 1996). Bacterial spoilage in refrigerated fish under aerobic storage condition is caused by Gram-negative psychrotrophic organisms such as *Pseudomonas* spp., *Alteromonas* spp., *Shewanella* spp. and *Flavobacterium spp* (Hobbs, 1991). The spoilage flora is effectively inhibited by atmosphere enriched with 20% or higher CO_2 concentration (Lopez-Galvez *et al.,* 1995). Ordonez *et al.* (2000) reported that the shelf-life of hake packaged in 40% CO_2 atmosphere could be extended for more than 14 days at 2ºC. Therefore, CO_2 enriched atmosphere has been increasingly used for the distribution and storage of seafood. Several methods can be used to modify atmosphere such as vacuum packaging, gas packaging and atmospheric modifier.

1.2.10.1 Vacuum packaging

Vacuum packaging is used extensively for shelf-life extension and keeping quality of fresh and processed fish. The product is placed in a low-oxygen permeability package. Air is removed under vacuum and the package is sealed. Under condition of a good vacuum, headspace O_2 is reduced to less than 1%, while CO_2 produced from tissue and microbial respiration eventually increases to 10-20% within the package headspace. This condition, that is low O_2 and elevated CO_2 levels, extends the shelf-life of fresh fish by inhibiting the growth of aerobic spoilage

microorganisms, particularly *Pseudomonas* spp. and *Aeromonas* spp. (Ashie *et al.,* 1996).

1.2.10.2 Gas packaging

Gas packaging is simply an extension of vacuum packaging technology and involves the evacuation of air, followed by addition of the appropriate gas mixture. Gases commonly used in gas package are N_2, O_2 and CO_2 (Oberlender *et al.,* 1983). Each gas plays a distinct and specific role in MAP of food system. N_2 is an inert gas that has no effect of food system and has no antimicrobial properties. It is used mainly as a filler to prevent package collapse in products that can absorb CO_2. It can also be used to replace O_2 in dried, high-fat products with low Aw to prevent chemical spoilage, e.g. oxidative rancidity problems, etc. O_2 is generally avoided in gas packaging mixtures for high fat fish. However, it may be used in low concentration in both high and low fat fish products to prevent anaerobic conditions and limit growth of potentially harmful anaerobes, especially *Clostridium botulinum.* CO_2 is the most important gas in the mixture. It is both bacteriostatic and fungistatic effect. It is also highly soluble in water and fat, where it forms carbonic acid. The bicarbonate ion, a dissociation product, changes cell permeability, and affected metabolic processes. According to this theory, lipids in the cell membrane are shifted, changing the interface with the external aqueous environment (Statham, 1984). The carbonic acid may lower pH, resulting in slight flavor changes in fish, and its absorption by the products may also cause package collapse (Ashie *et al.,* 1996).

1.2.10.3 Atmospheric modifier

Suitable atmosphere modifier may be used to provide the desired headspace atmosphere inside the package. The modifiers commercially available include O_2 absorber or scavengers, and ethanol generator (Ashie *et al.,* 1996). Most of the technologies used in the manufacture of the modifier were originated in Japan and the materials had been used in that country for many years before their introduction in the world.

1.2.10.4 Effect of MAP on preservation of fish and fish products

Modified atmosphere packaging (MAP) is widely used as a supplement to ice or refrigeration to delay spoilage and extend the shelf-life of fresh fishery products (Masniyom *et al.,* 2002). Seafood is highly perishable due to its autolytic enzymes and post-mortem pH changes that favor bacterial growth (Church and Parson, 1995). It is traditionally stored under refrigeration in air, which gives a shelf-life ranging from 2 to 10 days, depending upon species, harvest location and season (Stammen *et al.,* 1990). The use of gas packaging, especially elevated CO_2 levels, has been shown to inhibit normal spoilage bacteria including *Pseudomonas* spp., *Alteromonas* spp., *Shewanella* spp., *Moraxella* spp., and *Acinetobacter spp*. in fish from cold water. Thus, CO_2 atmosphere could extend the shelf-life by two or three folds (Stammen *et al.,* 1990). MAP, through the biostatic activity of CO_2, inhibits the growth of wide range of spoilage microorganisms. These include some pathogens such as *Staphylococcus aureus*, *Salmonella* spp., *Enterobacteriaceae* such as *Yersina enterocolitica* and *Escherichia coli*. However, the effectiveness of CO_2 inhibition is strongly related to storage temperature (Stammen *et al.,* 1990). Refrigerated seabass pretreated with polyphosphate and kept under MAP (80% CO_2, 10% N_2 and 10% O_2) had the lowest mesophilic bacterial count, especially when kept for a longer time ($P<0.05$) (Masniyom *et al.,* 2005). Mesophilic bacterial counts of samples pretreated with phosphate and kept under MAP increased more slowly than those stored under MAP without phosphate pretreatment, indicating that phosphates might show the synergistic effect on the retardation of bacterial growth in the sample kept under MAP (Masniyom *et al.,* 2005). However, the higher increase in TBARS value was observed in seabass slices stored under MAP, compared with those stored in air (Masniyom *et al.,* 2005). This was probably because carbonic acid formed might induce the denaturation of muscle proteins, leading to the release of free haem iron, a potential pro-oxidant in the muscle system. At high content of CO_2 (80%), carbonic acid formed in muscle probably caused the inactivation of antioxidative enzymes, e.g. gluthathione peroxidase, resulting in the higher oxidation in the muscle (Renerre *et al.,* 1996). Therefore, CO_2-enriched packaging effectively inhibited the spoilage caused by microorganisms, but it could not prevent the chemical deterioration, especially lipid

oxidation. Thus, the treatment with natural antioxidant like phenolic compounds could show synergistic effect in retardation of lipid oxidation of fish products kept under MAP. Refrigerated perch, sea trout, croaker and blue fish packed with CO_2 had a 45-55% increase in stability, mainly due to an extension in the lag phase of psychrotrophic organisms and their reduced growth rate in the logarithmic phase (Gray *et al.,* 1983). Fillets of trevalla (*Hyperoglyphe porosa*) packed in 100% CO_2 had a shelf-life of 8 to 16 days longer at 4 ºC than those stored under aerobic condition (Statham and Bremner, 1985). Lannelongue *et al.* (1982) found that the inhibitory effect of CO_2 on psychrotrophic, aerobic gram-negative spoilage bacteria in sword fish steak was proportional to the CO_2 tension in the packages. Maximum inhibition of growth was achieved with 100% CO_2. Hetero-fermentative*,* *Lactobacillus* spp. became a dominant part of the microflora of steaks stored in CO_2 enriched atmospheres. Growth of aerobic and anaerobic bacteria was slowest in sardines packed under 80% CO_2 and 20% N_2. Initial microflora were predominantly *Vibrionaceae*, *Moraxella* and *Acinetobacter* in the sardine, while microflora on sardine stored under 20% CO_2 and 80% N_2 were predominantly *Lactobacillus* and S*treptococcus*. Microflora on sardine stored for 10 days under 80% CO_2 and 20 % N2 was predominantly unidentifiable *cocci* as well as *Lactobacillus* spp. and S*treptococcus spp*. (Fujii *et al.,* 1990). Reddy *et al.* (1995) studied the shelf-life of MAP fresh tilapia fillets stored under refrigeration and temperature abuse conditions. The fillets packaged under 100% air had the shelf life of 9-13 days at a storage temperature of 4 ºC, but decreased to 3-6 days at 6 ºC. However, the shelf-life of MAP fillets stored at 4 ºC increased to 25 days when the lag phase and generation time of the bacteria were extended. Pastoriza *et al.* (1996) found that packaging under medium to high CO_2 concentration had some bacteriostatic effect on hake slice, prolonging their shelf-life to 3 weeks whereas air stored hake was sensorially rejected after 7 days of iced storage. Lopez-Galvez *et al.* (1998) found that the growth of microorganism in sole fillets was inhibited by higher CO_2 concentration (40%) with formation of lactic acid after 1 day of storage. The formation of TMA and other basic volatile was delayed by the use of CO_2 and the effect was higher in the 40% CO_2 atmosphere than in the 20% CO_2 atmosphere. Dhananjaya and Stroud (1994) reported that a CO_2:O_2:N_2 (60:20:20) mixture was more effective than CO_2:O_2:N_2 (40:30:30) gas mixture in extending the shelf-life of haddock

fillets stored in MAP. Therefore, the shelf-life of fish slices packed in CO_2 enriched atmospheres could be extended more effectively than that packed in air.

Mussels (*Mytilus galloprovincialis*) packaged in two modified atmospheres (MAP 1: 60% CO_2/20% N_2/20% O_2 and MAP 2: 60% CO_2/40% N_2) and under vacuum (VP) showed different quality. Better quality retention and greater shelf life of mussels packaged under MAP 1 was obtained as compared to MAP 2 and VP samples (Goulas, 2008). The 2- thiobarbituric acid (TBA) values of MAP 1 and air samples were signifi cantly higher than those of VP and MAP 2 samples. MAP 1 showed a greater inhibition effect on total viable count of mussel samples than all other packagings. Based primarily on odour scores, the MAP 1 samples remained acceptable up to 10–11 days, whereas, the MAP 2 and VP could maintain their quality up to 7–8 days. Air-packaged samples had the shortest shelf-life (5–6 days) (Goulas, 2008).

Fresh Atlantic salmon fillets superchilled (–2 °C) and MA (CO_2:N_2 60:40) packaged maintained a good quality with negligible microbial growth (<1000 colony-forming units [CFU]/g) for more than 24 days based on both sensory and microbial analyses (aerobic plate count, H_2S-producing, and psychrotrophic bacteria) (Sivertsvik *et al.,* 2003). Superchilled salmon stored in air had a 21-d sensory shelf life, whereas MA and air-stored fillets at chilled (+4 °C) conditions were spoiled after 10 d and 7 d, respectively. Modified atmosphere and superchilled conditions extended the shelf life of salmon fillets, and when combined, a synergistic effect was observed giving an additional effect. This was probably caused by increased dissolvement of CO_2 at the superchilled temperature. The combination of MA and superchilling gave a product of high quality in 24 day of storage with almost total bacterial growth inhibition.

The spoilage of chub mackerel stored under MAP (50% CO_2/50% N_2) was delayed, compared with those samples stored under vacuum or in air (Stamatis and Arkoudelos, 2007). Based on sensory evaluation, the MAP samples remained acceptable until day 12 and 10, the VP samples remained acceptable until day 10 and

8, while the air packaged chub mackerel samples remained acceptable until day 8 and 7 of storage at 3 and 6 °C, respectively (Stamatis and Arkoudelos, 2007).

Pre-rigor fillets of Atlantic salmon packed in air or under MAP (60% CO_2/40% N_2) with a CO_2 emitter had significantly lower bacterial growth, compared to fillets stored in air (Hansen *et al.,* 2009). MAP superchilled bottom fillets had lower bacterial counts, compared to the corresponding chilled fillets (Hansen *et al.,* 2009). Combining short-term superchilling and MAP with a CO_2 emitter prolonged the shelf-life of pre-rigor salmon fillets.

Fresh eel (*Anguilla anguilla*) received acceptable sensory scores during the first 11±1 days of storage in atmospheric air, 11±1 days of storage in vacuum and 18±1 days of storage in MAP (40% CO_2, 30% N_2 and 30% O_2) at 0 °C. Using the microbial quality indicators, the shelf life of eel packed in air, vacuum and MAP was estimated to be approximately 18, 28 and 34 days, respectively (Arkoudelosa *et al.,* 2007).

1.3 Objectives of study

1. To determine *in-vitro* antioxidative and lipoxygenase inhibitory activities of different phenolic compounds.

2. To investigate the impact of different phenolic compounds in prevention of lipid oxidation in different model systems.

3. To study the synergistic effect of selected phenolic compounds in combination with modified atmospheric package (MAP) on lipid oxidation and quality of striped catfish slices during refrigerated storage.

4. To study the effect of pH and sources of haemoglobin on lipid oxidation of washed fish mince.

5. To investigate the effect of selected phenolic compounds on haemoglobin mediated lipid oxidation of washed fish mince added without and with fish oil.

6. To determine the effect of natural extract containing phenolic compounds on lipid oxidation of different model systems.

7. To elucidate the preventive effect of selected phenolic compounds and natural extracts on lipid oxidation of fish emulsion sausage during refrigerated storage.

CHAPTER 2

COMPARATIVE STUDIES OF FOUR DIFFERENT PHENOLIC COMPOUNDS ON *IN VITRO* ANTIOXIDATIVE ACTIVITY AND THE PREVENTIVE EFFECT ON LIPID OXIDATION OF FISH OIL EMULSION AND FISH MINCE

2.1 Abstract

Antioxidative activities of different phenolic compounds (catechin, caffeic acid, ferulic acid and tannic acid) at various levels were determined by different assays. Among all the phenolic compounds tested, tannic acid exhibited the highest 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities and ferric reducing antioxidant power (FRAP). Nevertheless, catechin showed the highest metal chelating activity ($P<0.05$), whereas caffeic acid had the highest lipoxygenase (LOX) inhibitory activity ($P<0.05$). Impact of different phenolic compounds at a level of 100mg/l on lipid oxidation of menhaden oil-in-water emulsion and mackerel mince was investigated. Tannic acid showed the highest efficacy in retardation of lipid oxidation for both model systems as evidenced by the lower peroxide value (PV), conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) values. This was also related with the lower non-haem iron content in tannic acid treated samples. Tannic acid was therefore considered as the most potential natural antioxidant for controlling oxidation of fish oil-in-water emulsion and fish mince, whereas ferulic acid seemed to possess the lowest preventive effect on lipid oxidation.

2.2 Introduction

Fatty fish species are considered to be of great nutritional importance. This is mainly due to their naturally high content of essential n-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20: 5) and docosahexaenoic acid (22: 6). These fatty acids have been shown to have potential benefits for human health (Lee and Lip, 2003). Nevertheless, they are susceptible to oxidation, which is associated with the rancidity and loss in nutritive value (Hsieh and Kinsella, 1989; Frankel, 1998a). Apart from high amounts of PUFAs, the presence of haem pigments and trace amounts of metallic ions makes the fish, especially dark flesh fatty fish, prone to lipid oxidation (Hsieh and Kinsella, 1989). To retard such a quality loss, synthetic antioxidants have been used to decrease lipid oxidation during the processing and storage of fish and fish products (Boyd *et al.,* 1993). However, the use of synthetic antioxidants has raised questions regarding food safety and toxicity (Chang *et al.,* 1977). The use of natural antioxidants is emerging as an effective methodology for controlling rancidity and limiting its deleterious consequences. Natural phenolic compounds with antioxidant activity such as rosemary extract, tea catechin, tannins, etc. have been gaining an increasing attention due to their safety (Frankel, 1998b).

Phenolic compounds are bioactive substances widely distributed in plants and are important constituents of the human diet. Plant phenolics comprise a great diversity of compounds, such as flavonoids (anthocyanins, flavonols, flavones, etc.) and several classes of non-flavonoids (phenolic acids, lignins, stilbenes) (Harborne, 1989). Phenolic compounds vary in structure and the number of hydroxyl groups (Fig. 5), leading to the variation in their antioxidative activity. In general, phenolic compounds play a role as antioxidants through different mechanisms of action, such as scavenging of free radicals (Antolovich *et al.,* 2002), quenching of reactive oxygen species, inhibition of oxidative enzymes (Edenharder and Grunhage, 2003), chelation of transition metals or through interaction with biomembranes (Liao and Yin, 2000). Therefore, these compounds have been considered as the promising candidates as potential protectors against food oxidation and biological aging of tissues. Although the single phenolic compound have been proved as antioxidant, no comparative studies have been conducted among those phenolic compounds, which

possess different molecular properties, mode of action, stability etc. Additionally, different phenolic compounds may act as antioxidants at varying degrees in different food systems, depending on the polarity and molecular characteristics. Hydrophilic antioxidants could prevent the oxidation of bulk oil, while hydrophobic counterpart effectively retards lipid oxidation in oil-in-water emulsion (Frankel, 1998b). Thus, the present study aimed to determine *in vitro* antioxidative activities of different phenolic compounds (catechin, caffeic acid, ferulic acid and tannic acid) and to investigate their preventive effect on lipid oxidation of menhaden oil-in-water emulsion and mackerel mince during extended storage.

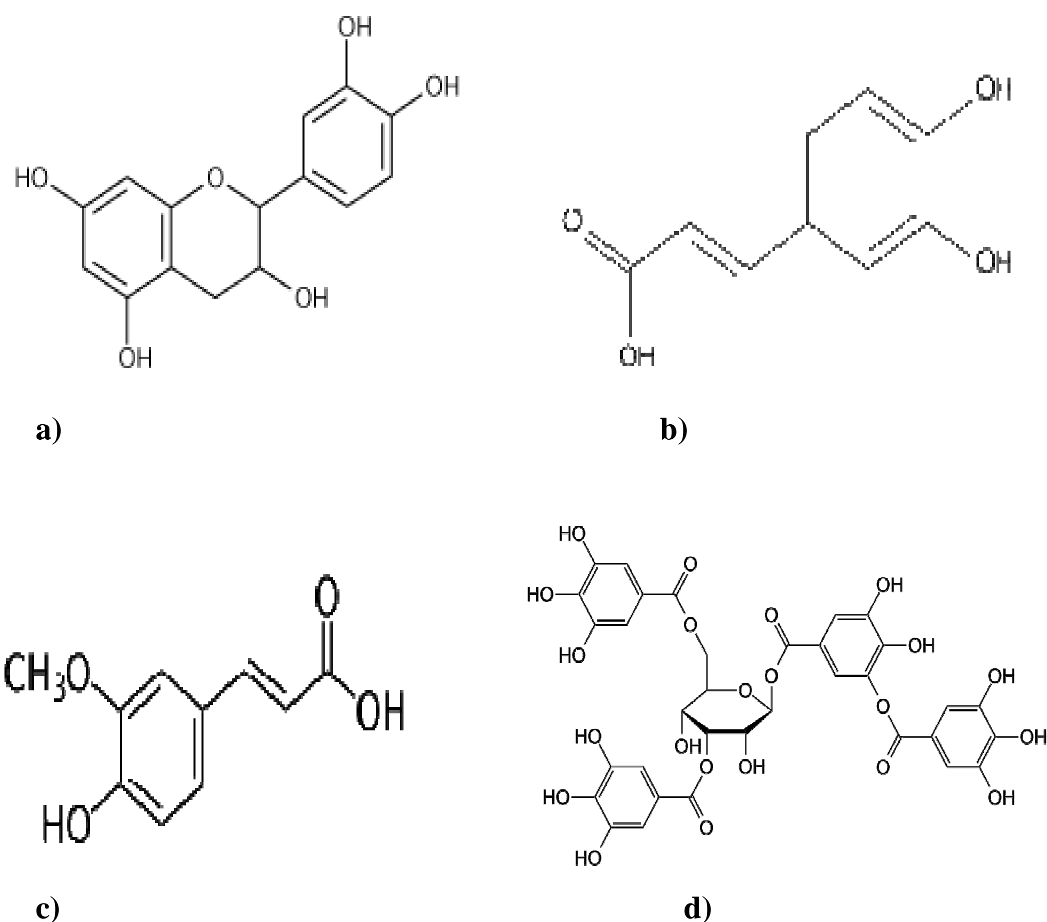


Figure 5. Structures of four phenolic compounds a) catechin, b) caffeic acid, c) ferulic acid and d) tannic acid.

2.3 Materials and methods

2.3.1 Chemicals and fish oil

Catechin, tannic acid, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picryl hydrazyl (DPPH), linoleic acid, soybean lipoxygenase-1 (EC 1.13.11.12, type 1), thioglycolic acid, bathophenanthroline disulfonic acid, ferrozine and menhaden oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, anhydrous sodium sulfate, potassium iodide, trichloroacetic acid, iron standard solution, ethanol and methanol were obtained from Merck (Damstadt, Germany). Caffeic acid, ferulic acid, disodium hydrogen phosphate, 2-thiobarbituric acid, 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride hexahydrate and potassium persulfate were procured from Fluka Chemical Co. (Buchs, Switzerland). Chloroform was purchased from Lab-Scan (Bangkok, Thailand).

2.3.2 Comparative study on *in vitro* antioxidative activity of different phenolic compounds

Four phenolic compounds including catechin, caffeic acid, ferulic acid and tannic acid were comparatively determined for their antioxidative activities by different *in vitro* assays. Prior to assay, phenolic compounds were added with distilled water and the mixtures were adjusted to pH 8-9 using 2 M NaOH until the compounds were completely dissolved. Thereafter, the obtained solution were adjusted to pH 7 using 2 N HCl and subjected to the assays for antioxidative activities.

2.3.2.1 DPPH radical scavenging activity

DPPH radical scavenging activity was determined as described by Wu *et al.* (2003) with a slight modification. Sample (1.5 ml) with the concentration range of 0.5-10 mg/l was added with 1.5 ml of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in dark for 30 min. The absorbance of the resulting solution was

measured at 517 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Sample blank at each concentration was prepared in the same manner except that ethanol was used instead of DPPH solution. A standard curve was prepared using Trolox in the range of 10–60 μM. The activity was calculated after the sample blank substraction and expressed as μmol Trolox equivalents (TE)/ml of phenolic compound.

2.3.2.2 ABTS radical scavenging activity

ABTS radical scavenging activity was assayed as per the method of Arnao *et al.* (2001) with a slight modification. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml ABTS solution with 50 ml methanol in order to obtain an absorbance of 1.1 ± 0.02 units at 734 nm using a UV-1601 spectrophotometer. Fresh ABTS solution was prepared for each assay. Sample (150 μl) with the concentration range of 0.5-10 mg/l was mixed with 2850 μl of ABTS solution and the mixture was left at room temperature for 2 h in dark. The absorbance was then measured at 734 nm using the spectrophotometer. Sample blank at each concentration was prepared in the same manner except that methanol was used instead of ABTS solution. A standard curve of Trolox ranging from 50 to 600 μM was prepared. The activity was expressed as μmol Trolox equivalents (TE)/ml of phenolic compound.

2.3.2.3 FRAP (Ferric reducing antioxidant power)

FRAP was assayed according to Benzie and Strain (1996). Stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 . 6H_2O$ solution. A working solution was prepared freshly by mixing 25 ml of acetate buffer, 2.5 ml of TPTZ solution and 2.5 ml of $FeCl_3 . 6H_2O$ solution. The mixed solution was incubated at 37 °C for 30 min in a water bath (Memmert, D-91126, Schwabach, Germany) and was referred to as FRAP solution. A sample (150 μl) with the concentration range of 0.5-

10 mg/l was mixed with 2850 μl of FRAP solution and kept for 30 min in dark at room temperature. The ferrous tripyridyltriazine complex (coloured product) was measured by reading the absorbance at 593 nm. Sample blank at each concentration was prepared by omitting $FeCl_3$ from FRAP solution and distilled water was used instead. The standard curve was prepared using Trolox ranging from 50 to 600 μM. The activity was expressed as μmol Trolox equivalents (TE)/ml of phenolic compound.

2.3.2.4 Metal chelating activity on ferrous ions (Fe^{2+})

The chelating activity towards Fe^{2+} was measured by the method of Boyer and McCleary (1987) with a slight modification. Sample (4.7 ml) with the concentration ranging from 100 to 200 mg/l was mixed with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The reaction mixture was allowed to stand for 20 min at room temperature. The absorbance was then read at 562 nm. The blank was prepared in the same manner except that distilled water was used instead of the sample. For sample blank at each concentration, $FeCl_2$ solution was excluded and distilled water was used instead. The chelating activity after sample blank substraction was calculated as follows:

Metal chelating activity (%) = $\{(A_{blank} - A_{sample})/A_{blank}\} \times 100$

where, A_{sample} is the absorbance of the sample and A_{blank} is the absorbance of blank.

2.3.3 Comparative study on lipoxygenase inhibitory activity of different phenolic compounds

The enzyme assay was performed as previously reported by Ha and Kubo (2005) with a slight modification. To study the inhibitory activity towards soybean lipoxygenase, 10 μl of different phenolic compounds at various concentrations (10, 25, 50 and 100 mg/l) was mixed with 20 μl of 0.1 M sodium borate buffer solution (pH 9.0) containing lipoxygenase (0.52 μM). The mixtures were allowed to stand at 25° C for 10 min, followed by the addition of 2.97 ml of 0.1 M sodium borate buffer (pH 9.0). To initiate the reaction, 30 μl of 3 mM linoleic acid were added. The resultant solution was mixed well, and the linear increase of

absorbance at 234 nm was measured after 5 min. One unit of lipoxygenase was defined as the increase in 0.1 unit of absorbance at 234 nm/min. The percentage inhibition was calculated as follows:

$$\text{Lipoxygenase inhibition (\%)} = \{(A_0 - A_1)/A_0\} \times 100$$

where, A_0 is the activity without inhibitor and A_1 is the activity in the presence of inhibitor (Banerjee *et al.,* 2002).

2.3.4 Comparative study of different phenolic compounds in prevention of lipid oxidation in fish oil-in-water emulsion and fish mince

2.3.4.1 Preparation of oil-in-water emulsion

Oil in water emulsions (250 ml) were prepared by homogenising 25 ml of menhaden oil with 225 ml 0.1 M acetate buffer (pH 5.4) containing different phenolic compounds (100 mg/l of emulsion) and 1 % lecithin as an emulsifier. The mixture was kept in an ice-bath during homogenisation with an Ultra-Turrax T25 high speed homogeniser (Janke and Kunkel, Staufen, Germany) at the speed of 13,500 rpm for 5 min. The emulsion was then sonicated with an Elma (S 30 H) sonicator (Kolpingstr, Singen, Germany) in an ice bath for 5 min. The prepared oil-in-water emulsion (225 ml) was transformed into 250 ml-erlenmeyer flask and kept at 30º C in dark. The control oil-in-water emulsion was prepared in the same manner except the distilled water was added instead of the solution of phenolic compounds. Samples were taken every 24 h for the determination of peroxide value (PV), conjugated dienes (CD) and thiobarbituric acid-reactive substances (TBARS).

2.3.5 Preparation of fish mince

Fish mince was prepared according to the method of Kamil *et al.* (2002) with a slight modification. Mackerel (*Rastrelliger kanagurta*) with an average weight of 100-150 g off-loaded 24 h after captures were purchased from the local market in Hat Yai, Thailand. The fish were kept in ice during the transportation. Upon arrival, fish were washed, filleted, de-skinned and minced using a mincer with a hole diameter of 5 mm. Fish mince obtained was divided into five portions (750 g each).

One portion without the addition of phenolic compounds was used as the control and 25 ml of distilled water was added instead. Other four portions were added with 25 ml of different phenolic compounds (pH 7) to obtain the final concentration of 100 mg/kg of mince. The mince was then thoroughly mixed in order to ensure the homogeneous distribution of phenolic solution in the mince. Different mince samples were packed in polyethylene bags, sealed and kept in ice using a mince/ice ratio of 1:2 (w/w). Molten ice was removed every day and the same quantity of ice was replaced. After the designated storage time (0, 3, 6, 9, 12 and 15 days), the samples were taken for analyses of peroxide value (PV), conjugated diene (CD), thiobarbituric acid-reactive substances (TBARS), haem iron and non-haem iron contents.

2.3.6 Lipid extraction and analysis

Lipid was extracted by the method of Bligh and Dyer (1959). Fish mince (25 g) was homogenised with 200 ml of a chloroform:methanol:distilled water mixture (1:2:1) at the speed of 9,500 rpm for 2 min at 4 °C using an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany). The homogenate was then added with 50 ml of chloroform and homogenised at 9500 rpm for 1 min. Then, 25 ml of distilled water were added and the mixture was homogenised again for 30 sec. The homogenate was centrifuged at 14,500 x g at 4 °C for 15 min using a RC-5B plus centrifuge (Beckman, JE-AVANTI, USA), and transferred into a separating flask. The chloroform phase was drained off into a 125 ml-erlenmeyer flask containing about 2–5 g of anhydrous sodium sulfate, shaken very well, and decanted into a round-bottom flask through a Whatman No. 4 filter paper (Whatman International, Ltd, Maidstone, England). The solvent was evaporated at 25 °C, using an EYELA rotary evaporator N-100 (Tokyo, Japan), and the residual solvent was removed by flushing with nitrogen.

2.3.6.1 Peroxide value (PV)

Peroxide value was determined according to the method of Sakanaka *et al.* (2004). To 50 µl of the oil extracted from mince or 50 µl of oil-in-water emulsion sample, 2.35 ml of 75% ethanol, 50 µl of 30% ammonium thiocyanate and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl were added and mixed thoroughly. After 3

min, the absorbance of the coloured solution was measured at 500 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). An increase in absorbance at 500 nm indicated the formation of peroxide (Yen and Hsieh, 1998).

2.3.6.2 Conjugated diene (CD)

Conjugated diene was measured according to the method of Frankel *et al.* (1996). Extracted oil or oil-in-water emulsion (0.1 ml) was dissolved in 5.0 ml of methanol and the absorbance was measured at 234 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Conjugated diene was measured as the increase in absorbance at 234 nm.

2.3.6.3 Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). Fish mince (0.5 g) or 0.5 ml of oil-in-water emulsion sample were mixed with 2.5 ml of a TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture was heated in a boiling water for 10 min to develop a pink colour, cooled with running tap water and then sonicated for 30 min followed by centrifugation at 5,000×g at 25 ºC for 10 min. The absorbance of the supernatant was measured at 532 nm. Standard curve was prepared using 1,1,3,3-tetramethoxypropane (malonaldehyde; MAD) at a concentration ranging from 0 to 10 ppm and TBARS were expressed as mg of MAD equivalents/kg sample.

2.3.6.4 Determination of haem iron content

Haem iron content was determined according to the method of Gomez-Basauri and Regenstein (1992a) with a slight modification. A fish mince sample (2 g) was weighed into a 50-ml polypropylene centrifuge tube and 20 ml of cold 40 mM phosphate buffer (pH 6.8) were added. The mixture was homogenised with an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany) at 13,500 rpm for 10 sec. The homogenate was centrifuged at 3000 × *g* for 30 min at 4 °C using a Sorvall RC 26 Plus refrigerated centrifuge (Sorvall, Norwalk, CT, USA). The supernatant was

filtered with Whatman No.1 filter paper (Whatman International, Ltd, Maidstone, England). Total haem pigment was determined by direct spectrophotometric measurement at 525 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Myoglobin content was calculated from the millimolar extinction coefficient of 7.6 and a molecular weight of 16,110. Haem iron content was calculated based on myoglobin, which contains 0.35% iron. The haem iron content was expressed as mg/100g sample.

2.3.6.5 Determination of non-haem iron content

Non-haem iron content was determined as described by Schricker *et al.* (1982) with a slight modification. The fish mince sample (1.0 g) was weighed into a screw cap test tube and 50 µl of 0.39% (w/v) sodium nitrite were added. A mixture (4 ml) of 40% trichloroacetic acid and 6 N HCl (ratio of 1:1 [v/v], prepared freshly) was added. The tightly capped tubes were placed in an incubator shaker at 65 °C (Memmert, D-91126, Schwabach, Germany) for 22 h and then cooled down at room temperature (28 -30 ºC) for 2 h. The supernatant (400 µl) was mixed with 2 ml of the nonhaem iron colour reagent (prepared freshly). After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenanthroline (0.162 g, dissolved in 100 ml of double deionised water with 2 ml thioglycolic acid [96–99%]); (2) double-deionised water; and (3) saturated sodium acetate solution.

The non-haem iron content was calculated from iron standard curve. The iron standard solution, ranging from 0 to 2 ppm (400 µl), was mixed with 2 ml of the non-haem iron colour reagent. The concentration of non-haem iron was expressed as mg /100g sample.

2.3.7 Statistical analysis

All experiments were run in triplicate. Completely randomized design (CRD) was used for this study. The experimental data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's

Multiple Range Test (Steel and Torrie, 1980). Data analysis was performed using a SPSS package (SPSS 14.0 for Windows, SPSS Inc, Chicago, IL, USA).

2.4 Results and discussion

2.4.1 *In vitro* antioxidant activity of different phenolic compounds

2.4.1.1 DPPH radical scavenging activity

DPPH radical scavenging activity of catechin, caffeic acid, ferulic acid and tannic acid at different concentrations is shown in Figure 6 (a). The activity of all phenolic compounds increased with increasing concentration ($P>0.05$). At the same concentration used, the descending order of DPPH radical scavenging activity of phenolic compounds tested was as follows: tannic acid > catechin > caffeic acid > ferulic acid ($P<0.05$). The high radical scavenging activity of tannic acid and catechin was probably attributed to the higher degree of hydroxylation in their structure. As a result, their capability to donate hydrogen to the free radical was more pronounced (Scherer and Godoy, 2009). The effect of antioxidants on DPPH radical scavenging is generally due to their hydrogen-donating ability (Siddhuraju and Becker, 2007). Tannins extracted from stem bark of *Cassia fistula* or from canola and rapeseed hulls possessed DPPH radical quenching capacity (Siddhuraju *et al.,* 2002; Amarowicz *et al.,* 2000).

The lower DPPH radical scavenging activity of ferulic acid was observed compared with other phenolic compounds tested ($P<0.05$). This might be attributed to the presence of adjacent substituted methoxyl group of a hydroxyl group in the aromatic ring, which reduced free radical scavenging capacity of ferulic acid (Siddhuraju and Becker, 2007; Rice-Evans *et al.,* 1996). Additionally, carboxyl group in ferulic acid might show the negative effect on its antioxidative activity. The carboxyl group is an electron-withdrawing group, which does not benefit the radical scavenging activity of the compound (Thiago Inacio *et al.,* 2008). Higher radical scavenging activity of caffeic acid has been reported in comparison with ferulic acid (Bratt *et al.,* 2003; Scherer and Godoy, 2009).

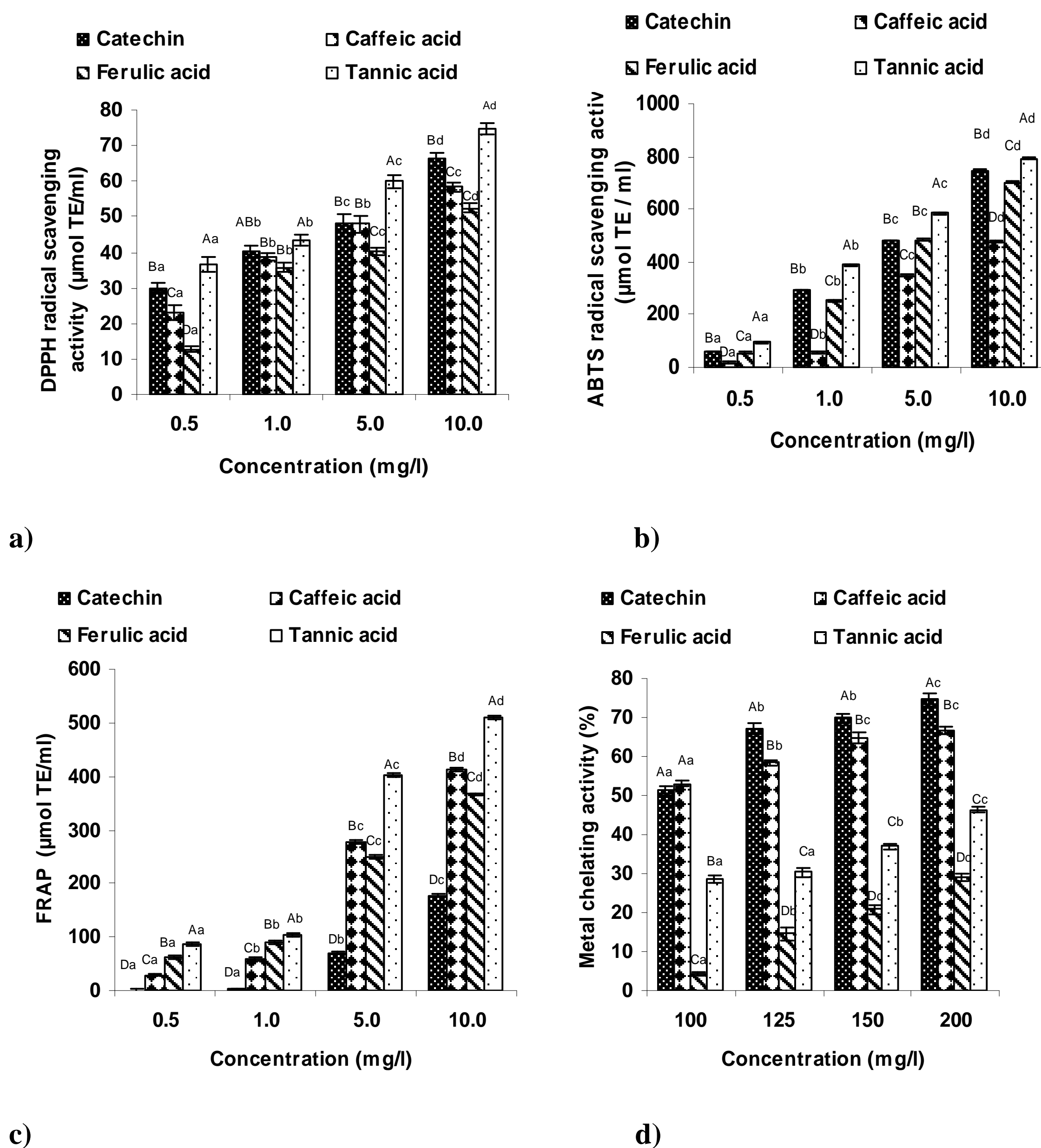


Figure 6. Antioxidative activity of different phenolic compounds at various levels as determined by DPPH radical scavenging (a), ABTS radical scavenging (b), FRAP (c) and metal chelating (d) assays. Bars represent the standard deviation (n=3). Different capital letters within the same concentration denote the significant differences ($P<0.05$). Different small letters within the same type of phenolic compound denote significant differences ($P<0.05$).

The presence of a second hydroxyl group in the ortho or para position is known to increase antioxidative activity due to the additional resonance stabilisation and *o*-quinone or *p*-quinone formation (Graf, 1992). Increasing the number of donated electrons helps caffeic acid in scavenging the DPPH radical more efficiently than ferulic acid (Medina *et al.,* 2007). Thus, the presence of higher numbers of hydroxyl groups present in catechin and tannic acid was most likely associated with the increased DPPH radical scavenging activity.

2.4.1.2 ABTS radical scavenging activity

ABTS radical scavenging activity of different phenolic compounds at various concentrations is presented in Figure 6 (b). ABTS radical scavenging activity of all phenolic compounds increased as the concentration increased ($P<0.05$). However, the activity varied with the types of phenolic compounds tested. Similar to the results of DPPH radical scavenging activity, tannic acid and catechin were more effective in scavenging ABTS radical, compared with caffeic acid and ferulic acid ($P<0.05$). Hagerman *et al.* (1998) reported that the high molecular weight phenolics such as tannic acid have more ability to quench ABTS radical and the effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific functional groups. Phenolic compounds capable of donating hydrogen atom were more effective in scavenging ABTS radical (Leong and Shui, 2002). It was noted that ferulic acid exhibited the higher ABTS radical scavenging activity than did caffeic acid ($P<0.05$), while the later showed the higher DPPH radical scavenging activity than did the former (Fig. 6 a and b). Though caffeic acid contains the higher number of hydroxyl groups, it exhibited the lower ABTS radical scavenging activity, in comparison with ferulic acid. Thus, it was more likely that the structure and side groups of phenolic compounds determined their ability in scavenging ABTS radical. The results suggested that different phenolic compounds had the varying capacity of scavenging the different radicals. As a consequence, different assays should be conducted to verify the antioxidant activity of various compounds, in which mode of action could be different.

2.4.1.3 FRAP (Ferric reducing antioxidant power)

Antioxidant potential of different phenolic compounds was estimated from their ability to reduce TPTZ– Fe (III) complex to TPTZ–Fe (II) complex as shown in Figure 6 (c). Among all phenolic compounds tested, tannic acid showed the highest FRAP ($P<0.05$), indicating that tannic acid could easily donate the electron to Fe (III), thus reducing it to Fe (II). The reducing capacity measures the ease of the compounds in donating electrons (Medina *et al.,* 2007). The results were in agreement with the highest DPPH and ABTS radical scavenging of tannic acid (Fig. 6 a and b). Lopes *et al.* (1999) and Andrade Jr *et al.* (2006) also reported that tannic acid was able to reduce Fe (III) to Fe (II).

Caffeic acid and ferulic acid showed the higher FRAP than did catechin, which had the higher DPPH and ABTS radical scavenging activities (Fig. 6 a,b). Caffeic acid can donate the highest number of electron (2.2 mol electron/mol phenolic), followed by ferulic acid (1.9 mol electrons/mol phenolic) and chlorogenic acid (1.6 mol electrons/mol phenolic) (Medina *et al.,* 2007). Gulcin (2006) reported that reducing power of caffeic acid was higher than that of BHA, BHT, α-tocopherol and trolox.

In the present study, catechin possessed a higher number of hydroxyl groups than ferulic acid and caffeic acid, but showed the lower FRAP ($P<0.05$). The result was in accordance with Medina *et al.* (2007) who recently discussed that the absolute antioxidant capacity cannot be predicted simply by determining the number of hydroxyl groups. The higher number of hydroxyl groups in the chlorogenic acid did not increase its reducing power (Medina *et al.,* 2007).

2.4.1.4 Metal chelating activity

Metal chelating activity of different phenolic compounds at various concentrations is depicted in Figure 6 (d). Catechin showed the higher metal chelating activity, followed by caffeic acid, tannic acid and ferulic acid at all concentrations tested ($P<0.05$) except at a level of 100 mg/l, where catechin had the activity similar to caffeic acid ($P>0.05$). Metal chelating activity of catechin was also reported by

Morel *et al.* (1993). Owing to polyhydroxylated structure, catechin could act as antioxidant either through the chelation of metals with redox property or by acting as scavenger of free radical (Rice-Evan *et al.,* 1996). The capacity of antioxidant for chelating metals is strongly dependent on the number of hydroxylic groups in ortho-position. Caffeic acid was reported to have ferrous chelating activity (Gulcin, 2006). It was noted that tannic acid possessed the poor metal chelating property, while it had the superior activity for other assays (Fig. 6 a,b,c). Lopes *et al.* (1999) found that antioxidant mechanism of tannic acid against $OH^{\bullet}$ formation via a Fenton reaction was mainly due to its iron chelating effect rather than $OH^{\bullet}$ scavenging activity.

The lowest metal chelating activity of ferulic acid was possibly due to the presence of only one hydroxyl group in its structure. The presence of a methoxy group, which hindered their chelating capacity, was also postulated. Methoxy groups could stabilise phenoxyl radicals owing to its electron-donating abilities but did not contribute to the chelating abilities (Danilewicz, 2003). Also, ferulic acid does not have a galloyl moiety, thus it cannot bind iron. Gulcin (2006) reported that ferulic acid possessed very less chelating activity against Fe (II). Thus, the efficiency in metal chelation varied with the type of phenolic compounds and did not relate with radical scavenging activity and reducing power.

2.4.2 Lipoxygenase inhibitory activity of different phenolic compounds

Percentage inhibition of lipoxygenase (LOX) by different phenolic compounds at various concentrations is shown in Figure 7. For all the phenolic compounds tested, inhibitory activity increased with increasing concentrations used ($P<0.05$). At the same concentration tested, caffeic acid showed the highest inhibitory activity towards LOX ($P<0.05$). Koshihara *et al.* (1984) and Vob *et al.* (1992) reported that caffeic acid inhibited LOX-5 with the IC_{50} value of 3.7 µM. Caffeic acid and its phenethyl ester are well recognised as natural inhibitors of LOX (Sudina *et al.* 1993). No differences in LOX inhibitory activity were observed among catechin, ferulic acid and tannic acid at 25 and 50 mg/l concentration ($P>0.05$). In general, the inhibitory activity of phenolic compounds towards LOX increases with the number of hydroxyl substituents (Laughton *et al.,* 1991; Sadik *et al.,* 2003). Though tannic acid contains a

larger number of hydroxyl groups in its structure, its LOX inhibitory activity was lower than caffeic acid ($P<0.05$). Due to the large molecular structure, tannic acid might not be able to bind to the active site of LOX effectively. The mechanism of LOX inhibition by phenolic compounds is most likely a combination of radical scavenging and binding to the hydrophobic active site of LOX and/or an interaction with the hydrophobic fatty acid substrate (Schurink, 2007).

Catechin and ferulic acid also showed LOX inhibitory activity in a concentration dependent manner. Green tea catechin displayed the inhibitory effects on both LOX-catalysed and Hb-catalysed oxidation of arachidonic acid and linoleic acid (Liu and Bonnie, 2004). Catechin mixtures prepared from tea effectively prevented the pro-oxidant activity of lipoxygenase (LOX) in fish skin extract (Mohri *et al.,* 1999). Mechanism of LOX-catalysed lipid oxidation is radical-based, where the fatty acid is oxidised by the ferric iron within the active site of the enzyme to form a fatty acid radical and a ferrous iron. This radical intermediate is then attacked by dioxygen to form the fatty acid hydroperoxide (Schurink, 2007). Therefore, the phenolic compounds like caffeic acid and tannic acid having higher reducing power and metal chelating activity showed the higher inhibitory effect towards LOX. The results revealed that all phenolic compounds tested could inhibit LOX at different degrees. Phenolic compounds might bind with LOX via hydrophobic interaction or hydrogen bond, leading to the conformational changes of LOX. This resulted in the loss in LOX activity, particularly when the higher levels of phenolic compounds were used.

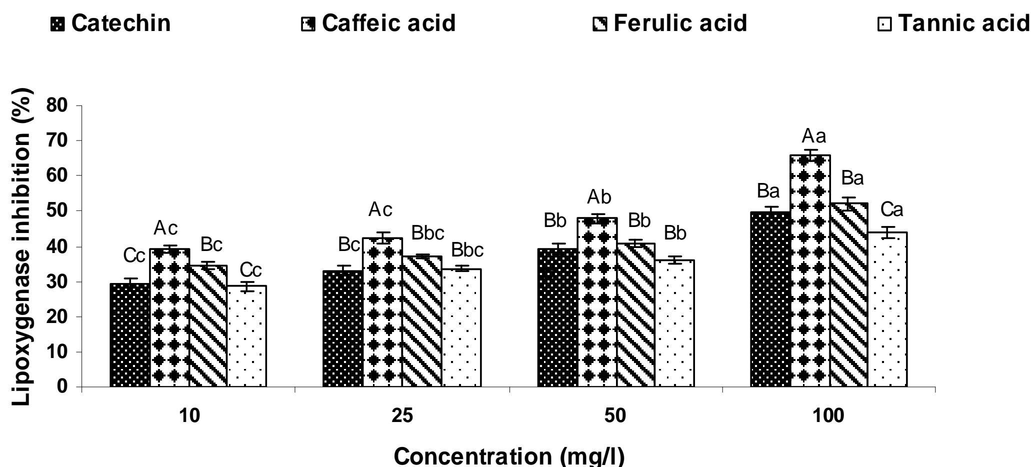


Figure 7. Lipoxygenase inhibitory activity of different phenolic compounds at various levels. Bars represent the standard deviation (n=3). Different capital letters within the same concentration denote the significant differences (P<0.05). Different small letters within the same type of phenolic compound denote significant differences (P<0.05).

2.4.3 Prevention of lipid oxidation in menhaden oil-in-water emulsion by different phenolic compounds

2.4.3.1 Changes in lipid oxidation products

PV of menhaden oil-in-water emulsion added without and with different phenolic compounds at a level of 100 mg/kg during the storage is presented in Figure 8 (a). The gradual increases in PV were observed in all samples during the first 48 h of storage. Thereafter, PV increased at a higher rate up to 96 h of storage (P<0.05). No marked change in PV was noticed during 96-168 h of storage (P>0.05). When comparing PV of all samples, it was found that the control sample contained the higher PV than did the samples added with phenolic compounds throughout the storage, particularly after 12 h of storage.

Tannic acid was more effective in lowering the increase in PV in menhaden oil-in-water emulsion than the other three phenolic compounds used in the present study (P<0.05). The higher efficiency of tannic acid in prevention of the

hydroperoxide formation correlated well with the higher DPPH and ABTS radical scavenging activities and reducing power (Fig. 6 a,b,c). Tannic acid contained a large number of hydrophobic portions, which could align themselves at the oil-water interface and functioned as a hydrogen donor or radical scavenger. This resulted in the retardation of the initiation and propagation stages, as evidenced by the lower PV.

Caffeic acid and ferulic acid showed similar effect on retarding the formation of PV in menhaden oil-in-water emulsion ($P>0.05$) (Fig. 8a) but their efficiency was lower than that of tannic acid ($P<0.05$). Nevertheless, caffeic acid and ferulic acid exhibited the higher ability in prevention of PV formation than did catechin ($P<0.05$). De Leonardis and Macciola (2003) reported that the antioxidant effectiveness of caffeic acid was better than that of BHA in hydrophobic phases such as cod liver oil.

The differences in ability in inhibiting PV formation among all phenolic compounds were more likely governed by their hydrophobicity/ hydrophilicity balance as well as their localisation in emulsion system. Catechin is the more polar antioxidant than tannic acid and caffeic acid (Medina *et al.,* 2007). Therefore, catechin was mostly found in the aqueous phase in close proximity to the transition metal ions present in the aqueous phase. Furthermore, phenolic compounds including, catechin could act as a pro-oxidants rather than an antioxidant in the presence of transition metal ions, particularly Fe^{3+}. Catechin might reduce transition metal ions ($Fe^{3+} \rightarrow Fe^{2+}$) and generated hydrogen peroxide through autoxidation, which drives the production of hydroxyl radicals *via* the Fenton reaction (Retsky *et al.,* 1993). Sorensen *et al.* (2008) also reported that the caffeic acid was found to be pro-oxidative in the omega-3-enriched oil-in-water emulsion irrespective of pH, emulsifier type and presence of iron. The formation of H-bonded complexes between catechin and water molecule at the water-oil interface or in the aqueous phase resulted in the less availability of catechin to act as antioxidant. Catechin showed a low inhibition on the formation of peroxides and TBARS in chilled horse mackerel (Medina *et al.,* 2007).

The impact of phenolic compounds on conjugated diene (CD) and thiobarbituric acid reactive substances (TBARS) formation in menhaden oil-in-water emulsion during the storage is shown in Figure 8 (b) and (c), respectively. After initiation stage, oxidation is propagated via hydrogen subtraction in the vicinity of double bonds. This propagation step implies the formation of isomeric hydroperoxides that frequently carry conjugated diene groups. This is the mechanism of formation of CD. CD and TBARS levels increased continuously in all samples throughout the storage of 168 h ($P<0.05$). Similar patterns in changes of CD and TBARS were noticeable in comparison with that of PV (Fig. 8a), in which tannic acid exhibited the highest efficacy in preventing the increase in PV. The increase in CD was coincidental with the increase in PV. All samples added with phenolic compounds showed the lower CD formation, compared with the control (Fig. 8b) ($P<0.05$). The inhibitory effect of tannic acid on CD formation was slightly higher than other phenolic compounds tested. This was due to the higher radical scavenging activity of tannic acid, thereby lowering the subsequent generation of reactive lipid radical, which can undergo further chain reaction. The increase in TBARS of emulsion indicated the formation of lipid oxidation products. This was dominant in the control samples and was retarded in the samples added with phenolic compounds, particularly tannic acid. The increase in TBARS values after 96 h of storage in the control samples were coincidental with the lower formation of PV. A decrease in the level of primary oxidation products is related to hydroperoxide degradation, producing secondary lipid peroxidation products (Undeland *et al.,* 1998). Thus, in menhaden oil-in-water emulsion, tannic acid acted as an efficient antioxidant while catechin showed the lowest preventive effect on lipid oxidation. The result confirmed the role of phenolic compounds in lowering the lipid oxidation in menhaden oil-in-water emulsion.

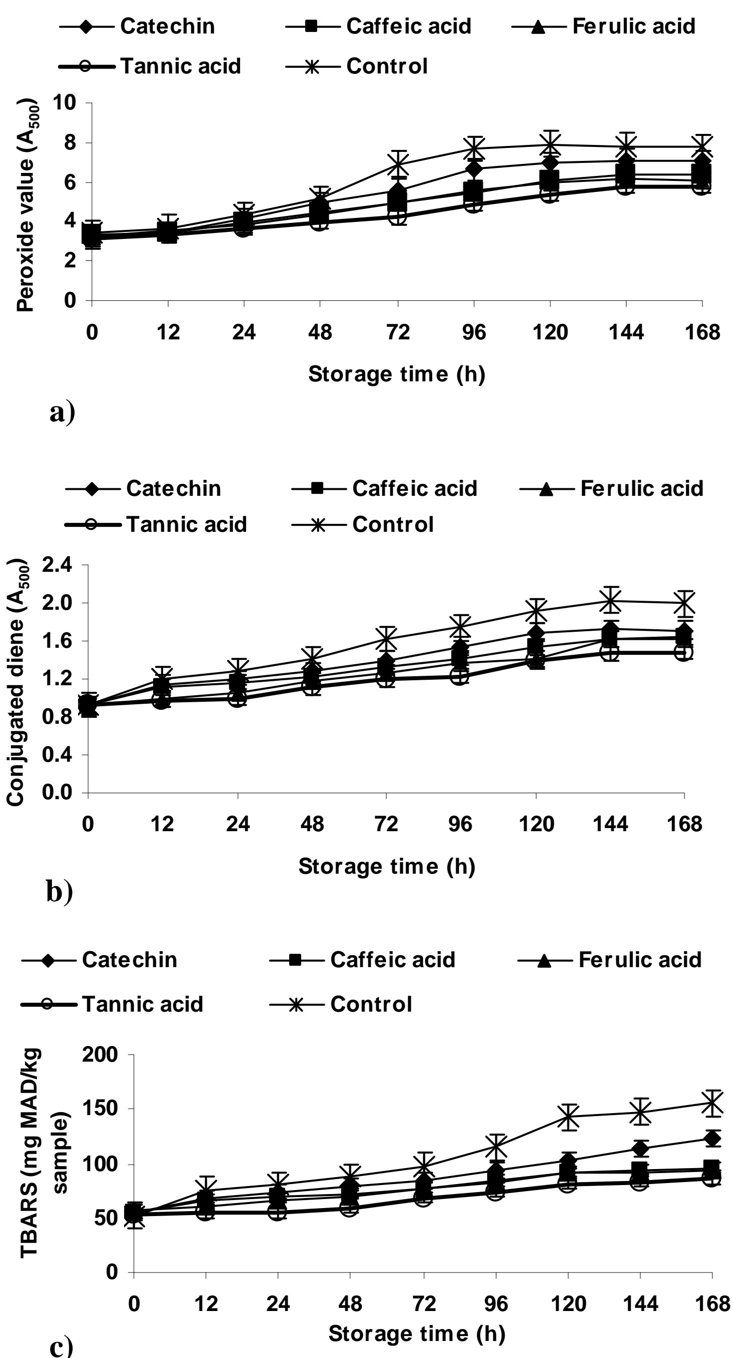


Figure 8. Effect of different phenolic compounds on the formation of lipid oxidation products in menhaden oil-in-water emulsion stored at 30ºC for a period of 168 h. Peroxide value (a), conjugated diene (b) and thiobarbituric acid reactive substances (TBARS) values (c). Bars represent the standard deviation (n=3). For all phenolic compounds, a final concentration of 100mg/l was used in the system.

2.4.4 Prevention of lipid oxidation in mackerel mince by different phenolic compounds

2.4.4.1 Changes in lipid oxidation products

The formation of PV in mackerel mince added without and with different phenolic compounds at a level of 100 mg/kg during iced storage is depicted in Fig. 9 (a). PV in the control sample increased drastically from day 0 to day 6 ($P<0.05$). Thereafter, no marked change in PV were found until the end of the storage period ($P>0.05$). PV of the control sample were higher than that of samples added with phenolic compounds throughout the storage ($P<0.05$). Samples added with tannic acid contained the lower PV than the control and those added with other phenolic compounds. The results indicated the efficient antioxidative activity of tannic acid in fish mince system. Tannic acid also showed higher antioxidant activity in most *in vitro* antioxidant assays (Fig. 6) as well as in menhaden oil-in-water emulsion (Fig. 8) ($P<0.05$). Free radical scavenging antioxidants including tannic acid interfere with the initiation or propagation steps of lipid oxidation reactions by scavenging lipid radicals and forming low-energy antioxidant radicals that do not readily promote oxidation of unsaturated fatty acids (Frankel, 1998b). Reducing capacity of phenolic antioxidants was realised as a key function for retarding and inhibiting lipid oxidation of fish tissues (Medina *et al.,* 2007).

PV of samples added with caffeic acid, ferulic acid and catechin was lower than that of the control samples ($P<0.05$), but was higher than that of samples added with tannic acid ($P<0.05$). Both caffeic acid and catechin showed similar efficiency in preventing the lipid oxidation in the mackerel mince, but the tannic acid was more effective antioxidant in the fish mince system. The results were in agreement with Alghazeer *et al.* (2008) and Tang *et al.* (2001) who reported that the incorporation of green tea catechin in frozen mackerel was effective in lowering PV and TBARS values as compared to the control during frozen storage. Tea catechin addition (300 mg/kg meat) effectively reduced lipid oxidation in cooked beef and chicken meat (Tang *et al.,* 2001) and in raw red meat, poultry and fish muscle (Tang *et al.,* 2001). Medina *et al.* (2007) found that caffeic acid employed at 10 ppm showed

similar antioxidative effectiveness in chilled horse mackerel muscle to propyl gallate at same concentration.

CD values of all samples increased gradually during storage up to 15 days of iced storage (Fig. 9b) ($P<0.05$). Among all phenolic compounds tested, ferulic acid showed the lower activity in prevention of CD formation and tannic acid tended to exhibit the highest preventive effect. PV of sample added with ferulic acid started to decline after day 9 of iced storage (Fig. 9a). These results indicated that ferulic acid was the least effective phenolic compound for the prevention of lipid oxidation in mackerel fish mince. This was coincidental with the lower ability of ferulic acid to chelate Fe^{2+} (Fig. 6d). Fe^{2+} has been known as the most important pro-oxidant in the fish muscle (Love, 1983). For other phenolic compounds tested, the preventive effect on lipid oxidation might be associated with the capacity of metal chelating in fish mince.

For TBARS, samples added with phenolic compounds as well as the control samples had the increased values as the storage time increased up to 12 days ($P<0.05$). The decreased TBARS value were found at day 15 of storage ($P<0.05$). This was probably due to the losses in oxidation products formed, particularly the volatile counterparts. Malonaldehyde and other short-chain carbon products of lipid oxidation are not stable and are decomposed to organic alcohols and acids, which are not determined by the TBA test (Fernandez *et al.,* 1997).

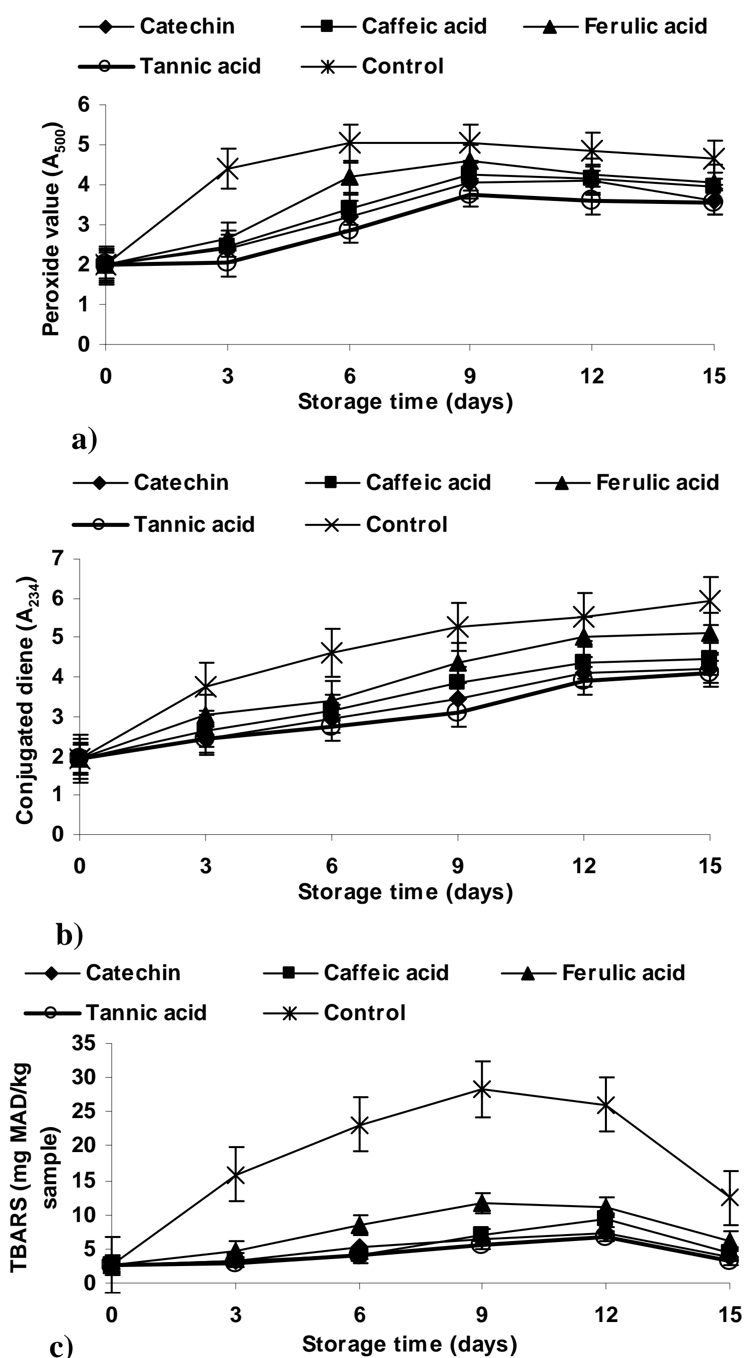
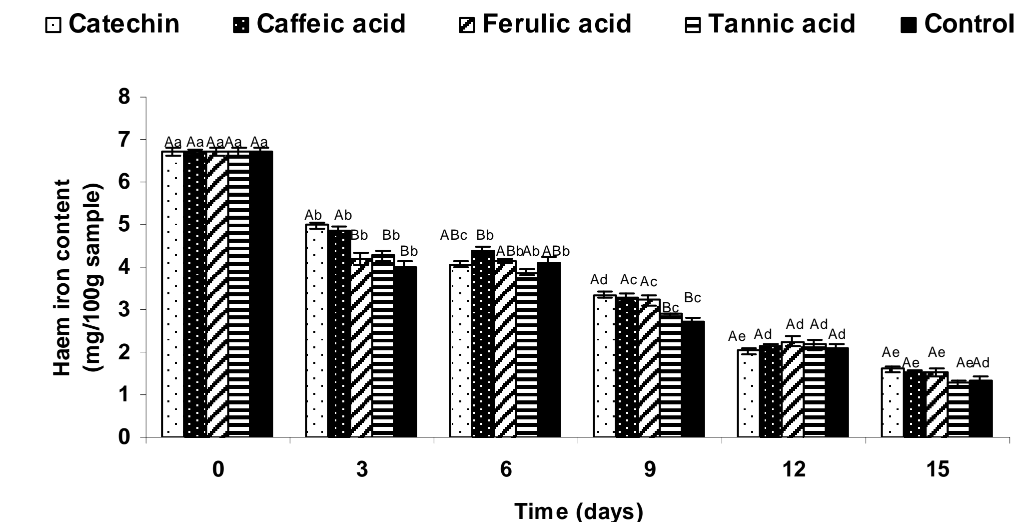


Figure 9. Effect of different phenolic compounds on the formation of lipid oxidation products in mackerel mince during iced storage for a period of 15 days. Peroxide value (a), conjugated diene (b) and thiobarbituric acid reactive substances (TBARS) values (c). Bars represent the standard deviation (n=3). For all phenolic compounds, a final concentration of 100mg/kg was used in the system.

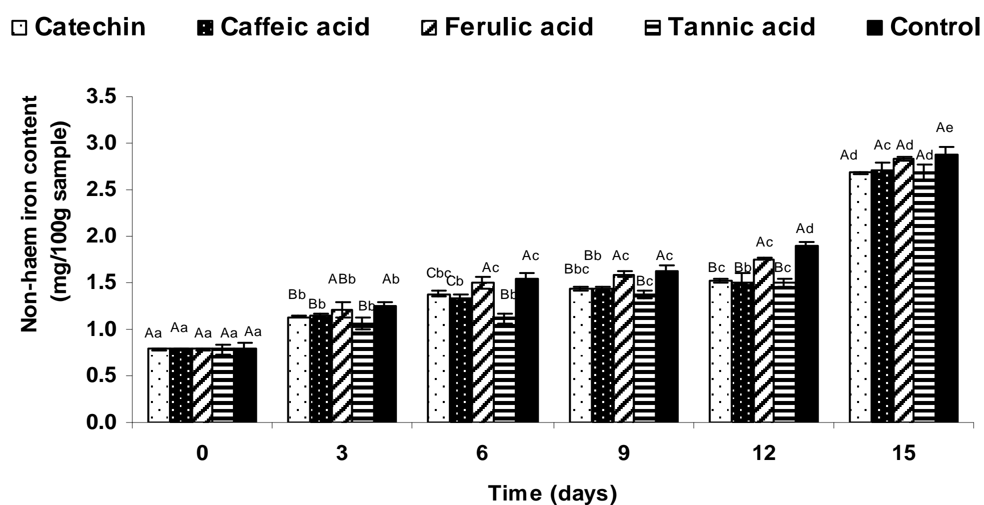
2.4.5 Changes in haem and non-haem iron contents

Changes in haem and non-haem iron contents in mackerel mince with and without the addition of different phenolic compounds during ice storage are presented in Figure 10 (a) and (b) respectively. Haem iron content of all samples decreased with increasing storage time up to 15 days of iced storage (P<0.05). There was no difference in haem iron content of samples added with different phenolic compounds and the control sample throughout the storage, except at day 3 and 9, when haem iron content of samples added with catechin and caffeic acid was higher than the control sample (P<0.05). Haem iron content decreased due to the haem breakdown, which resulted in the increase of non-haem iron content (Benjakul and Bauer, 2001). Additionally, the lowered haem pigment extractability with increasing storage time also resulted in the lower iron content of the haem extracted (Chaijan *et al.,* 2005). This is possibly associated with the higher lipid oxidation in dark muscle, which had a high fat content (Chaijan *et al.,* 2005). Benjakul and Bauer (2001) and Gomez-Basauri and Regenstein (1992a) reported that the decrease in haem iron content in the muscle was inversely related to non- haem iron content.

Non- haem iron content of all mackerel mince samples increased during the iced storage (P<0.05) (Fig. 10b). During 6-12 days of storage, the control sample and those added with ferulic acid showed the higher non- haem iron content than others (P<0.05). The lower metal chelating ability of ferulic acid might contribute to the high non- haem iron remaining in the mince. The continuous increase in non-haem iron content with increasing storage period was in agreement with the release of free iron from the muscle, which continued to degrade with the extended storage. Decker and Hultin (1990a, 1990b) suggested that the haem pigment or other iron-containing proteins are possibly denatured with increasing storage time, resulting in the release of iron. Deterioration of sub-cellular, organelles, e.g. mitochondria, and the release of cytochrome-C, could also be responsible for the increase in non-haem iron. As non-haem iron content of the control and sample added with ferulic acid was slightly higher than other samples, this could be related with the higher degree of oxidation in both samples.



a)



b)

Figure 10. Change in haem iron content (a) and non-haem iron content (b) in mackerel mince without and with different phenolic compounds during iced storage for a period of 15 days. Bars represent the standard deviation (n=3). Different capital letters within the same concentration denote the significant differences (P<0.05). Different small letters within the same type of phenolic compound denote significant differences (P<0.05). For all phenolic compounds, a final concentration of 100mg/kg was used in the system.

Brune *et al.* (1989) found that the absorption effects of tannin and catechin on non-haem iron content were more pronounced when there were 3 hydroxyl groups (galloyl) on the phenolic structure than when there were 2 hydroxyl groups catechol). Therefore, the ability in binding non-haem iron was governed by the structure and hydroxyl group of phenolic compounds and more likely affected the efficacy in inhibiting or preventing lipid oxidation mediated by free iron in the fish mince during iced storage.

2.5 Conclusions

Antioxidativity of phenolic compounds varied with the types and molecular structure. Tannic acid exhibited the antioxidative activity and could prevent lipid oxidation effectively in menhaden oil-in-water emulsion as well as in fish mince. This was most likely caused by its radical scavenging activity, LOX inhibitory activity as well as metal chelating activity, especially towards non-haem iron. Thus, the selection of suitable phenolic compound as an antioxidant is of great importance to maximize the prevention of lipid oxidation in different food model systems.

CHAPTER 3

SYNERGISTIC EFFECT OF TANNIC ACID AND MODIFIED ATMOSPHERIC PACKAGING ON THE PREVENTION OF LIPID OXIDATION AND QUALITY LOSSES OF REFRIGERATED STRIPED CATFISH SLICES

3.1 Abstract

Chemical, microbiological and sensorial changes of striped catfish *(Pangasius hypothalamus)* slices treated without and with tannic acid (100 and 200 mg/kg) were determined during 15 days of storage at 4 ºC in air and under modified atmospheric packaging (MAP, 60%N_2 / 35%CO_2 / 5%O_2). The slices consisted of 9.2 g lipid /100 g and the lipid contained 64.55% unsaturated fatty acids and 33.87 % saturated fatty acids. During the storage, the sample treated with 200 mg/kg tannic acid and stored under MAP (M_2) had the lowest peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) with the coincidental lowest non-haem iron content, indicating the retarded lipid oxidation. Fourier transform infrared (FTIR) spectra indicated primary oxidation products and free fatty acids in M_2 sample after 15 days. Conversely, these compounds were found at lower contents in the control samples kept in air without tannic acid treatment (A_0), suggesting that the deterioration was more advanced. Myosin heavy chain of A_0 was degraded by 17.85% after 15 days of storage, whereas no change was noticeable in M_2 compared with the fresh sample (F). Based on microbiological acceptability limit (10^7 cfu/g), the shelf-life of A_0 and M_2 was estimated to be 9 and 15 days, respectively. M_2 had the acceptable scores for all sensory attributes up to 15 days, while A_0 was acceptable when stored for 9 days. Therefore, tannic acid exhibited a synergistic effect with MAP on retarding lipid oxidation and microbial growth, thereby increasing the shelf-life of striped catfish slices during refrigerated storage.

3.2. Introduction

Fresh fish are highly perishable products and their deterioration is mainly from the biological reactions such as oxidation of lipids, protein degradation or decomposition mediated by endogenous or microbial enzymes. These activities lead to a short shelf-life of fish and other seafood products (Gobantes *et al.,* 1998). Fish contain a high amount of n-3 polyunsaturated fatty acid, especially eicosapentaenoic acid (20: 5 n-3) and docosahexaenoic acid (22: 6 n-3), which have been shown to have potential benefits for human health (Lee and Lip, 2003). Nevertheless, they are susceptible to oxidation, which is associated with the rancidity and loss in nutritive value (Frankel, 1998a). To alleviate the spoilage caused by microorganisms, frozen storage has been used widely. However, freezing and frozen storage are not able to terminate the chemical reactions, especially lipid oxidation and protein denaturation (Benjakul *et al.,* 2005). These reactions result in the decrease in functional properties, quality and consumer acceptability.

Modified atmosphere packaging (MAP) in combination with refrigeration has been proved to be an effective preservation method, in which the shelf-life extension and quality retention of a large variety of fresh chilled food products e.g. red meat, poultry, fruits, vegetables, etc have been achieved (Brody and Marsh, 1997). MAP was also used to extend the shelf-life of fish and fish products (Masniyom *et al.,* 2002; Ozogul *et al.,* 2004; Pastoriza *et al.,* 1998; Ruiz-Capillas and Moral, 2001, 2005). However, Masniyom *et al.* (2002) reported that the use of MAP with 60% and 80% CO_2 resulted in the increase in lipid oxidation of seabass slices during refrigerated storage. To lower the lipid oxidation, the treatment of fish with potential antioxidants is required. Phenolic compounds have been used as the natural antioxidant to retard lipid oxidation in foods. Tannins from *Osbeckia chinensis* were found to have potential antioxidative efficiency in the linoleic acid-thiocyanate system (Su *et al.,* 1998). Recently, Maqsood and Benjakul (2010a) found that tannic acid exhibited the highest antioxidative activity by different *in vitro* assays as well as in fish oil-in-water emulsion and fish mince. Tannic acid is affirmed as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) for the use as a direct additive in some food products such as baked goods and baking mixes,

alcoholic and non-alcoholic beverages, frozen dairy desserts and mixes, hard candy and cough drop as well as meat products (21 CFR184. 1097, U.S. Code of Federal Regulation, 2006). Therefore, the use of tannic acid in combination with MAP could be a promising means to lower lipid oxidation and extend the shelf-life of striped catfish slices, containing a high amount of fat, which has become an economically important species in Thailand.

To our knowledge, no information on synergistic effect of phenolic compound and MAP on lipid oxidation and shelf-life extension of refrigerated striped catfish slices have been reported. Thus, the objective of present work was to study the combined effect of tannic acid and MAP on the changes in chemical, microbiological as well as sensory properties of striped catfish slices during refrigerated storage at 4 ºC.

3.3 Material and methods

3.3.1 Chemicals

Tannic acid, thioglycolic acid, cumene hydroperoxide, β-mercaptoethanol and bathophenanthroline disulfonic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, anhydrous sodium sulphate, potassium iodide, trichloroacetic acid, iron standard solution, ethanol and methanol were obtained from Merck (Damstadt, Germany). Disodium hydrogen phosphate, 2-thiobarbituric acid, ammonium thiocyanate, ferrous chloride, sodium nitrite and saturated sodium acetate were purchased from Fluka Chemical Co. (Buchs, Switzerland). Plate count agar was obtained from Hi-media (Mumbia, India). Chloroform was procured from Lab-Scan (Bangkok, Thailand). All chemicals for electrophoresis were obtained from Bio-Rad (Richmond, CA, USA).

3.3.2 Fish preparation

Striped catfish (*Pangasius hypothalamus*) weighing 10-11 kg, off-loaded 24 h after capture and stored in ice, were purchased from the fish market in Hat Yai, Songkhla, Thailand. The fish were kept in ice during transportation to the Department of Food Technology, Prince of Songkla University. Upon arrival, fish were washed with tap water, filleted, deskinned and cut into slices with a thickness of 1-2 cm. The slices were placed in polyethylene bags and kept in ice until use. A portion of slices was taken for lipid extraction and analysis.

3.3.3 Lipid extraction and analysis

Lipid was extracted by the method of Bligh and Dyer (1959). Ground sample (25 g) was homogenised with 200 ml of chloroform:methanol:distilled water mixture (1:2:1) at the speed of 9,500 rpm for 2 min at 4 °C using an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany). The homogenate was then added with 50 ml of chloroform and homogenised at 9,500 rpm for 1 min. Subsequently 25 ml of distilled water was added and the mixture was homogenised again for 30 sec. The homogenate was centrifuged at 14,500 x g at 4 °C for 15 min using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA), and transferred into a separating flask. The chloroform phase was drained off into a 125 ml-erlenmeyer flask containing about 2–5 g of anhydrous sodium sulphate, shaken very well, and decanted into a round-bottom flask through a Whatman No. 4 filter paper (Whatman International, Ltd, Maidstone, England). The solvent was evaporated at 25 °C, using an EYELA rotary evaporator N-100 (Tokyo, Japan), and the residual solvent was removed by flushing with nitrogen.

3.3.3.1 Determination of fatty acid profile

Fatty acid profile of lipid from fish slices was determined as fatty acid methyl esters (FAMEs). The FAMEs were prepared according to the method of AOAC (2000). The prepared methyl esters were quantified by gas chromatography (GC). GC (Shimadzu, Kyoto, Japan) equipped with a flame ionisation detector (FID, at a split ratio of 1:20) was used. A fused silica capillary column (30 m × 0.25 mm),

coated with bonded polyglycol liquid phase, was used. The analytical conditions were: injection port temperature of 250 °C and detector temperature of 270 °C. The injection volume was 2 µl. The oven was programmed from 170 to 225 °C at a rate of 1 °C/min (no initial or final hold). Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated, based on the peak area ratio and expressed as gram fatty acid/100 g of lipid.

3.3.4 Tannic acid treatment and modified atmosphere packaging (MAP) of striped catfish slices

Striped catfish slices (50 g) were placed on the polystyrene trays (20 x 12 cm^2). Tannic acid (5 and 10 mg) was dissolved separately in 1 ml of distilled water and applied uniformly to prepared slices (50 g) to obtain a final concentration of 100 and 200 mg/kg, respectively. A tray containing fish slices was inserted in nylon/LLDPE bag (30 x 16 cm) (Asian Foams, Hat Yai, Thailand) with the thickness of 0.08 mm and gas permeability (CO_2, N_2 and O_2: 1.7 x 10^{-10}, 0.1 x 10^{-10} and 0.4 x 10^{-10} m^3.mm/cm^2.s.cmHg at 25 ºC, 1 atm pressure, respectively) and was packed with a fish/gas ratio of 1:3 (w/v) using a Henkovac type 1000 (Tecnovac, Italy). Prior to filling the gas, the bag was evacuated fully. Gas mixture containing 60% CO_2 / 35% N_2 / 5% O_2 with a pressure of 5 kg/cm^2 was filled in the bag. Fish slices treated without and with tannic acid (100 and 200 mg/kg) and packed in air were prepared and designated as A_0, A_1 and A_2, respectively. Those packed under MAP were designated as M_0, M_1 and M_2, respectively. For the control samples (A_0 and M_0), same quantity (1 ml) of distilled water was added to the slices. All samples were stored at 4 ºC and taken for chemical, microbiological and sensory analysis every 3 days for 15 days, except sensory analysis was omitted at day 12.

3.3.5 Chemical analysis

3.3.5.1 Peroxide value

Peroxide value was determined as per the method of Richards and Hultin (2002) with a slight modification. Ground sample (1 g) was homogenised at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v). Homogenate was then filtered using Whatman No. 1 filter paper. Two millilitre of 0.5% NaCl was then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3,000 x g for 3 min to separate the sample into two phases. Two millilitre of cold chloroform/methanol (2:1) were added to 3 ml of the lower phase. Twenty-five microlitre of ammonium thiocyanate and 25 µl of iron (II) chloride were added to the mixture (Shantha and Decker, 1994). Reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A standard curve was prepared using cumene hydroperoxide at a concentration range of 0.5-2 ppm.

3.3.5.2 Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Ground sample (0.5 g) was mixed with 2.5 ml of a TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture was heated in a boiling water bath (95-100 ºC) for 10 min to develop a pink color, cooled with running tap water and then sonicated for 30 min, followed by centrifugation at 5,000 ×g at 25 ºC for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (MAD) at the concentration ranging from 0 to 10 ppm and TBARS were expressed as mg of MAD equivalents/kg of sample.

3.3.5.3 Determination of haem iron content

The haem iron content was determined according to the method of Gomez-Basauri and Regenstein (1992a) with a slight modification. Ground sample (2 g) was transferred into a 50 ml-polypropylene centrifuge tube and 20 ml of cold 40 mM phosphate buffer (pH 6.8) were added. The mixture was homogenised with a

homogeniser at a speed of 13,500 rpm for 10 sec. The homogenate was centrifuged at 3000 × *g* for 30 min at 4 °C. The supernatant was filtered with Whatman no.1 filter paper. Total haem pigment was determined by direct spectrophotometric measurement at 525 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). Myoglobin content was calculated from the millimolar extinction coefficient of 7.6 and a molecular weight of 16,110. Haem iron was calculated based on myoglobin, which contains 0.35% iron. The haem iron content was expressed as mg/100g sample.

3.3.5.4 Determination of non- haem iron content

Non-haem iron was determined as described by Schricker *et al.* (1982) with a slight modification. Ground sample (1 g) was placed in a screw cap test tube and 50 µl of 0.39% (w/v) sodium nitrite was added. A mixture (4 ml) of 40% trichloroacetic acid and 6 N HCl (ratio of 1:1 [v/v], prepared freshly) was added. The tightly capped tubes were placed in an incubator shaker at 65 ºC (Memmert, D-91126, Schwabach, Germany) for 22 h and then cooled down at room temperature (25-30 ºC) for 2 h. The supernatant (400 µl) was mixed with 2 ml of the non-haem iron colour reagent (prepared freshly). After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenanthroline disulfonic acid (0.162 g, dissolved in 100 ml of double deionised water with 2 ml thioglycolic acid [96–99%]); (2) double-deionised water; and (3) saturated sodium acetate solution. Non-haem iron content was calculated from iron standard curve. The iron standard solution, ranging from 0 to 2 ppm, (400 µl) was mixed with 2 ml of the non-haem iron colour reagent. The concentration of non-haem iron was expressed as mg /100 g sample.

3.3.5.5 Fourier transform infrared (FTIR) spectral analysis

Lipid extracted from the fresh sample (F) and A_0 and M_2 stored for 15 days were used for FTIR analysis. FTIR analysis of striped catfish lipid was performed in a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany). Prior to

analysis, the crystal cell was cleaned with acetone, wiped dry with soft tissue and the background scan was run. For spectra analysis, the sample (200 μl) was applied directly onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectral, in the range of 4000–400 cm^{-1} (mid-IR region) with automatic signal gain, were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean, empty cell at 25 ºC. Analysis of spectral data was carried out using the OPUS 3.0 data collection software programme (Bruker Co., Ettlingen, Germany).

3.3.5.6 SDS - polyacrylamide gel electrophoresis (SDS–PAGE)

Fresh striped catfish muscle (F) and A_0 and M_2 stored for 15 days were subjected to SDS–PAGE according to the method of Laemmli (1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution were added to the sample (3 g). The mixture was then homogenised using a homogeniser at a speed of 13,500 rpm for 2 min. The homogenate was incubated at 85 ºC for 1 h to dissolve total protein. The samples were centrifuged at 3500 × *g* for 20 min to remove undissolved debris. The samples (15 µg protein) were loaded onto the polyacrylamide gel made of 10% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel, using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% methanol (v/v) and 7.5% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid. Wide range molecular weight marker was used for estimation of molecular weight of proteins.

Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis system). The intensity of protein band of interest was expressed relative to that found in fresh sample (F).

3.3.6 Microbiological analysis

Fish slices (25 g) were collected aseptically in a stomacher bag and 10 volumes of sterile saline solution (0.85 g/100 ml) were added. After homogenising in a Stomacher blender (Stomacher M400, Seward Ltd., Worthington, England) for 1 min, a series of 10-fold dilutions was made using normal saline solution (0.85%) for microbiological analyses. Mesophilic and psychrophilic bacterial counts were determined by plate count agar (PCA) with the incubation at 35 ºC for 2 days (Hasegawa, 1987) and 7 ºC for 7 days (Cousin *et al.,* 1992), respectively. Microbial counts were expressed as log cfu/g.

3.3.7 Sensory analysis

The sensory evaluation was performed by 30 untrained panelists, who were the graduate students in Food Science and Technology programme with the age of 25-33 years and were familiar with fish consumption. The assessment was conducted for the odour, colour, taste and texture of cooked samples using a 9-point hedonic scale (Mailgaad *et al.,* 1999): 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much; 9, like extremely. The fish samples were covered with aluminium foil and cooked in steaming pot until the core temperature of each sample reached 70 ºC (Masniyom *et al.*, 2002). Stick water was drained and samples were allowed to cool to room temperature (25–28 ºC) prior to evaluation.

3.3.8 Statistical analysis

All experiments were run in triplicate. Completely randomized design (CRD) was used for this study. The experimental data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's New Multiple Range Test (Steel and Torrie, 1980). For pair comparison, *T*-test was used. Data analysis was performed using a SPSS package (SPSS 14.0 for Windows, SPSS Inc, Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Lipid content and fatty acid profile of striped catfish slices

Striped catfish slices had a lipid content of 9.2 g/100 g sample. Eymard *et al.* (2009) reported that horse mackerel muscle contained a lipid content of 2.4 g/100 g muscle, which was almost 4-fold lower than that found in striped catfish slices. Fatty acid composition of fresh striped catfish slices is shown in Table 4. Lipid of fresh striped catfish slices contained 33.8% saturated fatty acids (SFA), 42.60% mono-unsaturated fatty acids (MUFA) and 21.95% of poly-unsaturated fatty acids (PUFA). The unsaturated fatty acids were approximately 2-fold higher than that of saturated fatty acids (Table 4), suggesting that lipids of striped catfish slices were susceptible to oxidation. The predominant fatty acid was oleic acid (C18:1 *n*-9) (37.60 %), followed by palmitic acid (C16:0) (24.80 %) and linoleic acid (C18:2 *n*-6) (17.13 %). Among PUFA, linoleic acid (C18:2 *n*-6) was the most abundant, followed by linolenic acid (C18:3 *n*-3). Significant amount of n-3 fatty acid, especially docosahexaenoic acid (C22:6 *n*–3) (DHA) and eicosapentaenoic acid (C20:5 *n*–3) (EPA) were found. DHA was present at higher content than EPA. Kolakowska *et al.* (2002) and Chaijan *et al.* (2006) reported that DHA is usually more abundant than EPA (up to 2–3 times). The results revealed that striped catfish was an excellent source of fish oil or lipid, containing high content of n-3 fatty acids. Intake of n-3 polyunsaturated fatty acids from fish oil is associated with a reduction in sudden cardiac death in patients with ischemic heart disease (Lee and Lip, 2003).

Table 4. Fatty acid profile of lipid extracted from striped catfish slices.

Fatty acids	**Formula**	**Fatty acid content (g/100g lipid)**
Lauric acid	C12:0	0.19
Myristic acid	C14:0	0.90
Pentadecanoic acid	C15:0	0.15
Palmitic acid	C16:0	24.80
Heptadecanoic acid	C17:0	0.22
Stearic acid	C18:0	7.39
Arachidic acid	C20:0	0.16
Behenic acid	C22:0	0.06
∑ Saturated fatty acids (SFA)		**33.87**
Myristoleic acid	C14:1	0.10
Palmitoleic acid	C16:1 n-7	3.88
Cis-10-Heptadecanoic acid	C17:1	0.09
Cis-9-Octadecanoic acid	C18:1 n-9	37.60
Cis-11-Eicosenoic acid	C20:1 n-9	0.87
Cis-13-Docosenoic acid	C22:1 n-9	0.06
∑ Monounsaturated fatty acids (MUFA)		**42.60**
Cis-9,12-Octadecadienoic acid	C18:2 n-6	17.13
Cis-9,12,15-Octadecatrienoic acid	C18:3 n-3	0.87
Cis-6,9,12-Octadecatrienoic acid	C18:3 n-6	0.39
Cis-5,8,11,14-Eicosatetraenoic acid	C20:4 n-6	0.97
Cis-8,11,14-Eicosatrienoic acid	C20:3 n-6	0.72
Cis-11,1-Eicosadienoic acid	C20:2 n-6	0.57
Cis-5,8,11,14,17-Eicosapentaenoic acid	C20:5 n-3 (EPA)	0.13
Cis-11,14,17-Eicosatreinoic acid	C20:3 n-3	0.07
Eicosatetraenoic acid	C20:4 n-3	0.08
Cis-4,7,10,13,16,19-Docosahexaenoic acid	C22:6 n-3 (DHA)	0.65
Docosapentaenoic acid	C22:5 n-3	0.19
Docosapentaenoic acid	C22:5 n-6	0.18
∑ Polyunsaturated fatty acids (PUFA)		**21.95**

3.4.2 Effect of tannic acid combined without and with MAP on the chemical changes of striped catfish slices during refrigerated storage

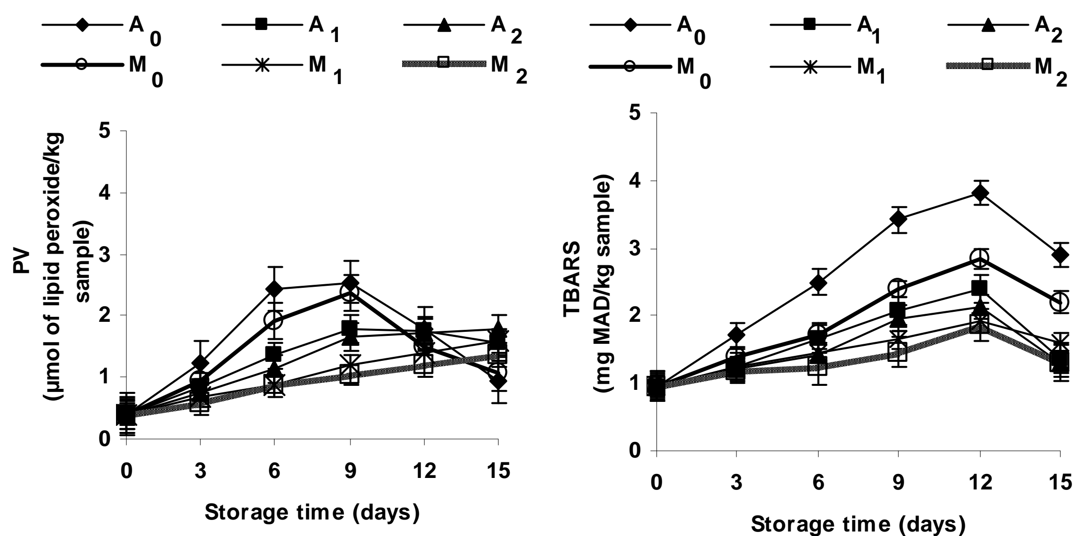
3.4.2.1 Changes in PV and TBARS

Changes in PV and TBARS values of refrigerated striped catfish slices treated without and with tannic acid (100 and 200 mg/kg) stored in air and under MAP during 15 days of storage at 4 ºC are presented in Figure 11 (a) and (b), respectively. Gradual increase in PV was observed in all samples throughout the storage of 15 days ($P < 0.05$) except for the control kept in air (A_0) and the control stored under MAP (M_0), in which PV decreased markedly after 9 days of storage ($P<0.05$). Hydroperoxide formed as primary oxidation products at higher levels in both A_0 and M_0 might undergo the decomposition into secondary oxidation products. A decrease in the level of primary oxidation products is related to hydroperoxide degradation, producing secondary lipid peroxidation products (Boselli *et al.,* 2005). When comparing the PV of all samples, it was found that A_0 contained the highest PV, while M_2 contained the lowest PV within the first 12 days of storage ($P<0.05$). The result indicated the synergistic effect of MAP and tannic acid treatment, especially at a level of 200 mg/kg on the retardation of propagation stage of lipid oxidation in refrigerated striped catfish slices. Free radical scavenging antioxidants including tannic acid interfere with the initiation or propagation steps of lipid oxidation reactions by scavenging lipid radicals (Maqsood and Benjakul, 2010a) and forming low-energy antioxidant radicals that do not readily promote oxidation of unsaturated fatty acids (Frankel, 1998b).

TBARS values of all samples increased as the storage time increased up to day 12 ($P<0.05$). Thereafter, the decrease in TBARS values were obtained ($P<0.05$), probably due to the losses in secondary oxidation products formed, particularly the low molecular weight volatile compounds. Malonaldehyde and other short-chain carbon products of lipid oxidation are not stable and are most likely decomposed to organic alcohols and acids, which are not determined by the TBA test (Fernandez *et al.,* 1997; Borneo *et al.,* 2009). Alghazeer *et al.* (2008) reported the gradual increase of PV and TBARS in frozen mackerel and instant decrease in both

PV and TBARS after 10 weeks of storage. Additionally, the decrease in TBARS was also probably due to the interaction of thiobarbituric acid (TBA)-reactive substances with proteins (Fernandez *et al.,* 1997). This finding was in agreement with Goulas and Kontominas (2007) who reported that TBARS values in MAP and air stored sea bream fillet at 4 ºC increased gradually up to a certain point during storage, followed by either a decrease in values or a lower increase rate.

The samples, M_1 and M_2, displayed the lower TBARS values as compared to those stored in air or that kept under MAP without tannic acid treatment (M_0) throughout the storage period of 15 days ($P<0.05$). Therefore, the use of tannic acid in combination with MAP was more effective in retardation of the lipid oxidation in striped catfish slices than MAP (M_0) alone. TBARS values of the samples A_1 and A_2 were found to be lower than that of M_0 throughout the storage of 15 days. The result indicated that tannic acid alone was more effective than MAP in retarding lipid oxidation in striped catfish slices stored at refrigerated temperature ($P<0.05$). The application of antioxidants (rosemary extract) to the surface of sea bream fillets stored under MAP delayed lipid oxidation and could improve the sensory quality (Gimenez *et al.,* 2004). Goulas and Kontominas (2007) also reported the effect of antioxidant (oregano essential oil) and MAP (40% CO_2 / 30% O_2 / 30% N_2) on the retardation of TBARS formation of refrigerated sea bream fillets. Arashisar *et al.* (2004) found the highest TBARS value in rainbow trout fillets packaged with 30% O_2 as compared to the MAP with no oxygen. Thus, the tannic acid at a level of 200 mg/kg in combination with MAP was more effective in retarding lipid oxidation of striped catfish slices during refrigerated storage.



a) b)

Figure 11. Changes in PV (a) and TBARS (b) of striped catfish slices treated without and with tannic acid stored in air and under MAP (60%N_2 / 35%CO_2 / 5%O_2) during refrigerated storage. Bars represent the standard deviation (n=3).

3.4.2.2 Changes in FTIR spectra

FTIR spectra of crude lipid extracted from fresh (F) striped catfish slice and A_0 and M_2 samples stored for 15 days are illustrated in Figure 12. FTIR spectrum for fresh sample (F) consisted of several peaks with different wavenumbers (Fig. 12a). Band with wavenumber of 667.36 cm^{-1} is associated with out-of-plane O-H bend (Brian, 1998); a band with wavenumber of 754.62 cm^{-1} resulted from the overlapping of the methylene rocking vibration and out-of-plane bending vibration of *cis*-disubstituted olefins / alkenes (Guillen *et al.,* 2004). A weak band with wavenumber of 969.29 cm^{-1} was due to bending vibration out-of-plane of *trans* disubstituted olefinic groups (Guillen *et al.,* 2004), a band with wavenumber of 1095.35 cm^{-1} was associated with the stretching vibration of the C-O groups in esters (Guillen *et al.,* 2004). The bands situated at wavenumber of 1162.79 cm^{-1} and 1215.70 cm^{-1} were both associated with the stretching vibration of the C-O group in esters and with the bending vibration of the CH_2 groups and both were related to the proportion of saturated acyl groups in oil samples (Guillen and Cabo, 1997). A band with wavenumber of 1376.99 cm^{-1} was

due to symmetric bending vibrations of CH_3 groups (Guillen *et al.,* 2004) and a band having wavenumber of 1464.40 cm^{-1} was due to the bending vibrations of the CH_2 and CH_3 aliphatic groups (Guillen *et al.,* 2004). Two stretched bands at wavenumbers of 2853.62 cm^{-1} and 2923.58 cm^{-1} were due to the methylene asymmetrical and symmetrical stretching vibration, respectively (Guillen and Cabo, 1997; Guillen *et al.,* 2004). Both the methylene asymmetrical stretching bands at approximately 2923.58 cm^{-1} and the methylene symmetrical stretching band near 2853.62 cm^{-1} were obviously present in most of the lipid samples (Guillen *et al.,* 2004). Also, a band near 3010.84 cm^{-1} was due to the stretching vibration of the *cis* olefinic C-H double bonds (Guillen and Cabo, 1997; Guillen *et al.,* 2004). The results suggested that the oil from striped catfish slices mainly contained triglycerides with low amounts of free fatty acids.

A stretching vibrational band of the C=O carboxylic group representing free fatty (FFA) acids was observed at wavenumber of 1739.29 cm^{-1}. A small band at wavenumber of 1657.16 cm^{-1} was assigned to the disubstituted *cis* C=C of unsaturated acyl groups (Fig. 12b) (Guillen *et al.,* 2004; Van de Voort *et al.,* 1994b). The lower amplitude of these bands was found in A_0 in comparison with M_2 samples stored for 15 days (Fig. 12b). The result suggested that FFA produced in A_0 underwent oxidation, leading to the lower amounts retained in the sample as evidenced from the high TBARS values in A_0 (Fig. 11b). For M_2, FFA might be formed by lipases, but they were most likely less prone to oxidation in the presence of tannic acid and under MAP. As a result, FFA was still found at the high level in M_2. For fresh sample (F), these peaks were less pronounced, probably due to the lower lipase activity in the fresh sample at day 0.

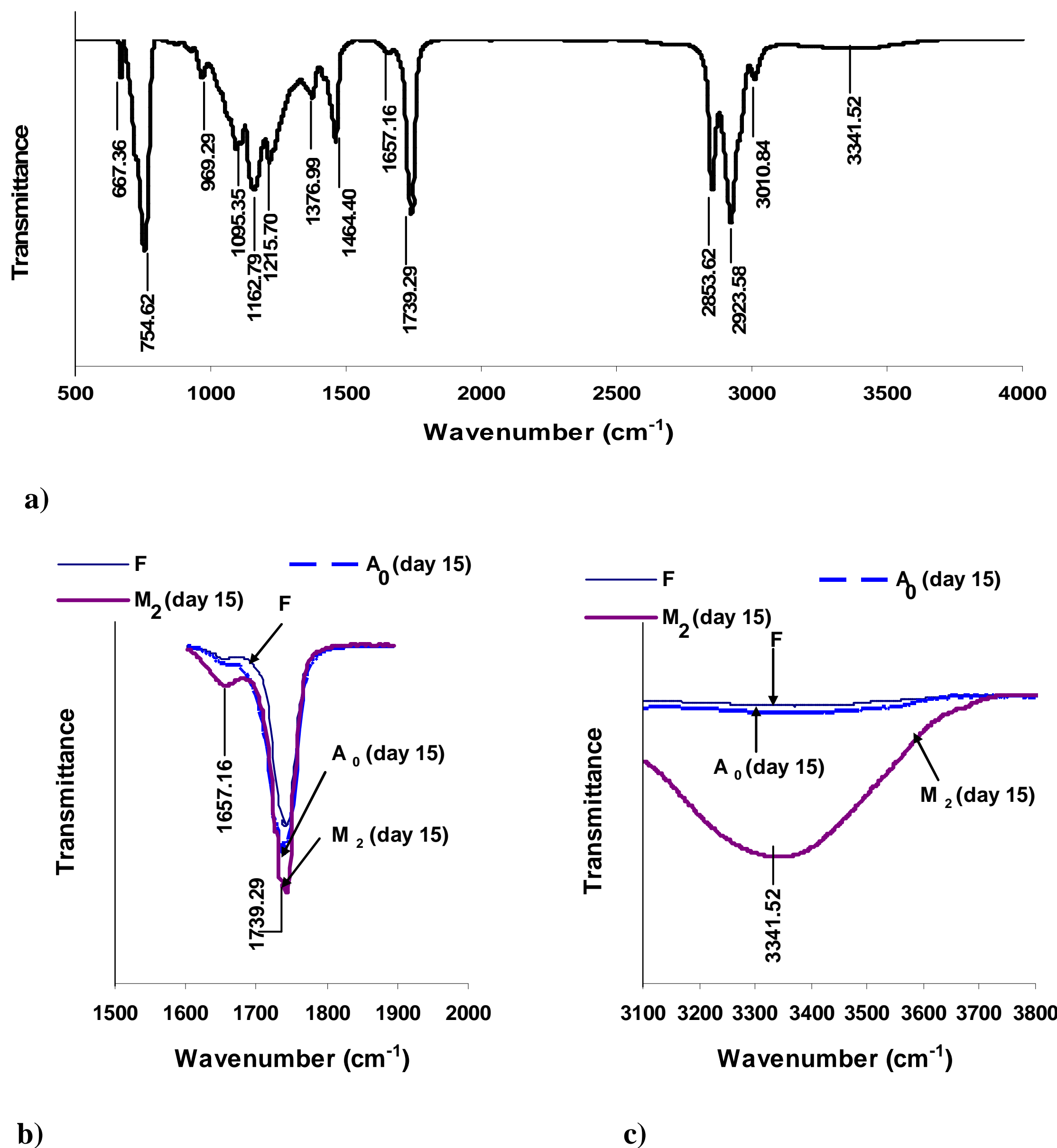


Figure 12. FTIR spectra of crude oil extracted from fresh striped catfish muscle (a) and the change in the transmittance in the region of 1500-2000 cm^{-1} (b) and 3100 -3600 cm^{-1} (c) of fresh sample (F), sample without tannic acid treatment and stored in air (A_0) and those treated with tannic acid (200 mg/kg) and stored under MAP (M_2) after 15 days of refrigerated storage.

A band situated at wavenumber of 3341.52 cm^{-1} was due to the hydroperoxide formation in the sample (Fig. 12c). In oxidized oils, a band of hydroperoxides is detected approximately at the wavenumber of 3444 cm^{-1} (Ismail *et al.,* 1993). Van de Voort *et al*. (1994b) reported a band having wavenumber of 3800-3100 cm^{-1} in the ATR/FTIR spectra, referred to the OH stretching region. Hydroperoxide moieties exhibit the characteristic absorption bands between 3600-3400 cm^{-1} due to their –OO-H stretching vibrations (Van de Voort *et al.,* 1994a). In fish lipid oxidation process, the broadening of the bands between the wavenumber of 3600 and 3300 cm^{-1} is mainly due to the hydroperoxide formed during the fish lipid oxidation (Guillen *et al.,* 2004; Chaijan *et al.,* 2006). The hydroperoxide band at wavenumber of 3341.52 cm^{-1} in M_2 showed a higher amplitude, compared to that of A_0 stored for 15 days (Fig. 12c), which was coincidental with the results of spectrophotometric determination of PV (Fig. 11a). The hydroperoxide formed in A_0 might undergo the intensive decomposition, yielding the secondary lipid oxidation products. On the other hand, the oxidation of M_2 was still in the primary stage, in which the hydroperoxides were still accumulated in the sample at day 15. Thus, the FTIR spectra revealed the formation of FFA and hydroperoxide in oil extracted from striped catfish slices during 15 days of refrigerated storage.

3.4.2.3 Change in haem and non-haem iron contents

Changes in haem and non- haem iron contents in refrigerated striped catfish slices treated without and with tannic acid and packed in air or under MAP are shown in Figure 13 (a) and (b), respectively. Haem iron content of all samples decreased gradually up to day 6 of storage. Subsequently, a marked decrease was observed until the end of storage (day 15) ($P<0.05$). Haem iron content of A_2 and M_2 was higher than that of the control sample (A_0 and M_0) without tannic acid treatment throughout the storage ($P<0.05$). Decrease in haem iron content with increasing storage time was probably due to a haem breakdown, resulting in the increase in non-haem iron content (Benjakul and Bauer, 2001). Haem proteins became less soluble as the fish undergoes deterioration, leading to the decreased haem iron content (Chen, 2003; Chaijan *et al.,* 2005). Lower haem iron content in the control sample (A_0 and

M_0), reflecting higher non-haem iron content, might be associated with the higher degree of oxidation in these samples.

Non-haem iron content of all samples increased gradually up to day 6 of storage with the subsequent increase at the higher rate up to 15 days of storage ($P<0.05$) (Fig. 13b). The increase in non-haem iron content with increasing storage period was coincidental with the release of free iron from the muscle (Fig. 13a). Decker and Hultin (1990a) suggested that haem pigment or other iron-containing proteins are possibly denatured with increasing storage time, resulting in the release of iron. Deterioration of sub-cellular, organelles, e.g. mitochondria, and the release of cytochrome C could also be responsible for the increase in non-haem iron (Decker and Hultin, 1990a). After 6 days of storage, the increase in lipid oxidation was concomitant with the increasing non-haem iron content for the control samples, A_0 and M_0.

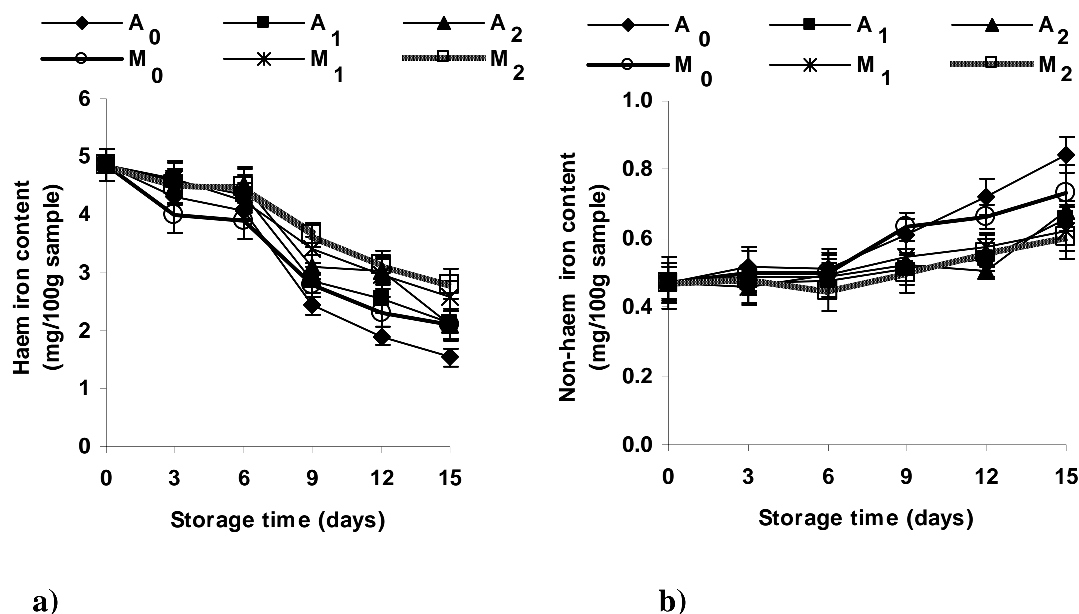


Figure 13. Changes in haem (a) and non-haem iron (b) contents of striped catfish slices treated without and with tannic acid stored in air and under MAP (60%N_2 / 35%CO_2 / 5%O_2) during refrigerated storage. Bars represent the standard deviation (n=3).

During storage, non-haem iron was released owing to the deterioration by microbial spoilage, which might accelerate the oxidation process in the muscle (Chaijan *et al.,* 2005). On the contrary, the non-haem iron content of the samples treated with tannic acid was lower than the samples without tannic acid treatment. This was mainly due to the ability of tannic acid to chelate iron, particularly in the free form (Lopes *et al.,* 1999). It was noted that A_0 contained a higher content of non-haem iron than did M_0 at day 15 of storage. A higher amount of non-haem iron released in A_0 was associated with the accelerated oxidation. Among the transition metals, iron is known as the most important pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen peroxide and lipid peroxides to reactive free radicals via the Fenton reaction ($Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$) (Miller *et al.,* 1996). Therefore, the use of tannic acid with metal chelating ability in conjunction with MAP could lower the lipid oxidation mediated by transition metal ions.

3.4.2.4 Changes in protein patterns

Protein patterns of fresh (F) striped catfish muscle and A_0 and M_2 samples stored at 4 ºC for 15 days are shown in Figure 14. All samples contained myosin heavy chain (MHC) and actin as the major proteins. Additionally, all samples also consisted of α-tropomyosin, β-tropomyosin and troponin T. In general, there was no difference between protein patterns of fresh sample (F) and M_2 stored for 15 days. However, a slight degradation of MHC was noticed in A_0 after 15 days of storage. Band intensity of MHC in A_0 after 15 days of storage decreased by 17.85%, whereas no change in protein pattern was found in M_2 stored for 15 days, compared with that of fresh sample. Masniyom *et al.* (2004) also found no marked change in protein pattern of seabass slices stored under MAP, compared with fresh sample. The degradation of MHC and actin in A_0 at the end of storage (day 15) was most likely attributed to endogenous and microbial proteinases (Masniyom *et al.,* 2004). Moreover, protein oxidation can also lead to protein degradation (Park *et al.,* 2007). Thus, the tannic acid treatment in combination with MAP could prevent protein degradation of striped catfish slices effectively, mainly due to their role in inhibiting microbial growth. This

could result in the maintenance of textural property of the striped catfish slices after extended storage.

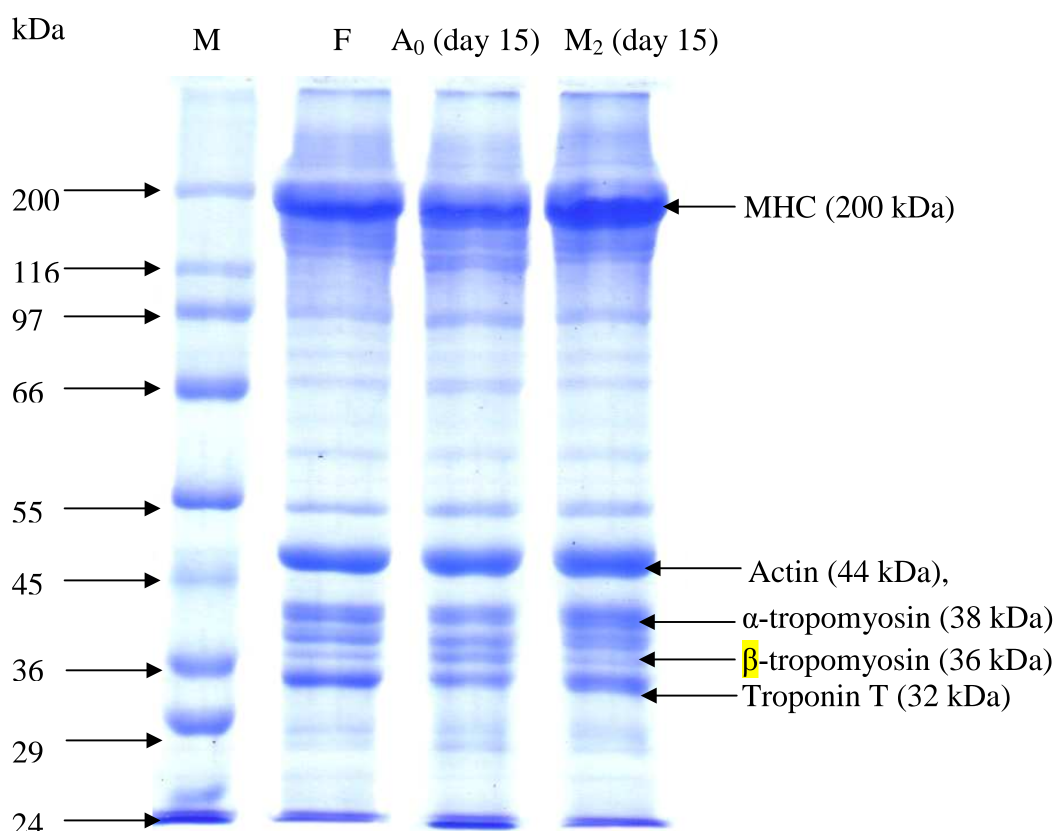


Figure 14. SDS-PAGE pattern of muscle protein from fresh striped catfish muscle (F) and sample without tannic acid treatment and stored in air (A_0) and those treated with tannic acid (200 mg/kg) and stored under MAP (M_2) after 15 days of refrigerated storage.

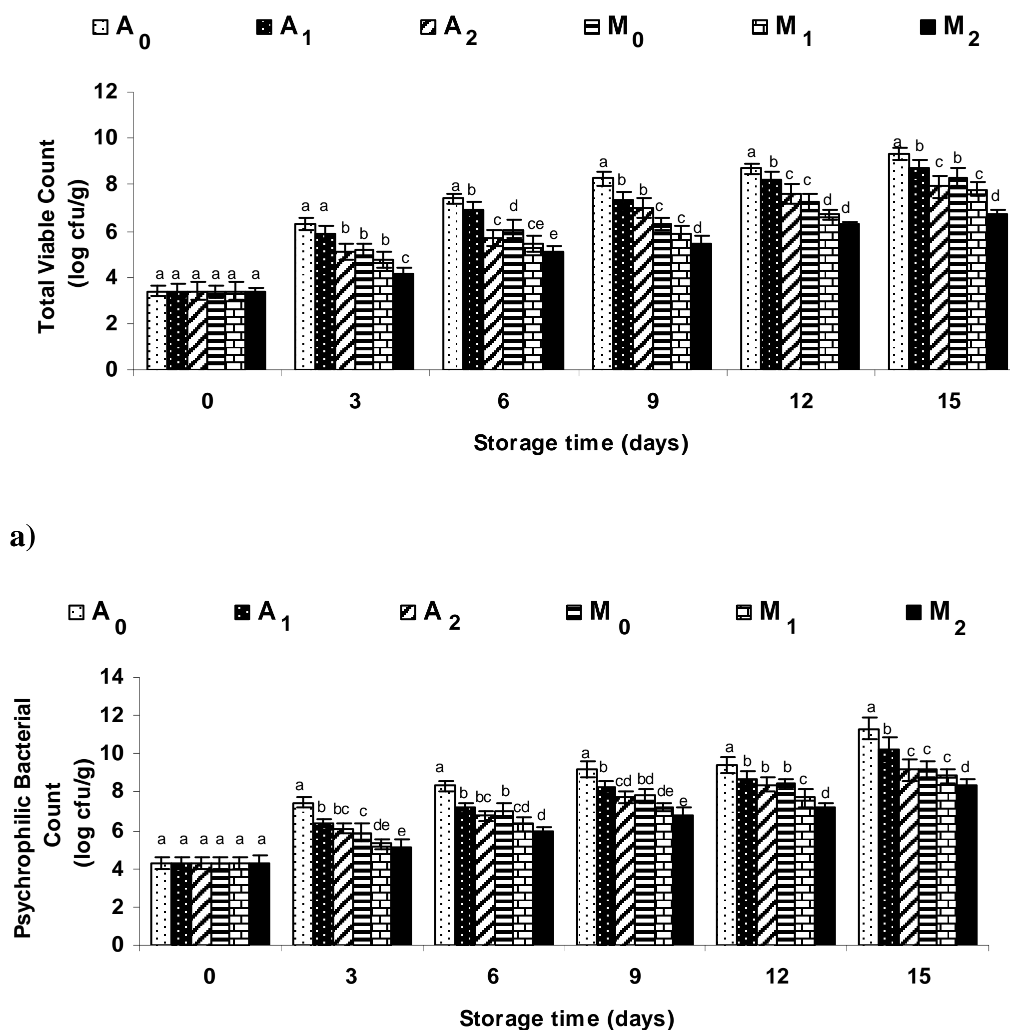
3.4.3 Effect of tannic acid in combination without and with MAP on the microbiological changes of striped catfish slices during refrigerated storage

Total viable count (TVC) and psychrophilic bacterial count (PBC) of the refrigerated striped catfish slices treated without and with tannic acid and packed in air or under MAP are depicted in Figure 15 (a) and (b), respectively. TVC and PBC of all samples increased with increasing time of storage ($P<0.05$). TVC and PBC of the striped catfish slices stored in air increased rapidly from an initial value of 2.56 x

10^3 to 2.43 x 10^9 cfu/g and from 2.28 x 10^4 to 2.09 x 10^{11} cfu/g, respectively at the end of storage (day 15). Generally, M_2 showed the lowest TVC and PBC at all storage times tested ($P<0.05$). TVC and PBC of A_0 was higher than those of M_0 ($P<0.05$), indicating that MAP alone was effective in retarding the microbial growth of striped catfish slices during refrigerated storage. However, the synergistic effect on retardation of microbial growth was obtained when tannic acid was used in combination with MAP. Tannic acid at higher concentration rendered the higher efficiency in retarding the growth of microorganisms. Air-stored hake slice showed higher TVC than MAP (50% CO_2 / 45% N_2 / 5% O_2) stored hake after 2 days of iced storage (Pastoriza *et al.,* 1998). Bacteria grew more quickly in sardines stored in air, followed by those in vacuum packaging, and the lowest counts were found in sardine stored under MAP (Stamatis and Arkoudelos, 2007). Storage at low temperature in combination with the presence of 60% CO_2 inhibited both TVC and psychrophilic bacterial growth in smoked trout fillets stored at 4 ºC (Sukran *et al.,* 2006). CO_2 (30-65%) has been reported to delay spoilage of fresh fish by inhibiting psychrophilic, aerobic and gram-negative bacteria (Huss, 1972; Ruiz-Capillas and Moral, 2001). PBC exceeded TVC for all the samples at each sampling time, indicating more pronounced growth of psychrophilic bacteria in striped catfish slices than the mesophilic bacteria at refrigerated temperature. At day 0, PBC was higher than TVC, suggesting that psychrophilic bacteria were the predominant microorganisms in striped catfish handled in ice after capture. Similar finding was reported for pearl spot and rainbow trout stored under MAP at refrigerated temperatures (Ravi Sankar *et al.,* 2008; Arashisar *et al*., 2004).

TVC values of A_0 exceeded 10^7 cfu/g, considered as the upper limit for the fish to be safe for consumption (ICMSF, 1986), at day 12 of storage at 4 ºC. Under MAP conditions, TVC of samples did not reach this value throughout the 15 days of storage. Thus, from microbiological point of view, shelf-life of A_0 (air stored striped catfish slices without tannic acid treatments), M_0 (MAP stored samples without tannic acid treatment) and M_2 (MAP stored samples with tannic acid (200 mg/kg) treatment) was 9, 12 and 15 days, respectively. Pastoriza *et al.* (1998) found that air-stored hake slices had a shelf-life of 7 days, whereas it was extended up to 14 days for MAP-

stored fish (50% CO_2 / 45% N_2 / 5% O_2) and to 16 days for MAP-stored fish dipped in NaCl. Tannic acid could act as antimicrobial agent (Zaidi-Yahiaoui *et al.,* 2008). Taguri *et al.* (2004) reported the antibacterial activity of hydrolysable tannins against *Salmonella aureus and Staphylococcus*. Thus, the use of tannic acid at high level (200 mg/kg) in combination with MAP exhibited the synergistic effect on the retardation of microbial growth of striped catfish slices stored at the refrigerated temperature.



b)

Figure 15. Changes in total viable count (a) and psychrophilic bacterial count (b) in striped catfish slices treated without and with tannic acid stored in air and under MAP (60%N_2 / 35%CO_2 / 5%O_2). Bars represent the standard deviation (n=3). Different small letters denotes the significant difference between the samples at the same storage time (P<0.05).

3.4.4 Sensory evaluation

Hedonic scores of all attributes including colour, odour, texture and taste of the control sample kept in air (A_0) and sample treated with tannic acid (200 mg/kg) stored under MAP (M_2) are presented in Table 5. The scores of all sensory attributes continuously decreased with increasing storage time ($P<0.05$). There was no difference in the scores of all sensory attributes between A_0 and M_2 up to day 3 of refrigerated storage ($P>0.05$). Thereafter, A_0 exhibited the lower scores for all attributes, especially colour and odour, as compared to M_2 ($P<0.05$). The change in colour and development of rancidity and off-odour in A_0 at day 9 was in accordance with the exceeded limit of the bacterial counts (Fig. 15 a and b) and increased TBARS values (Fig. 11b). Bacteria play a major role in fish spoilage by generating off-colours, off-odours and an unacceptable visual appearance of fish (Pastoriza *et al.,* 1998). Bak *et al.* (1999) reported that rancidity scores for frozen shrimps were significantly higher for samples packed under atmospheric air, compared to samples packed under MAP. Considering a score of 5 as the acceptable limit, the shelf-life of striped catfish slices treated with tannic acid (200 mg/kg) and kept under MAP (M_2) was 15 days. The texture scores for sample packed under MAP and treated with tannic acid (200 mg/kg) (M_2) were higher than that of control samples (A_0) ($P<0.05$). This was in agreement with the lower protein degradation in M_2 sample (Fig. 14). The higher odour and colour scores were also found in M_2. This was related with the lower lipid oxidation in M_2, in which rancidity was developed to a lower extent. Additionally, the deterioration of M_2 was lowered due to the lower content of oxidation products formed, which might cause non-enzymatic browning reaction (Tazi *et al.,* 2009).

Table 5. Hedonic scores of cooked striped catfish slices without tannic acid treatment and stored in air (A_0) and those treated with tannic acid (200 mg/kg) and stored under MAP (60% N_2 / 35% CO_2 / 5% O_2) (M_2).

Attributes	Sample	Storage time (days)			
		0	3	9	15
Colour	A_0	7.83 ± 0.74^{Aa}	7.03 ± 0.61^{Ba}	4.80 ± 1.03^{Cc}	NA
	M_2	7.90 ± 0.99^{Aa}	7.33 ± 0.98^{Ba}	6.83 ± 1.11^{Ca}	5.60 ± 0.96^{D}
Odour	A_0	7.70 ± 0.83^{Aa}	6.83 ± 0.91^{Ba}	3.83 ± 0.95^{Cd}	NA
	M_2	7.90 ± 0.99^{Aa}	7.27 ± 0.98^{Ba}	6.17 ± 1.11^{Ca}	5.30 ± 1.05^{D}
Taste	A_0	7.70 ± 0.74^{Aa}	7.00 ± 0.83^{Ba}	5.50 ± 0.94^{Cb}	NA
	M_2	8.03 ± 0.80^{Aa}	7.10 ± 0.88^{Ba}	6.20 ± 1.24^{Ca}	5.53 ± 1.15^{D}
Texture	A_0	7.90 ± 0.61^{Aa}	6.93 ± 0.94^{Ba}	5.23 ± 1.38^{Cbc}	NA
	M_2	8.07 ± 0.82^{Aa}	7.30 ± 1.17^{Ba}	6.40 ± 1.33^{Ca}	5.73 ± 0.92^{D}

Values are mean ± SD of scores of 30 panelists. Different small letters in the same column within same attribute denote the significant difference ($P<0.05$). Different capital letters in the same row denote significant difference ($P<0.05$). NA denotes “not analysed”.

3.5 Conclusion

Shelf-life of refrigerated striped catfish slices could be extended effectively by using tannic acid (200 mg/kg) in combination with MAP (60% N_2 / 35% CO_2 / 5% O_2), which could retard the lipid oxidation and microbial growth during the extended storage. Thus, tannic acid acted synergistically with MAP to maintain the quality of refrigerated striped catfish slices.

CHAPTER 4

EFFECT OF BLEEDING ON LIPID OXIDATION AND QUALITY CHANGES OF ASIAN SEABASS (*LATES CALCARIFER*) MUSCLE DURING ICED STORAGE

4.1 Abstract

Lipid oxidation, microbial load and fishy odour development in the slices of bled and un-bled Asian seabass during 15 days of iced storage were comparatively investigated. Bled samples showed the lower peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) throughout the storage period ($P<0.05$). Bleeding effectively lowered total haem and non-haem iron contents in Asian seabass slices. The release of non-haem iron was pronounced in un-bled samples during the storage. Solid phase micro extraction-gas chromatography mass spectrometric (SPME-GCMS) analysis revealed that the bled samples stored in ice for 15 day contained the lower amount of volatile compounds. Heptanal, the major volatile compound detected in the un-bled samples, was 4-fold higher than that of bled counterparts. Aldehydic compounds including hexanal, octanal, nonanal and nonenal contents were also higher in the former. Bled samples had the lower fishy odour, compared with the un-bled counterparts during the storage ($P<0.05$). The lower total viable counts (TVC) and psychrophilic bacterial counts (PBC) were observed in bled samples, in comparison with un-bled ones ($P<0.05$). Thus, bleeding was a potential means in retarding lipid oxidation, fishy odour development and microbial growth of Asian seabass slices during storage in ice.

4.2 Introduction

Lipid oxidation is a major cause of quality deterioration in muscle-based foods, where flavour, colour, texture and nutritional value can be negatively affected (Kanner, 1994; Gandemer and Meynier, 1995). The haem proteins including haemoglobin (Hb) and myoglobin are potent catalysts of lipid oxidation in muscle foods (Kanner, 1994). In contrast to mammalian meat, Hb is a major contributor to lipid oxidation in fish and fish products, since the blood is not practically removed prior to processing (Richards and Hultin, 2002). Hb found in the blood of fish can accelerate lipid oxidation, which results in development of off-odour (Richards and Hultin, 2002). During Hb autoxidation, active oxygen can be generated (Richards and Hultin, 2002). After being slaughtered, the iron atom in the haem ring of the haem proteins is primarily in the ferrous (+2) state. Conversion of ferrous haem protein to met (+3) haem protein (metHP) is a process known as autoxidation. Autoxidation appears to be a critical step to enhance lipid oxidation since metHP reacts with peroxides to stimulate the formation of compounds capable of initiating and propagating lipid oxidation (Everse and Hsia, 1997). Additionally, haem or iron can be released from the Hb during post-mortem handling and storage, thereby promoting lipid oxidation (Richards and Hultin, 2002). Fish blood plasma contained 1.2-3 % lipid, mainly found as lipoproteins (Richards and Hultin, 2002), which could be a source of oxidizable lipid. However, many components in blood plasma were able to inhibit lipid oxidation (Richards and Hultin, 2002).

Generally, bleeding of fish is carried out to eliminate most of blood from the tissues. Immediate bleeding of live fish delays rigor-mortis as well as softening compared to the flesh of untreated fish during iced storage (Mochizuki *et al.,* 1998; Ando *et al.,* 1999; Terayama and Yamanaka, 2000). Without bleeding, the residual blood in fish tissues is one of the main factors inducing the development of undesirable odour and unpleasant flavour of the fish flesh during ice storage (Flechtenmacher, 1975). Terayama and Yamanaka (2000) reported that mechanically bled skipjack meat was highly valued due to its bright red colour and lack of fishy odour as compared with the un-bled fish. Bleeding was also shown to retard lipid oxidation of minced trout muscle during storage at 2 ºC (Richards and Hultin, 2002).

However, no information regarding the effects of bleeding on postmortem changes of Asian seabass, an economically important species of Asean countries exists. Thus, the objective of this study was to investigate the effect of bleeding on the retardation of lipid oxidation, and development of fishy odour and microbial growth in the intact Asian seabass slice during iced storage.

4.3 Materials and Methods

4.3.1 Chemicals

Thioglycolic acid, cumene hydroperoxide, bathophenanthroline disulfonic acid, pentanal, hexanal, heptanal, octanal and nonanal were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, potassium iodide, trichloroacetic acid, iron standard solution, ethanol and methanol were obtained from Merck (Damstadt, Germany). Disodium hydrogen phosphate, 2-thiobarbituric acid, ammonium thiocyanate, ferrous chloride, sodium nitrite and saturated sodium acetate were purchased from Fluka Chemical Co. (Buchs, Switzerland). Plate count agar was obtained from Hi-media (Mumbia, India). Chloroform was procured from Lab-Scan (Bangkok, Thailand).

4.3.2 Bleeding and preparation of Asian seabass

Live Asian seabass were purchased from the Songkhla Lake, Songkhla, Thailand. Fish were transported in the seawater to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, live fish were bled from caudal vein by cutting the tail. Fish were left in seawater to bleed out completely for 1 h. The un-bled fish were killed by ice-shocking. Both bled and un-bled samples were washed with tap water, descaled, deheaded, filleted and cut into slices of 2-3 cm thickness (Fig. 16). The slices from bled and un-bled Asian seabass were packed separately in polythene bags, sealed and kept in ice using a slice/ice ratio of 1:2 (w/w). Molten ice was removed every day and the same quantity of ice was replaced. All samples were taken for chemical analysis every 3 days for 15 days. Microbiological analysis was also performed at day 0, 3, 9 and 15, while the evaluation of fishy odour

was carried out at day 0, 9 and 15. For the analysis of volatile compounds, bled and unbled-samples stored in ice for 15 days were used.

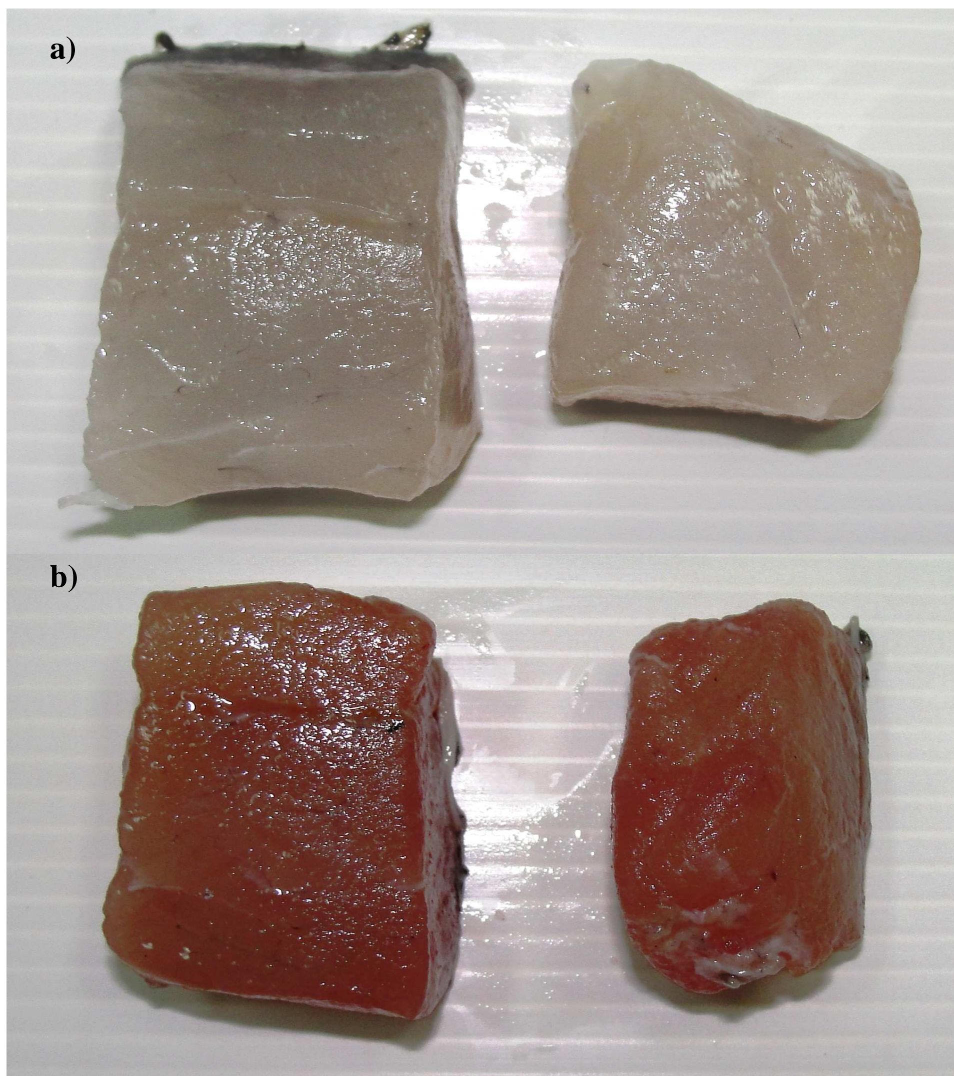


Figure 16. Photograph of the slices from bled (a) and un-bled (b) Asian seabass.

4.3.2 Chemical analysis

4.3.2.1 Peroxide value

Peroxide value (PV) was determined as per the method of Richards and Hultin (2002) with a slight modification. Ground sample (1 g) was mixed with 11 ml of chloroform/methanol (2:1, v/v). The mixture was homogenised at a speed of 13,500

rpm for 2 min using an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany). Homogenate was then filtered using Whatman No. 1 filter paper (Whatman International, Ltd, Maidstone, England). Two millilitre of 0.5% NaCl was then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s using a Vortex-Genie2 mixer 4 (Bohemia, NY, USA) and then centrifuged at 3,000 x g for 3 min using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA) to separate the sample into two phases. Two millilitre of cold chloroform/methanol (2:1) were added to 3 ml of the lower phase. Twenty-five microlitre of 30 % ammonium thiocyanate and 25 µl of 20mM iron (II) chloride were added to the mixture (Shantha and Decker, 1994). Reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A standard curve was prepared using cumene hydroperoxide at the concentration range of 0.5-2 ppm.

4.3.2.2 Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Ground sample (0.5 g) was mixed with 2.5 ml of a TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture was heated in a boiling water for 10 min to develop a pink colour, cooled with running tap water and then sonicated for 30 min using an Elma (S 30 H) sonicator (Kolpingstr, Singen, Germany). The mixture was then centrifuged at 5,000 ×g at 25 ºC for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (MAD) at the concentration ranging from 0 to 10 ppm and TBARS were expressed as mg of MAD equivalents/kg sample.

4.3.2.3 Determination of total haem content

Total haem content was determined according to the method of Gomez-Basauri and Regenstein (1992a) with a slight modification. Ground sample (2 g) was transferred into a 50 ml-polypropylene centrifuge tube and 20 ml of cold 40 mM phosphate buffer (pH 6.8) were added. The mixture was homogenised with a homogeniser at a speed of 13,500 rpm for 10 sec. The homogenate was centrifuged at

3000*g* for 30 min at 4 °C. The supernatant was filtered with a Whatman no.1 filter paper. Total haem content was determined by direct spectrophotometric measurement at 525 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan).

4.3.2.4 Determination of non-haem iron content

Non-haem iron content was determined as described by Schricker *et al.* (1982) with a slight modification. Ground sample (1 g) was placed in a screw cap test tube and 50 µl of 0.39% (w/v) sodium nitrite was added. A mixture (4 ml) of 40% trichloroacetic acid and 6 N HCl (ratio of 1:1 [v/v], prepared freshly) was added. The tightly capped tubes were placed in an incubator shaker at 65 ºC (Memmert, D-91126, Schwabach, Germany) for 22 h and then cooled down at room temperature (25-30 ºC) for 2 h. The supernatant (400 µl) was mixed with 2 ml of the non-haem iron colour reagent (prepared freshly). After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The colour reagent was prepared by mixing a 1:20:20 ratio (w/v/v) of: (1) bathophenanthroline disulfonic acid (0.162 g, dissolved in 100 ml of double deionised water with 2 ml thioglycolic acid [96–99%]); (2) double-deionised water; and (3) saturated sodium acetate solution.

Non-haem iron content was calculated from iron standard curve. The iron standard solution, ranging from 0 to 2 ppm, (400 µl) was mixed with 2 ml of the non-haem iron colour reagent. The concentration of non-haem iron was expressed as mg /100 g sample.

4.3.2.5 Determination of volatile compounds

Volatile lipid oxidation compounds in Asian seabass slices were determined by solid phase micro extraction-gas chromatography mass spectrometry (SPME-GCMS) (Iglesias and Medina, 2008).

4.3.2.5.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 3 g of slice was homogenised at a speed of 13,500 rpm for 2 min with 8 ml of saturated NaCl in ultra pure water. The mixture was centrifuged at 2000 x g for 10 min at 4 ºC. The supernatant (6 ml) was heated at 60 ºC with equilibrium time of 10 h in a 20 ml headspace vial. Finally, the SPME fiber (50/30 µm DVB/Carboxen^{TM}/PDMS StableFlex ^{TM}) (Supelco, Bellefonte, PA, USA) was exposed to the head space of the vial containing the sample extract and the volatile compounds were allowed to absorb in the SPME fiber for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

4.3.2.5.2 GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatography coupled with HP 5972 mass selective detectors equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m x 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC and finally an increase of 15 ºC/min to a final temperature of 250 ºC and hold for 10 min. Helium was employed as a carrier gas, with a constant flow of 1.0 ml/min. Injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 265 ºC. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250 ºC. Initially, a full scan mode data was acquired to determine appropriate masses for the later acquisition in selected ion monitoring (SIM) mode under the following conditions: mass range: 25-500 amu and scan rate: 0.220 s/scan. All the analyses were performed with ionization energy of 70 eV, filament emission current at 150 µA and the electron multiplier voltage at 500 V.

4.3.2.5.3 Analyses of volatile compounds

Identification of the volatile compounds in the samples was based on the retention times of individual aldehydic standards including pentanal, hexanal, heptanal, octanal and nonanal. Identification of the compounds was also done by consulting ChemStation Library Search (Wiley 275.L) using Probability-Based Matching (PBM) algorithm developed by Prof. Fred McLafferty and co-workers at Cornell University. Quantitative determination was carried out by using an internal calibration curve that was built using stock solutions of the compounds in ultra-pure water saturated in salt and analyzing them by the optimized HS-SPME method. Quantification limits were calculated to a single-to-noise (S/N) ratio of 10. The identified volatile compounds related with lipid oxidation were presented in the form of normalized area under peak of each identified compound.

4.3.3 Microbiological analysis

Fish slices (25 g) were collected aseptically in a stomacher bag and 10 volumes of sterile saline solution (0.85 g/100 ml) were added. After homogenising in a Stomacher blender (Stomacher M400, Seward Ltd., Worthington, England) for 1 min, a series of 10-fold dilutions was made using normal saline solution (0.85%) for microbiological analysis. Mesophilic and psychrophilic bacterial counts were determined by plate count agar (PCA) with the incubation at 35 ºC for 2 days (Hasegawa, 1987) and 7 ºC for 7 days (Cousin *et al.,* 1992), respectively. Microbial counts were expressed as log cfu/g.

4.3.4 Sensory analysis

The sensory evaluation was performed by 11 trained panelists, who were the graduate students in Food Science and Technology programme with the age of 25-33 years and were familiar with fish consumption. Panelists were trained in two sessions using a 10 point scale, where 0 represented no fishy odour and 10 represented the strongest fishy odour. Reference samples were prepared by storing Asian seabass slices packed in polythene bags in ice for 0, 5, 10 and 15 days representing the score of 0, 3, 7 and 10, respectively. Slices were placed in a glass cup (diameter of 4 cm)

and covered with aluminium foil. Panelists were asked to sniff the headspace above the samples after opening the cover and score the samples.

4.3.5 Statistical analysis

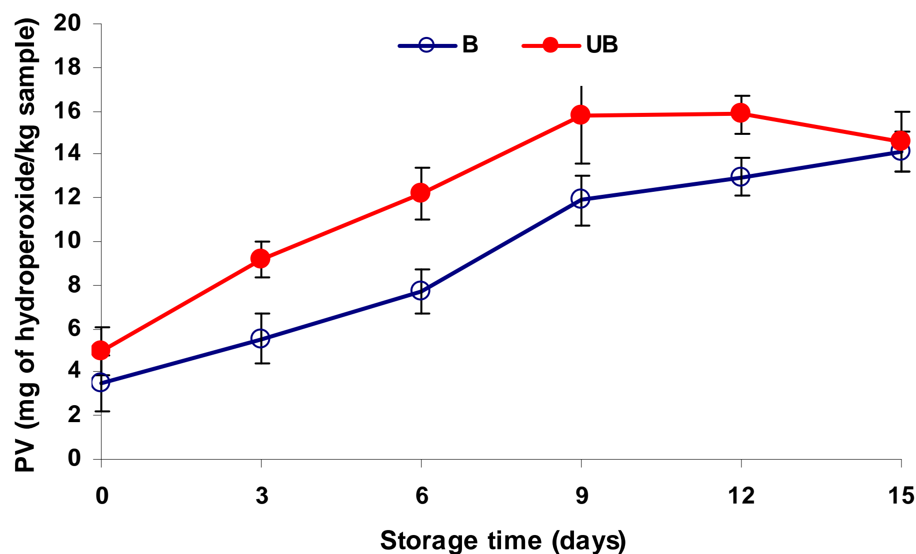
All experiments were run in triplicate. Completely randomized design (CRD) was used for this study. The experimental data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's New Multiple Range Test (Steel and Torrie, 1980). For pair comparison, *T*-test was used. Data analysis was performed using a SPSS package (SPSS 14.0 for Windows, SPSS Inc, Chicago, IL, USA).

4.4 Results and discussion

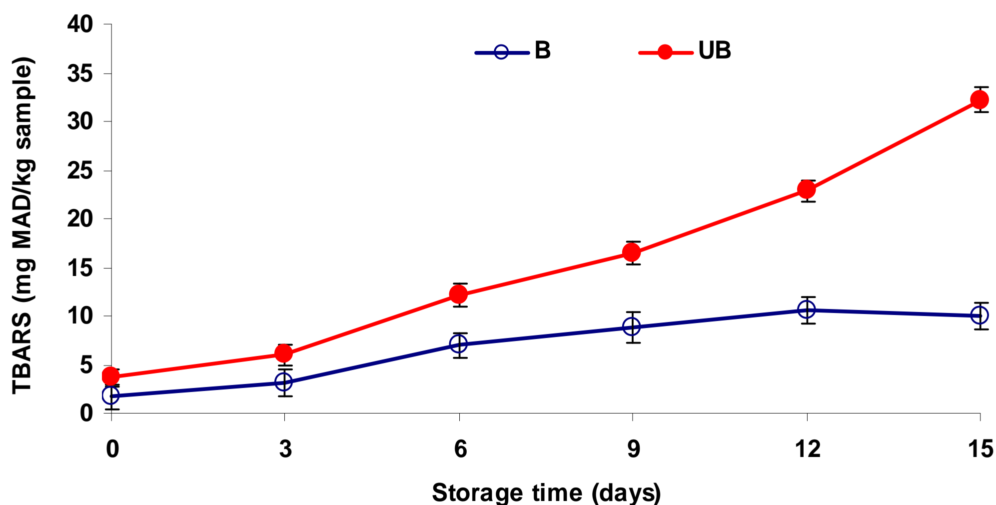
4.4.1 Effect of bleeding on chemical changes of Asian seabass slices during iced storage

4.4.1.1 Changes in PV and TBARS

Changes in PV and TBARS values of slices from bled and unbled Asian seabass during iced storage are shown in Figure 17 (a) and (b), respectively. Continuous increase in PV was observed in bled samples throughout the storage of 15 days ($P<0.05$). For un-bled samples, the increase in PV was noticeable during the first 9 days. Thereafter, no change in PV was found up to day 15 of storage ($P>0.05$). When comparing PV of both samples, it was found that PV of un-bled samples were higher than that of bled samples during the first 12 days of storage ($P<0.05$). The results suggested that the initiation and propagation of lipid oxidation were more pronounced in the un-bled samples when compared with bled samples. After 9 days of storage, hydroperoxide in the un-bled samples might undergo decomposition into the secondary oxidation products at a similar rate with its formation. This might result in constant PV found in un-bled samples after day 9 of storage. A decrease in the level of primary oxidation products is related to hydroperoxide degradation, leading to the formation of secondary lipid peroxidation products (Boselli *et al.,* 2005).



a)



b)

Figure 17. Changes in PV (a) and TBARS (b) in the slices from bled and un-bled Asian seabass during iced storage. Bars represent the standard deviation (n=3).

TBARS values of the bled and un-bled samples increased as the storage time increased up to 15 days (P<0.05), except for the former, in which TBARS kept constant after day 12 (P>0.05). Un-bled samples displayed the formation of TBARS at

higher rates compared with the bled samples throughout the storage ($P<0.05$). The result was in accordance with Richards and Hultin (2002) who reported that TBARS values were greater in un-bled minced trout than bled counterparts during storage for 15 days at 2 ºC.

The malonaldehyde and 4-hydroxyhexenal contents in the bled skipjack were lower than that found in the control (un-bled) sample after 2 days of storage at 0 ºC (Sakai *et al.,* 2006). Hb in fish muscle was reported to accelerate lipid oxidation (Richards and Hultin, 2002; Terayama and Yamanaka, 2000; Richards *et al.,* 2002; Richards and Li, 2004). Apart from Hb, the fish blood also contain large amount of white blood cells, which can also generate superoxide, hydrogen peroxide and hydroxyl radical (Gabig and Babior, 1981) and lipoxygenase products (Pettitt *et al.,* 1989), which are known to enhance the lipid oxidation. Therefore, bleeding of fish decreased the Hb content and white blood cells were removed from the muscle. Consequently, lipid oxidation in the bled samples could be retarded as evidenced by the lower PV and TBARS in the bled samples.

4.4.1.2 Effect of bleeding on the formation of secondary lipid oxidation products in the Asian seabass slices

Formation of secondary lipid oxidation products in the slices prepared from bled and un-bled Asian seabass obtained at day 15 of iced storage is depicted in Figure 18. Fish muscle contained high amount of polyunsaturated fatty acids (PUFAs), which are prone to oxidation. Oxidation of PUFAs is known to produce volatile aldehydic compounds including hexanal, heptanal, octanal, etc (Yasuhara and Shibamoto, 1995). Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to follow lipid oxidation in a number of foods, including muscle foods (Ross and Smith, 2006). Among all the aldehydic compounds, heptanal, octanal and hexanal were found as the major aldehydes in Asian seabass slices. Pentanal, nonanal and 4-ethylbenzaldehyde were also found in both samples. Aldehydes have been used as the indicators of lipid oxidation because they possess low threshold values and are the major contributors to the development of off-

flavor and odour (Ross and Smith, 2006; Boyd *et al.,* 1992). Additionally, they also are the degradation products of microbiological metabolism reactions (Girard and Nakai, 1994). Aldehydes generated from oxidation reactions of fatty acids are frequently related with the off-odours (McGill *et al.,* 1974). Propanal and heptanal can serve as a reliable indicator of flavour deterioration for fish products, while hexanal contributes to the rancidity in meats (Augustin *et al.,* 2006; Ross and Smith, 2006). For all aldehydic compounds except pentanal, the higher amounts were found in the un-bled samples compared with the bled counterparts. The peak area of heptanal was 4-folds higher in un-bled samples (22.03 %), compared with that of bled samples (6.6 %) ($P < 0.05$) (Fig. 18). Certain fishy odour in the cold stored cod, cold stored butter and rancid soybeans have been reported to be caused by the presence of hepatanal in these products (Karahadian and Lindsay, 1989). The results indicated that lipid oxidation and greater decomposition of hydroperoxides formed was more pronounced in the un-bled samples. Autoxidation of polyunsaturated fatty acids and triglycerides in fish leads to formation of aldehydes which can produce off-odours, thereby limiting the shelf-life of the fish (Boyd *et al.,* 1992). Rancidity developed from the autoxidation of lipids leads to unacceptability of the product by consumer depending on the oxidation level occurred (Iglesias and Medina, 2008).

Various alcohols were detected in slices from both bled and un-bled samples. Alcohols were the secondary products produced by the decomposition of hydroperoxide (Girand and Durance, 2000). Similar levels of alcohols were found in both samples, however, 1-hexanol was absent in the bled samples (Fig. 18). 8-carbon alcohols are known to be present in all species of fish (Josephson *et al.,* 1984). 1-octen-3-ol, which imparts a desirable mushroom-like odor besides green and plant-like aromas in fish (Josephson *et al.,* 1986), tends to be higher in bled samples, compared with un-bled samples. Un-bled samples also had the higher 3, 5, octadien-2-one than bled counterpart. Ketone is another secondary lipid oxidation product derived from the decomposition of hydroperoxide in the chilled fish muscle (Iglesias and Medina, 2008). In general, the lower amount of secondary oxidation products including aldehydes, alcohols and ketones in the bled samples was in accordance with the lower PV and TBARS (Fig. 17 a and b).

The peak area of 2-ethylfuran found in un-bled samples was higher when compared with bled counterparts (Fig. 18). 2-ethylfuran is formed by the decomposition of 12-hydroperoxide of linolenate (18:3n-3), the 14-hydroperoxide of eicosapentaenoate (20:5n-3) and the 16-hydroperoxide of docosahexaenoate (22:6n-3), which can undergo ß-cleavage to produce a conjugated diene radical which can react with oxygen to produce a vinyl hydroperoxide. Cleavage of the vinyl hydroperoxide by loss of a hydroxyl radical, forms an alkoxyl radical, that undergoes cyclisation, thus producing 2-ethylfuran (Medina *et al.,* 1999). Thus, the bleeding was found to be effective in retarding the formation of secondary lipid oxidation products in Asian seabass slices during iced storage.

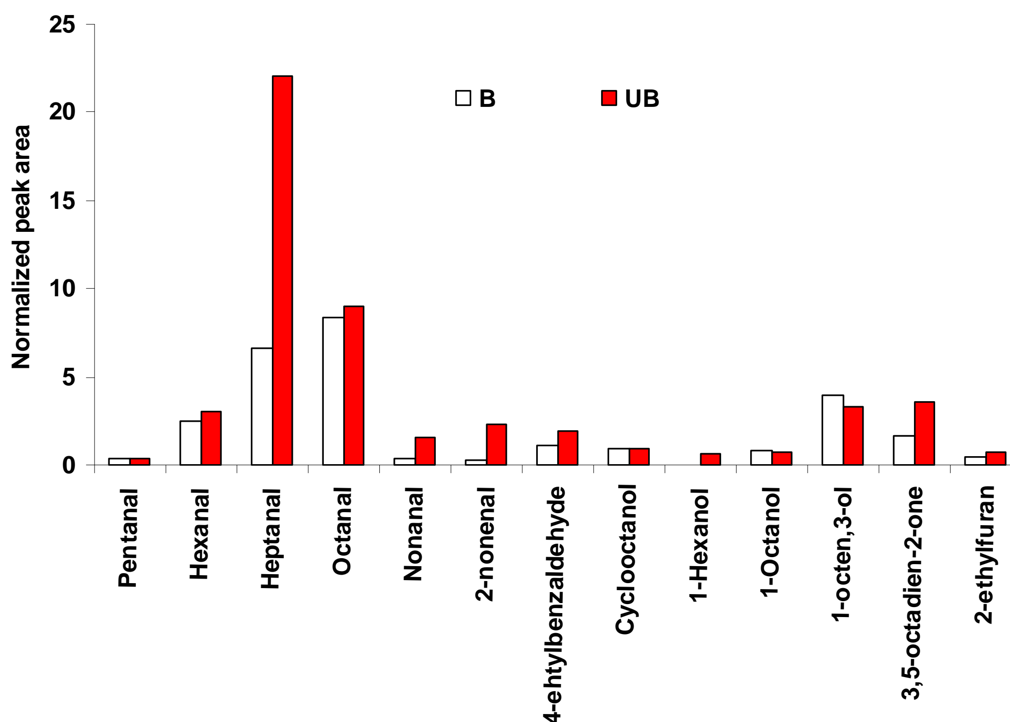


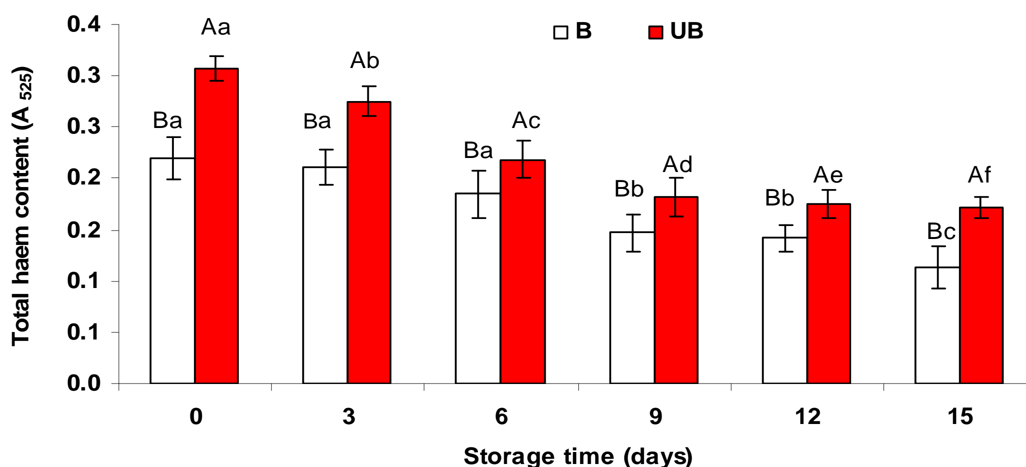
Figure18. Normalized peak area of the secondary lipid oxidation compounds identified by SPME-GCMS technique in the slices from bled and un-bled Asian seabass stored in ice for 15 days.

4.4.1.3 Changes in total haem and non-haem iron contents

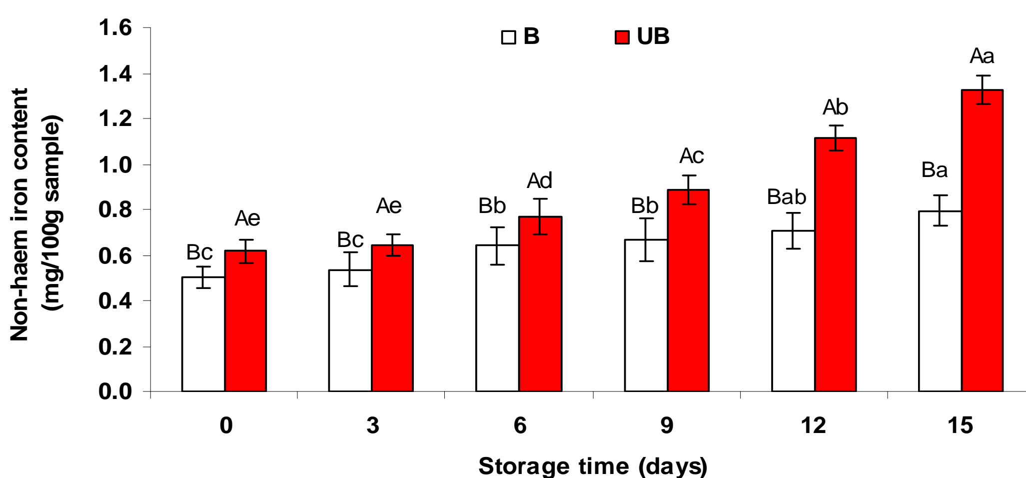
Changes in total haem and non-haem iron contents of slices from bled and un-bled Asian seabass during iced storage are shown in Figure 19 (a) and (b), respectively. At day 0, bled samples showed the lower total haem content than that of

un-bled counterparts ($P<0.05$). This confirmed that the blood was effectively removed from the muscle via bleeding. Blood contains a high amount of Hb, a major haem protein. Hb is made up of four polypeptide chains with each chain containing one haem group; each haem consisted of an iron atom coordinated inside the haem ring (Richards *et al.,* 2007). Total haem content of un-bled samples decreased with increasing storage time throughout the storage of 15 days ($P<0.05$). However, total haem content of the bled sample were not changed during the first 6 days of storage ($P>0.05$). Thereafter, a gradual decrease was found until the end of storage period ($P<0.05$) (Fig. 19a). Decreases in total haem content with increasing storage time were probably due to a haem breakdown, resulting in the release of non-haem iron (Benjakul and Bauer, 2001). Alternatively, the released iron can stimulate lipid oxidation (Tappel, 1955). Haem proteins became less soluble as the fish undergoes deterioration, leading to the decreased total haem content (Chen, 2003; Chaijan *et al.,* 2005). During the storage, total haem content of the un-bled samples was higher than that of the bled samples ($P<0.05$). Haem has been implicated as a potent promoter of lipid oxidation primarily through the decomposition of lipid hydroperoxides to form free radicals capable of abstracting hydrogen atoms from fatty acids and hence initiate lipid oxidation (Tappel, 1955). Thus, bleeding of Asian seabass resulted in the removal of haem from the muscle to some degree.

For non-haem iron content, bled samples had the lower non-haem iron content than un-bled counterparts at day 0. No changes in non-haem iron content were observed in both samples within the first 3 days of storage ($P>0.05$). Slight increase in non-haem iron content was found in bled samples, whereas the marked increase was obtained in un-bled counterparts after 3 days of storage (Fig.19b). The increase in non-haem iron content with increasing storage period was coincidental with the decrease in total haem content during the storage (Fig. 19a).



a)



b)

Figure 19. Changes in total haem content (a) and non-haem iron content (b) in the slices from bled and un-bled Asian seabass during iced storage. Bars represent the standard deviation (n=3). Different capital letters within the same storage time denote the significant differences (P<0.05). Different small letters within the same sample denote significant differences (P<0.05).

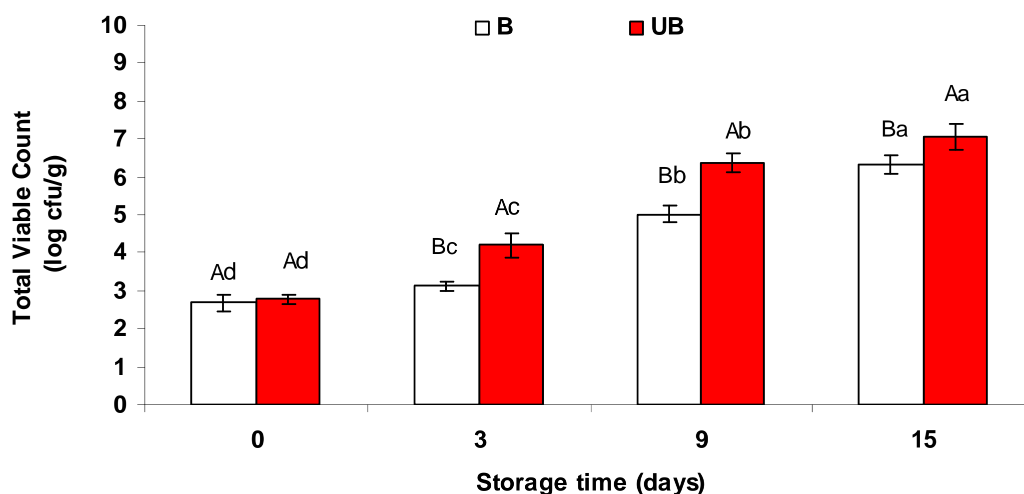
These results suggested that the disruption of porphyrin ring more likely occurred during extended storage. As a result, the release of free iron became pronounced, especially with the longer storage time. Decker and Hultin (1990a)

suggested that haem pigment or other iron-containing proteins were possibly denatured with increasing storage time, resulting in the release of iron. The increase in non-haem iron of fish muscle during refrigeration is a reflection of the degradation of haem (Gomez-Basauri and Regenstein, 1992b). Damage of porphyrin ring during storage was suggested as the cause of the breakdown of haem molecules and the release of iron (Gomez-Basauri and Regenstein, 1992b). Non-haem iron content of the un-bled samples was higher than that of bled samples throughout the storage ($P<0.05$). The higher non-haem iron content correlates well with the higher rate of lipid oxidation in the un-bled samples as reflected by higher PV and TBARS (Fig. 17 a and b).

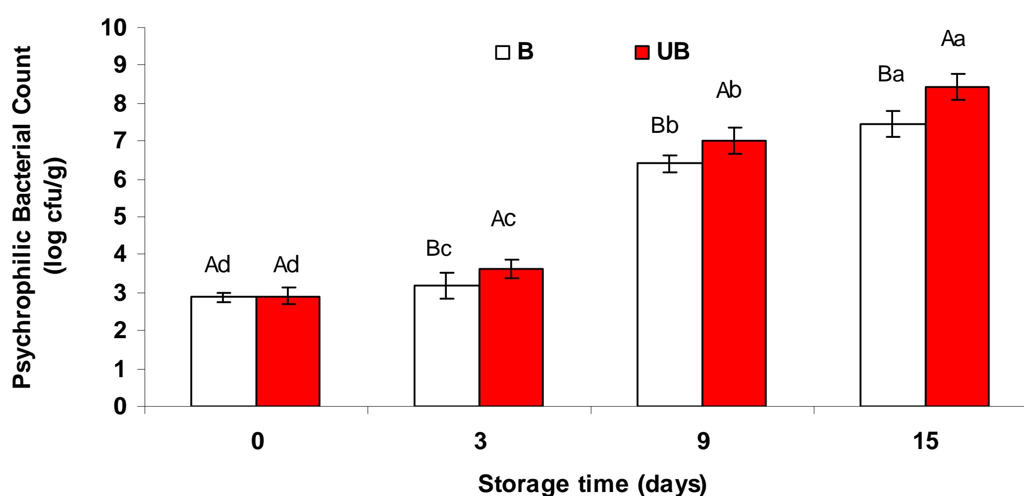
Non-haem iron has been reported to act as prooxidant in the fish muscle (Kanner *et al.,* 1991). Metal ion, especially Fe^{3+} and Fe^{2+}, has shown their proxidative activity in the muscle system (Love, 1983). The increase of non-haem iron could have some important consequences affecting both the nutritional and technological properties of fish muscle. The degradation of haem iron would reduce the nutritional value of fish in terms of bioavailability of iron, since haem iron is more available than non-haem iron (Hunt and Roughead, 2000). Thus, bleeding could be a means to lower the contents of total haem and non-haem iron contributing to lipid oxidation of Asian seabass slices during extended storage.

4.4.2 Effect of bleeding on microbiological changes of Asian seabass slices during iced storage

Total viable count (TVC) and psychrophilic bacterial count (PBC) of the slices from bled and un-bled Asian seabass during iced storage are depicted in Figure 20 (a) and (b) respectively. TVC and PBC of slices from both bled and un-bled samples increased with increasing time of storage ($P<0.05$). TVC and PBC of un-bled samples increased rapidly from the initial values of 0.63×10^3 to 1.21×10^7 and from 0.84×10^3 to 2.69×10^8 cfu/g, respectively.



a)



b)

Figure 20. Changes in total viable count (TVC) (a) and psychrophilic bacterial count (PBC) (b) in the slices from bled and un-bled Asian seabass during iced storage. Bars represent the standard deviation (n=3). Different capital letters within the same storage time denote the significant differences (P<0.05). Different small letters within the same sample denote significant differences (P<0.05).

TVC and PBC of un-bled samples were higher than that of bled samples throughout the storage ($P<0.05$), suggesting that bleeding was effective in retarding the growth of bacteria in the slices. Nevertheless, bleeding had no impact on both TVC and PBC in slices at day 0. Thereafter, the both TVC and PBC were found in higher number in un-bled samples when compared with bled counterparts ($P<0.05$). The blood enriched with nutrients for microbial growth could induce the enumeration of bacteria, which were contaminated from skin, gills, viscera or environment during dressing of the Asian seabass. *Micrococcus*, *Corynebacterium, Bacillus, Pseudomonas, Vibrio, Aeromonas* and *Flavobacterium-Cytophaga* were prevalent in skin and gill of healthy Asian seabass (Loganathan *et al.,* 1989). Blood of fish such as carp, big head carp and grass carp has an average protein content of 27.5 % and blood glucose level of 65 % (Atanasova *et al.,* 2008). Fish blood agar (FBA) prepared from blood of Nile tilapia was proved as a good medium for the growth of bacteria like *Streptococcus iniae*, *Acinetobacter calcoaceticus*, *Staphylococcus epidermidis*, and *Edwardsiella tarda* (Pasnik *et al.,* 2005). Thus, the blood present in the un-bled sample provides a suitable substrate for the growth of microorganisms as evidenced by higher TVC and PBC throughout the storage of 15 days. TVC values of un-bled samples exceeded 10^7 cfu/g, the upper limit for the fish to be safe for consumption (ICMSF, 1986), at day 15 of iced storage, while bled samples did not reach this limit up to 15 days. At day 9 and 15, it was found that PBC was higher than TVC in both samples. Psychrophilic bacteria became dominant and the inhibition of mesophilic bacteria occurred at the low temperature was presumed. From the microbiological point of view the shelf-life of bled and un-bled samples was 15 and 9 day, respectively. Therefore, bleeding could retard the growth of bacteria by devoiding the nutrients for their growth.

4.4.3 Effect of bleeding on fishy odour development of Asian seabass slices during iced storage

Fishy odour intensity of the slices prepared from bled and un-bled Asian seabass during iced storage are depicted in Figure 21. Fishy odour intensity of both bled and un-bled samples increased with increasing storage time ($P<0.05$). Un-

bled samples had higher intensity of fishy odour than that of the bled samples throughout the storage (P<0.05). The greater development of fishy odour in un-bled samples correlated well with the higher formation of TBARS (Fig 17b) and secondary lipid oxidation compounds (Fig. 18) in the un-bled samples. Richards and Hultin (2002) reported that mince from un-bled trout had the greater rancidity scores than the bled trout during 15 days of storage at 2 ºC. Bleeding of Atlantic mackerel reduced the rancidity development during storage at 2 ºC. At day 8, around 40 % of mince from bled Atlantic mackerel had a rancid odour as compared to 100 % of mince from un-bled sample (Richards and Hutlin, 2002). Bled Asian seabass slices stored for 9 days had the fishy odour similar to that of fresh slices (day 0). The results suggested that the bleeding could retard the development of fishy odour which was mainly associated with the lipid oxidation. The off-odour developed in the fish muscle due to lipid oxidation was considered as fishy (Fu *et al.,* 2009). At day 15, the intensity of fishy odour developed in the un-bled samples was higher. It was due to off-odour contributed by both lipid oxidation and microbial spoilage in the un-bled samples. Bacteria play a major role in fish spoilage by generating off-odours and an unacceptable visual appearance of fish (Pastoriza *et al.,* 1998). Thus, the bleeding reduced the fishy odour development to some extent during iced storage of Asian seabass slices.

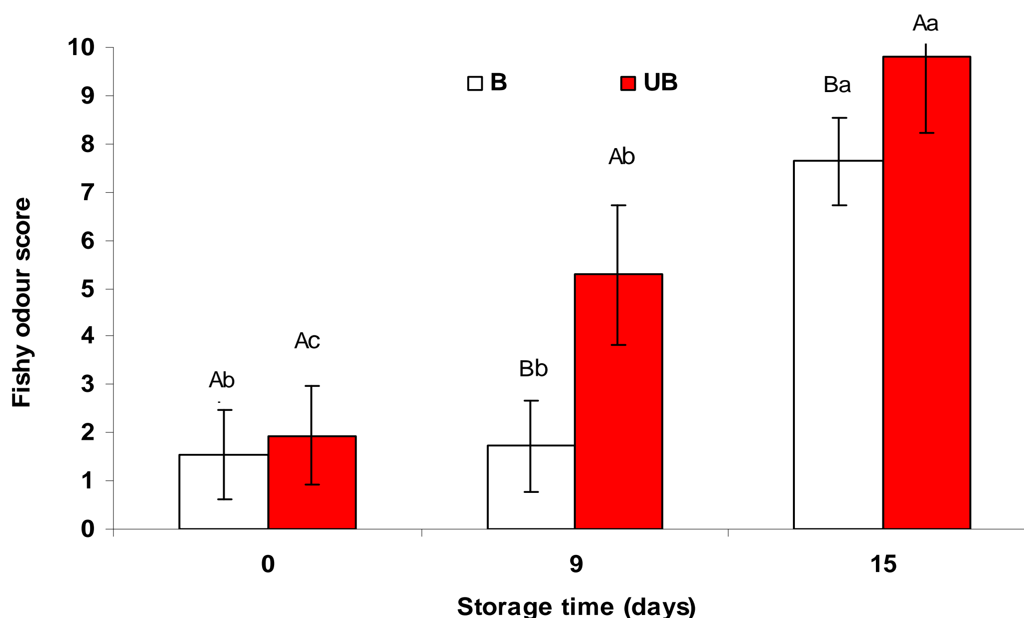


Figure 21. Fishy odour scores of the slices prepared from bled and un-bled Asian seabass during iced storage. Bars represent the standard deviation (n=11). Different capital letters within the same storage time denote the significant differences (P<0.05). Different small letters within the same sample denote significant differences (P<0.05).

4.5 Conclusion

Oxidation of lipid mediated by haem along with microbial growth was the main cause of the development of fishy odour in the Asian seabass slices during iced storage. Thus, the bleeding of the Asian seabass can be effective practice to retard lipid oxidation, fishy odour development as well as the growth of microorganism during iced storage.

CHAPTER 5

COMPARATIVE STUDIES ON MOLECULAR CHANGES AND PRO-OXIDATIVE ACTIVITY OF HAEMOGLOBIN FROM DIFFERENT FISH SPECIES AS INFLUENCED BY pH

5.1 Abstract

Oxygenation, autoxidation as well as pro-oxidative activity of haemoglobins from tropical fish (Asian seabass, tilapia and grouper) as influenced by different pH (6, 6.5 and 7) were comparatively studied. Relative oxygenation of all haemoglobins decreased in the acidic conditions. Haemoglobin from seabass was more oxygenated and stable against autoxidation at both pH 6 and 7, compared to those from tilapia and grouper. Haemoglobin from tilapia and grouper was fully oxidised at pH 6 after 120 h. Lipid oxidation of washed Asian seabass mince added with haemoglobin from various fish at different pH (6, 6.5 and 7) was monitored during 10 days of iced storage. Haemoglobins accelerated lipid oxidation more effectively at pH 6, compared with pH 6.5 and 7 as indicated by the higher peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). At the same pH values, haemoglobins from tilapia and grouper were more pro-oxidative than that from Asian seabass as evidenced by the higher PV and TBARS ($P<0.05$). Volatile lipid oxidation compounds detected by gas chromatography–mass spectrometry (GC-MS) were also formed at higher rate in the washed mince added with haemoglobin from tilapia or grouper with coincidental stronger fishy odour development, compared to the control and that added with haemoglobin from Asian seabass. Thus, lipid oxidation in fish muscle was more likely governed by haemoglobin, whose pro-oxidative activity varied depending upon the pH as well as molecular properties of haemoglobin.

5.2 Introduction

Lipid oxidation is a major cause of quality deterioration in fish muscle. Deterioration of flavour, odour, colour, texture, and the production of toxic compounds can arise from lipid oxidation (Kanner, 1994). Haemoglobin has been known to be an effective catalyst of lipid oxidation. Additionally, haemoglobin can be a source of activated oxygen associated with its autoxidation, and haem or iron can be released from the protein to promote lipid oxidation (Richards and Hultin, 2002). The iron atom in the haem ring of haem proteins is primarily in the ferrous (+2) state. Conversion of ferrous haem protein to met (+3) counterpart is a process known as autoxidation (Richards and Dettmann, 2003). Autoxidation appears to be a critical step in the ability of haem proteins to stimulate lipid oxidation since methaemoglobin reacts with peroxides to stimulate the formation of compounds capable of initiating and propagating lipid oxidation (Everse and Hsia, 1997). It was reported that human haemoglobin stimulated the oxidation of linoleic acid only slightly at pH 7.4, but the rate was increased considerably at pH 6.5 (Gutteridge, 1987). Nevertheless, pH values below neutrality are typical of post mortem fish muscle and vary with the fish species. As a consequence, different fish can be prone to lipid oxidation at different degrees, depending on the post mortem pH.

Acceleration of lipid oxidation by pH reduction could be due to enhanced autoxidation of haemoglobin at reduced pH (Tsuruga *et al.,* 1998). Haemoglobin autoxidation causes the production of superoxide anion radical and methaemoglobin from ferrous oxyhaemoglobin (Misra and Fridovich, 1972). Dismutation of superoxide anion radical can produce hydrogen peroxide, which activates methaemoglobin as an initiator of lipid peroxidation (Harel and Kanner, 1986). Lowering the oxygenation of haemoglobin was found to enhance the autoxidation of haemoglobin (Balagopalakrishna *et al.,* 1996). Binding of oxygen by haemoglobins in rainbow trout decreased as pH was lowered (Binotti *et al.,* 1971). Thus, lowering pH more likely prevents oxygenation of haemoglobin and can promote lipid oxidation via acceleration of haemoglobin autoxidation.

Fish haemoglobins operate under much greater variations in their environmental conditions than haemoglobins from air breathing vertebrates (Weber, 2000). Depending on fish species and developmental stage of the fish, there are sometimes more than one electrophoretically distinct form of haemoglobin with varying responses to pH and catalytic properties (Riggs, 1970). A more rapid rate of autoxidation was detected in haemoglobins from cold water fish as compared to warm water fish (pH 7, 20 °C) (Wilson and Knowles, 1987). Hagfish had the lower haemoglobin autoxidation rates as compared to carp, tuna, and lamprey. Knowledge regarding the pro-oxidative activity of haemoglobin from different fish species can be useful in developing the species-specific antioxidative strategies to retard the lipid oxidation and increase the shelf-life of fish and fish products. Nevertheless, only haemoglobins from temperate or cold water fish have been evaluated for their pro-oxidative activities. No information on molecular properties and pro-oxidative activity of haemoglobins from tropical fish has been reported. Thus, the aim of this study was to evaluate autoxidation rate and pro-oxidative activity of haemoglobins from different tropical fish species, including freshwater, brackish and marine water fish, in the washed mince, as affected by pH.

5.3 Materials and methods

5.3.1 Chemicals

Cumene hydroperoxide, streptomycin sulphate, sodium heparin, pentanal, hexanal, heptanal, octanal and nonanal were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, potassium iodide, tris [hydroxy-methyl] aminomethane (Tris) and trichloroacetic acid were obtained from Merck (Damstadt, Germany). Disodium hydrogen phosphate, 2-thiobarbituric acid, ammonium thiocyanate and ferrous chloride were procured from Fluka Chemical Co. (Buchs, Switzerland). Chloroform and methanol were obtained from Lab-Scan (Bangkok, Thailand). All chemicals used were of analytical grade.

5.3.2 Fish supply and bleeding

Live Asian seabass (*Lates calcarifer*), tilapia (*Oreochromis niloticus*) and grouper (*Epinephelus itajara*) were purchased from fish farms, Songkhla, Thailand. All live fish were transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, the fish were bled from the caudal vein after the tail was cut off. Blood (15 ml) was collected in a 20 ml glass test tube rinsed with 150 mM NaCl containing sodium heparin (30 U/ml). The collected blood was stirred continuously in order to avoid the coagulation of blood.

5.3.3 Preparation of haemolysates

Haemolysates were prepared according to the method of Richards and Hultin (2000). Four volumes of cold 1.7 % NaCl in 1 mM Tris buffer, pH 8.0, were added to heparinised blood. This mixture was centrifuged at 700 x *g* for 10 min at 4 °C using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA). Plasma was then removed. Red blood cells were washed by suspending three times in 10 volumes of 1 mM Tris buffer (pH 8.0) and centrifuging at 700 *g* for 10 min. Cells were lysed in 3 volumes of 1 mM Tris buffer, pH 8.0, for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before centrifugation at 28,000 *g* for 15 min at 4 °C. Prepared haemolysate was stored at -40 °C and was thawed just before it was used.

5.3.4 Quantification of haemoglobin

Haemoglobin content was determined following the method described by Richards and Hultin (2000) with a slight modification. The haemolysate was diluted in 50 mM Tris buffer (pH 8.0) to obtain the absorbance of 0.5-0.6 at 415 nm. Approximately 1 mg of sodium dithionite was added to the sample (2 ml) and mixed thoroughly. The sample was bubbled with oxygen gas with a purity of 99.5 % to 100 % (TTS Gas Agency, Hat Yai, Songkhla, Thailand) for 10 min. Prepared sample (2 ml) was transferred to a 3 ml disposable cuvette. The absorbance of the sample was then measured at 415 nm against a blank, which contained only buffer, using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The haemoglobin concentration

was calculated by Lambert-Beer's law using a millimolar extinction coefficient of 125 for oxyhaemoglobin at pH 8 (Antoni and Brunoni, 1971).

5.3.5 Haemoglobin oxygenation

The affinity of different haemoglobins for oxygen was determined by scanning the haemolysate solutions (6 µM) in 50 mM sodium phosphate buffer with a pH range of 6 to 8 from 640 to 500 nm using a UV-1601 spectrophotometer (Pazos *et al.,* 2005a). The blank was also prepared as previously described. The absorbance at the peak (574 nm) minus the absorbance at the valley (560 nm) was recorded and this difference was used as a measure of oxygenation. The large differences indicated that the haemoglobin was highly oxygenated (Pelster and Weber, 1991).

5.3.6 Haemoglobin autoxidation

Haemoglobin autoxidation was monitored as per the method described by Pazos *et al.* (2005a) with some modifications. Different haemolysates were diluted to a final concentration of 6 µM in 50 mM sodium phosphate buffer, pH 6 and 7. The diluted haemolysates (5 ml) were transferred to the test tubes and stored in the ice. To follow haemoglobin autoxidation over the specified period of time, different haemolysate solutions, pH 6 and 7 were monitored for the changes in spectra from 500 to 630 nm up to 5 days of iced storage. The blanks were prepared using only the buffer having the same pH with the samples.

5.3.7 Preparation of washed mince

Live fresh Asian seabass (*Lates calcarifer*) with an average weight of 1 kg, purchased from a fish farm, Songkhla, Thailand and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, fish were washed with chilled tap water, descaled, filleted, de-skinned and minced using a mincer with a hole diameter of 5 mm. The mince was washed twice in ice cold distilled water at a mince-to-water ratio of 1:3 (w/ w) by stirring continuously with a plastic rod for 2 min. The mixture was allowed to stand for 15 min, followed by dewatering on a fiberglass screen. The mince was then mixed with 50 mM sodium

phosphate buffer (pH 6.0, 6.5, and 7.0) at a mince/buffer ratio of 1:3 and homogenised using an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany) for 1 min. It was allowed to stand for 15 min and then centrifuged at 15000 x *g* for 20 min at 4 °C. The resulting pellet was referred to as "washed mince". The final moisture content of the washed mince was adjusted to 88 %. The final pH was monitored by using a pH-meter (Sartorious North America, Edgewood, NY, USA) and was adjusted, if required.

5.3.8 Effect of haemolysate addition on lipid oxidation of washed mince at different pH

To study the impact of haemolysate from different fish species on lipid oxidation, a known volume (10 ml) of different haemolysates were added into washed mince (100 g) with different pH to obtain a final concentration of 6 μM of Hb/ kg of washed mince. For the control samples, the haemolysate was replaced by the same volume of distilled water. Streptomycin sulphate was added in the washed mince to obtain a final concentration of 200 ppm in order to inhibit the microbial growth. The samples were mixed manually. Washed mince (100 g) containing haemolysate from different fish species with different pH (6, 6.5 and 7) was packed in the separate polyethylene bags (14 x 8 cm^2). Three different polyethylene bags were randomly taken for each sampling time. The samples were stored in ice using a mince/ice ratio of 1:2 (w/w). Molten ice was removed every day and the same quantity of ice was replaced. Samples were taken for peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) determination every 2 days up to 10 days, whilst the evaluation of fishy odour was carried out at day 0, 2, 6 and 10. Volatile compounds in the samples were determined at the end of storage study (day 10).

5.3.9 Analyses

5.3.9.1 Peroxide value

Peroxide value (PV) was determined as per the method of Richards and Hultin (2002) with a slight modification. Washed mince (1 g) was mixed with 11 ml of chloroform/methanol mixture (2:1, v/v). The mixture was homogenised at a speed of

13,500 rpm for 2 min. Homogenate was then filtered using Whatman No. 1 filter paper (Whatman International, Ltd, Maidstone, England). Two millilitres of 0.5 % NaCl were then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s using a Vortex-Genie2 mixer 4 (Bohemia, NY, USA) and then centrifuged at 3,000 x g for 3 min to separate the sample into two phases. Two millilitres of cold chloroform/methanol (2:1) mixture were added to 3 ml of the lower phase. Twenty-five microlitres of 30 % ammonium thiocyanate and 25 µl of 20 mM iron (II) chloride were added to the mixture (Shantha and Decker, 1994). Reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A standard curve was prepared using cumene hydroperoxide at the concentration range of 0.5-2 ppm.

5.3.9.2 Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Washed mince (0.5 g) was mixed with 2.5 ml of a TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture was heated in a boiling water for 10 min to develop a pink colour, cooled with running tap water and then sonicated for 30 min using an Elma (S 30 H) sonicator (Kolpingstr, Singen, Germany). The mixture was then centrifuged at 5,000 ×g at 25 ºC for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (MAD) at the concentration ranging from 0 to 10 ppm and TBARS were expressed as mg of MAD equivalents/kg sample.

5.3.9.3 Determination of volatile compounds

Volatile lipid oxidation compounds in washed mince without and with the addition of haemoglobin from different fish species and stored in ice for 10 days were determined by solid phase micro extraction-gas chromatography mass spectrometry (SPME-GCMS) (Iglesias and Medina, 2008).

5.3.9.3.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 3 g of sample was homogenised at a speed of 13,500 rpm for 2 min with 8 ml of ultra pure water. The mixture was centrifuged at 2000 x g for 10 min at 4 ºC. The supernatant (6 ml) was heated at 60 ºC with equilibrium time of 10 h in a 20 ml headspace vial. Finally, the SPME fibre (50/30 µm DVB/Carboxen^{TM}/PDMS StableFlex ^{TM}) (Supelco, Bellefonte, PA, USA) was exposed to the head space of the vial containing the sample extract and the volatile compounds were allowed to absorb in the SPME fibre for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

5.3.9.3.2 GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatography coupled with HP 5972 mass selective detectors equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m x 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC and finally an increase of 15 ºC/min to a final temperature of 250 ºC and hold for 10 min. Helium was employed as a carrier gas, with a constant flow of 1.0 ml/min. Injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 265 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 250 ºC. Initially, a full scan mode data was acquired to determine appropriate masses for the later acquisition in selected ion monitoring (SIM) mode under the following conditions: mass range: 25-500 amu and scan rate: 0.220 s/scan. All the analyses were performed with ionisation energy of 70 eV, filament emission current at 150 µA and the electron multiplier voltage at 500 V.

5.3.9.3.3 Analyses of the volatile compounds

Identification of the volatile compounds in the samples was based on the retention times of individual aldehydic standards including pentanal, hexanal, heptanal, octanal and nonanal. Identification of the compounds was also done by consulting ChemStation Library Search (Wiley 275.L) using Probability-Based Matching (PBM) algorithm developed by Prof. Fred McLafferty and co-workers at Cornell University. Quantitative determination was carried out by using an internal calibration curve that was built using stock solutions of the compounds in ultra-pure water and analysing them by the optimised HS-SPME method. Quantification limits were calculated to a single-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing three replicates of each sample. The identified volatile compounds related with lipid oxidation were presented in the form of normalised area under peak of each identified compound.

5.3.9.4 Sensory analysis

The sensory evaluation was performed by 8 trained panelists, who were the graduate students in Food Science and Technology programme with the age of 25-33 years and were familiar with fish consumption. Panelists were trained in two sessions using a 10 point scale, where 0 represented no fishy odour and 10 represented the strongest fishy odour. Reference samples were prepared by storing Asian seabass slices packed in polythene bags in ice for 0, 5, 10 and 15 days representing the score of 0, 3, 7 and 10, respectively. To test the samples, the panelists were asked to open the sealable polyethylene bags and sniff the headspace above the samples. The samples were then scored.

5.3.10 Statistical analysis

All experiments were run in triplicate. Completely randomized design (CRD) was used for this study. The experimental data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's New Multiple Range Test (Steel and Torrie, 1980). For pair comparison, *T*-test was

used. Data analysis was performed using a SPSS package (SPSS 14.0 for Windows, SPSS Inc, Chicago, IL, USA).

5.4 Results and discussions

5.4.1 Effect of pH on oxygenation and autoxidation of haemoglobins from various fish

The relative oxygenation of haemoglobin from Asian seabass, tilapia and grouper at pH ranging from 6 to 8 is shown in Figure 22. The affinity for oxygen of all haemoglobins decreased with decreasing pH ($P < 0.05$). A marked decrease was observed when pH shifted from 7 to 6.5. A similar pattern of oxygen affinity in pH range of 6 to 8 was found in cod haemoglobin (Pazos *et al,* 2005a) and trout haemoglobin (Richards and Hultin, 2000). The oxygenation of haemoglobin generally decreases in the acidic environment, known as the Bohr effect (Stryer, 1988a). Therefore, ability of different haemoglobins to bind O_2 was more pronounced in the neutral pH or slightly alkaline pH range. At the same pH tested, haemoglobins from various fish species showed different affinity to bind oxygen. Haemoglobin from Asian seabass exhibited the higher affinity for oxygen than those from grouper and tilapia, respectively. Undeland *et al.* (2004) also found that the haemoglobin from mackerel, menhaden, flounder and pollock showed differences in their affinity to bind oxygen. Among all, menhaden haemoglobin had the highest affinity for oxygen. The results suggested that the postmortem pH most likely had the impact on the change in haemoglobin, particularly their oxygenation. The pH change of fish muscle might determine the molecular properties of haemoglobins and have influence on the quality of fish muscle. Generally, pH of fish muscle gradually decreases at the beginning of storage, mainly associated with the lactic acid produced via anaerobic respiration (Hultin and Kelleher, 2000). However, the slight increase in pH has been reported in fish muscle, caused by the formation of volatile bases produced by microorganisms, particularly during the spoilage period (Benjakul *et al.,* 2003).

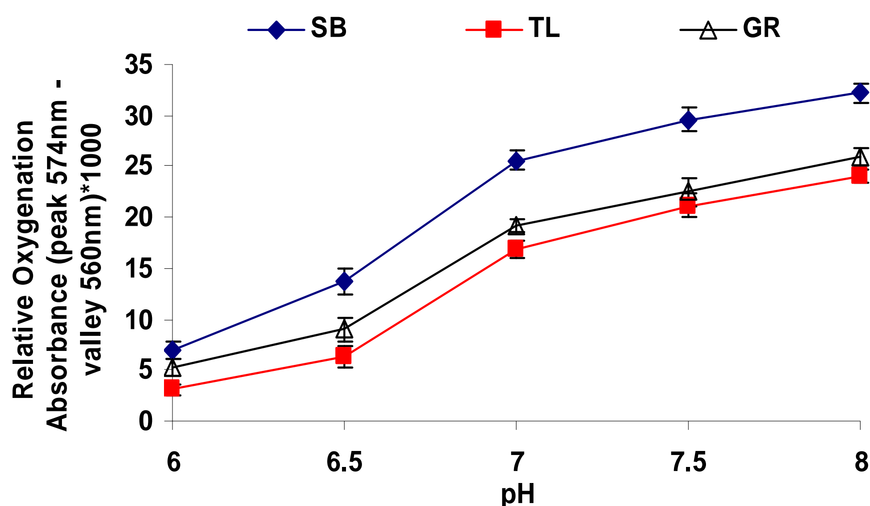


Figure 22. Relative oxygenation of haemoglobin from Asian seabass (SB), tilapia (TL) and grouper (GR) as a function of pH (a). The haemoglobin concentration was 6 µM. Bars represent the standard deviation (n=3).

Autoxidation of haemoglobin at pH 6 and 7 as a function of time is depicted in Figure 23. Haemoglobins from all fish were less oxygenated at pH 6, compared to pH 7 (Fig. 22), as indicated by the large differences between the peak and valley. The autoxidation rates of all haemoglobins were monitored by obtaining spectra in the wavelength of 500 to 630 nm. The decrease in both absorbance at 574 nm (peak) and 560 nm (valley) of the haemoglobin is the indication of formation of methaemoglobin from oxyhaemoglobin (Richards and Dettmann, 2003). All haemoglobins had the greater stability to oxidation at pH 7 than at pH 6 (Fig 23). Tilapia and grouper haemoglobins had a noticeable spectral change after 72 h (Fig. 23). At time 120 h, the visible spectra of methaemoglobin were established in both tilapia and grouper haemoglobin at pH 6, which is characteristic of haem release from the haemoglobins and / or porphyrin destruction. However at pH 7, some reduced form still remained in all samples as mirrored by the positive difference in absorbance between 574 nm (peak) and 560 nm (valley). The spectra of Asian seabass haemoglobin shifted to the lower absorbance with increasing incubation time, however, it was not fully oxidised to methaemoglobin after 120 h at both pH. The

results suggested the higher stability of Asian seabass haemoglobin, compared to those from tilapia and grouper. Lower autoxidation rate of Asian seabass haemoglobin correlated well with its higher oxygenation, in comparison with haemoglobin from tilapia and grouper. Small decrease in the absorbance at the peak and valley along with the positive difference of absorbance between 574 nm (peak) and 560 nm (valley) was found in Asian seabass haemoglobin, when compared with those from tilapia and grouper (Fig. 22 and 23). Therefore, Asian seabass haemoglobin was more resistant to autoxidation, compared with those from tilapia and grouper.

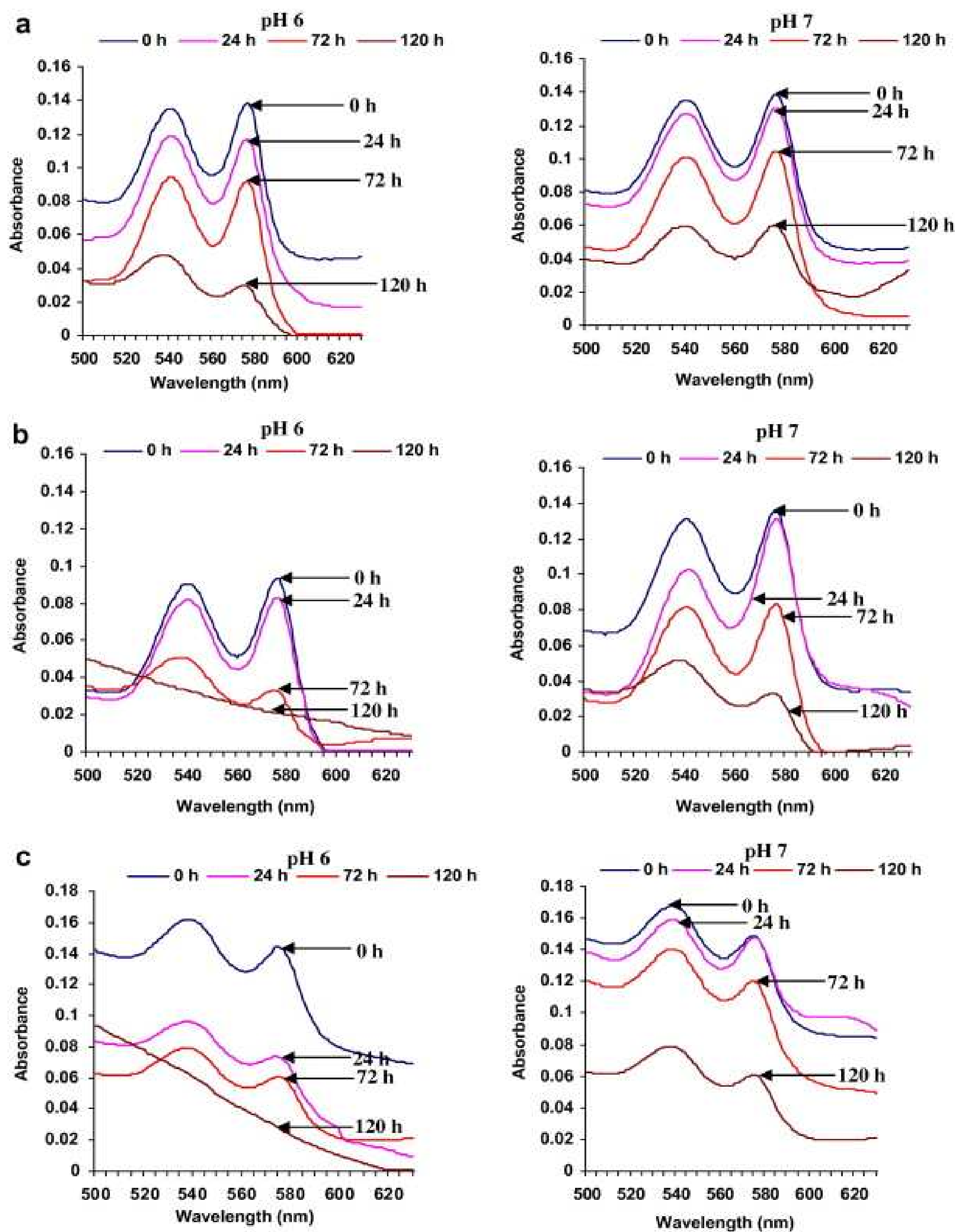


Figure 23. Spectral changes of haemoglobin from Asian seabass (a), tilapia (b) and grouper (c) exposed to pH 6 and 7 as a function of time. The haemoglobin concentration was 6 μM.

5.4.2 Effect of haemoglobin from different fish species on lipid oxidation of washed mince at different pHs

Changes in PV and TBARS of washed mince added with haemoglobin from different fish species at various pH (6, 6.5 and 7) during 10 days of iced storage are shown in Figure 24 and 25, respectively. After 2 days of storage, the sharp increases in both PV and TBARS were noticeable in all samples added with haemoglobin from different fish species. The lowest PV and TBARS were obtained in the control. This indicated the pro-oxidative activity of fish haemoglobin in washed mince. Haemoglobins from all three fish species catalysed the lipid oxidation at different degrees, depending on pH. The lipid oxidation rate was more pronounced at pH 6 and 6.5, compared with pH 7, as reflected by higher PV and TBARS at pH 6 and 6.5 (Fig. 24 and 25). The results correlated well with the greater autoxidation of all these haemoglobins at pH 6 than pH 7 (Fig. 22 and 23). The higher degree of lipid oxidation in all samples added with haemoglobins at acidic pH was in agreement with the findings of Undeland *et al.* (2002) and Undeland *et al.* (2004). This might be related to the lower degree of oxygenation (Bohr or Root effect) and the increased rates of autoxidation at pH 6, compared with pH 7. The concomitant increase in lipid oxidation rates in washed mince at pH 6 suggested a possible role of deoxyhaemoglobin as an effective catalyst of lipid oxidation (Richards and Hultin, 2000). Richards *et al.* (2002) reported that deoxyhaemoglobin acted as a stronger oxidation catalyst than oxyhaemoglobin because it has a haem crevice that is more accessible (Levy and Rifkind, 1985). The iron atom of the porphyrin group inside the crevice is also kicked out of the plane when deoxygenation occurs (Stryer, 1988a). This may allow the iron to more easily interact with lipid hydroperoxides, creating more free radicals to facilitate both haemoglobin autoxidation and lipid oxidation. Haemoglobin autoxidation can also promote lipid oxidation through the production of superoxide anion radical and methaemoglobin (Richards and Hultin, 2003). Haemoglobin autoxidation can also result in the formation of oxygen radicals ($O_2^{\bullet -}$) (Shikama, 1998), protein radicals (e.g., hypervalent ferryl-haemoglobin (Fe^{4+}==O)) and lipid radicals ($LOO^{\bullet}$ and $LO^{\bullet}$) (Ryter and Tyrrell, 2000).

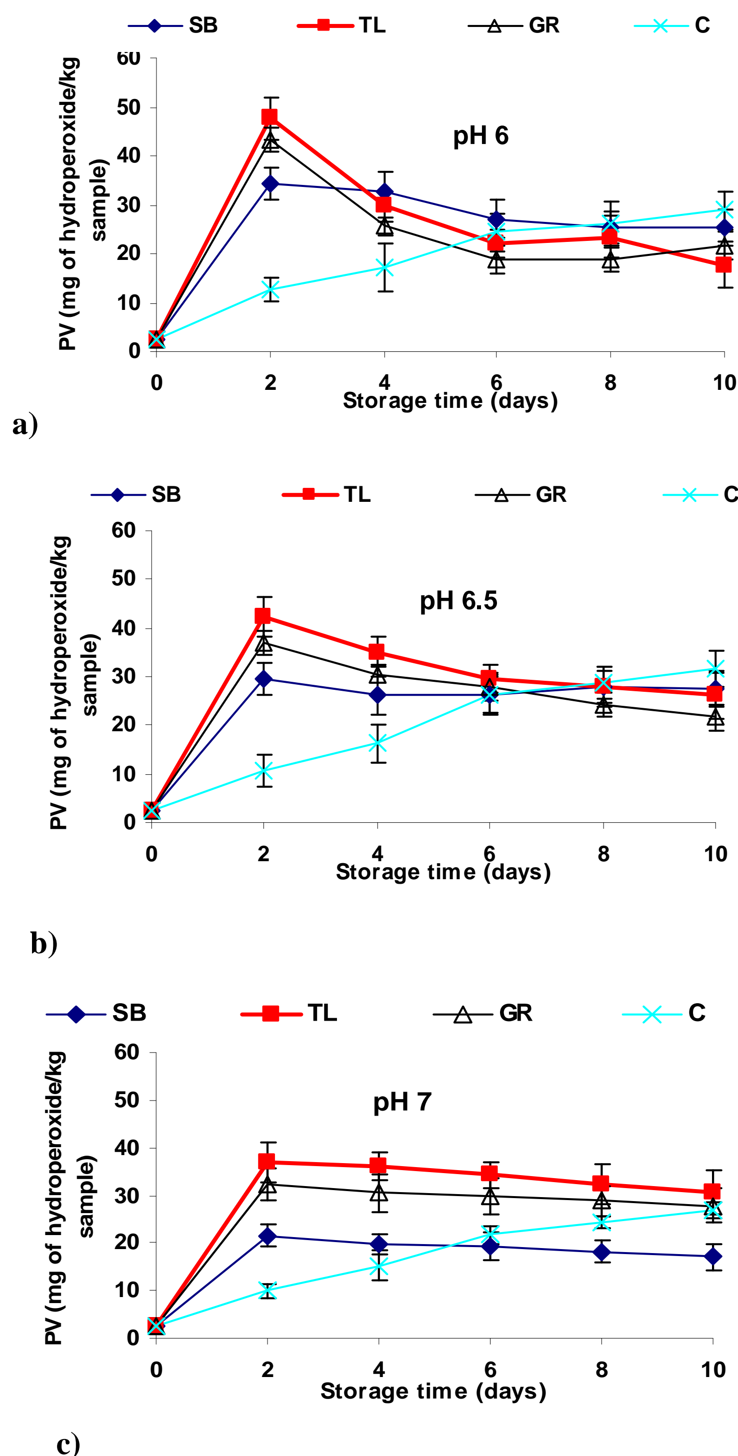


Figure 24. Changes in peroxide value (PV) of washed mince at pH 6 (a), 6.5 (b) and 7 (c) and added without and with 6 µM haemoglobin from Asian seabass, tilapia or grouper during 10 days of iced storage. Bars represent the standard deviation (n=3). SB: Asian seabass, TL: tilapia, GR: grouper and C: control (without addition of haemoglobin).

Methaemoglobin can release its haem group 60-folds greater than oxy- and deoxyhaemoglobin (Hargrove *et al.,* 1997). Furthermore, human haemoglobin is dissociated into dimers 10 times more quickly at pH 6.2 than at pH 7.5 (Dumoulin *et al.,* 1997) and dimers undergo autoxidation 16 times faster than tetramers (Griffon *et al.,* 1998). The lowered prooxidative activity of haemoglobin with increasing pH was also reported by Richards and Hultin (2000).

During 4-10 days of storage, the continuous decrease in PV was noticeable in all the samples added with haemoglobins (P<0.05). This suggested the decomposition of hydroperoxide formed, especially within the first 2 days of storage. A decrease in the level of primary oxidation products is related to hydroperoxide degradation, producing secondary lipid oxidation products (Boselli *et al.,* 2005). However, gradual increase in PV was observed in the control samples (without addition of haemoglobin) throughout the storage of 10 days (P<0.05). For the samples added with haemoglobins from the same fish species, the higher PV was found at pH 6, compared to pH 6.5 and 7 (P<0.05). This confirmed the pro-oxidative activity of haemoglobin at acidic pH. When comparing PV of all samples tested at the same pH, it was noted that the sample added with haemoglobin from tilapia had the highest PV, while the lowest PV was found in the samples added with haemoglobin from Asian seabass (P<0.05).

For TBARS values, the sharp increase was also found at day 2 of storage in all samples (P<0.05) except the control, in which the TBARS remained constant throughout the storage (P>0.05). All samples added with haemoglobin from different fish had no change in TBARS after 2 days of storage for all pHs tested, except for the sample added with haemoglobin from grouper at pH 7, which showed the continuous increase in TBARS during the storage of 10 days (P<0.05). When the effect of pH on TBARS formation was compared, it was found that the sample at pH 6 exhibited the highest TBARS, regardless of sources of haemoglobin. The constant TBARS could indicate that these secondary oxidation products might react further with free amino acids, proteins and peptides in the washed mince system to form Schiff's base (Dillard and Tappel, 1973). Furthermore, some volatile compounds were lost during the storage. For the control samples, there was no difference in TBARS at

all pHs throughout the storage of 10 days (P>0.05). PV of the control samples at all pH values increased continuously throughout the storage period (P<0.05), indicating the lipid oxidation was still in propagation stage till the end of storage period. A slight increase in TBARS values of the control samples at different pH were found with the increasing storage time (P<0.05). Thus, the enhanced autoxidation and decreased oxygenation of all haemoglobins from different fish species at acidic pH resulted in the higher lipid oxidation in the washed mince. Among all haemoglobins tested, that from tilapia was found to be more active in catalysing lipid oxidation in washed mince followed by haemoglobin from grouper and Asian seabass, respectively (P<0.05). Rapid autoxidation of tilapia and grouper haemoglobins to methaemoglobin was closely associated with their pronounced pro-oxidative activity in washed mince, compared with that from Asian seabass. Also, the haemoglobins from tilapia and grouper had low affinity for oxygen than haemoglobin from seabass (Fig. 22). Haemoglobins with low oxygen affinity are easily oxidised (Astatke *et al.,* 1992). When autoxidation takes place, the formation of methaemoglobin and the superoxide anion radical ($O_2^{\bullet -}$) were formed. Additionally, $O_2^{\bullet -}$ dismutates to H_2O_2, which subsequently reacts with methaemoglobin to form the hypervalent ferrylhaemoglobin radical known to initiate lipid oxidation (Kanner and Harel, 1985).

The habitat of the fish can strongly influence the stability of haemoglobin in relation to autoxidation (Powers, 1972). Haemoglobins of fish from sluggish water have non-Bohr effect, while those from active streams possess haemoglobins with Bohr effect (Riggs, 1970; Powers, 1972). Richards and Hultin (2003) reported that haemoglobin from herring and mackerel was more pro-oxidative than haemoglobin from trout. Frequent migration of herring and mackerel was related with more unstable and pro-oxidative haemoglobins. However, the present study showed that active migratory fish like Asian seabass had less pro-oxidative haemoglobin. Other biological and genetical factors as well as inhabiting environment might play an essential role in the properties of haemoglobin in the fish (Undeland *et al.,* 2004).

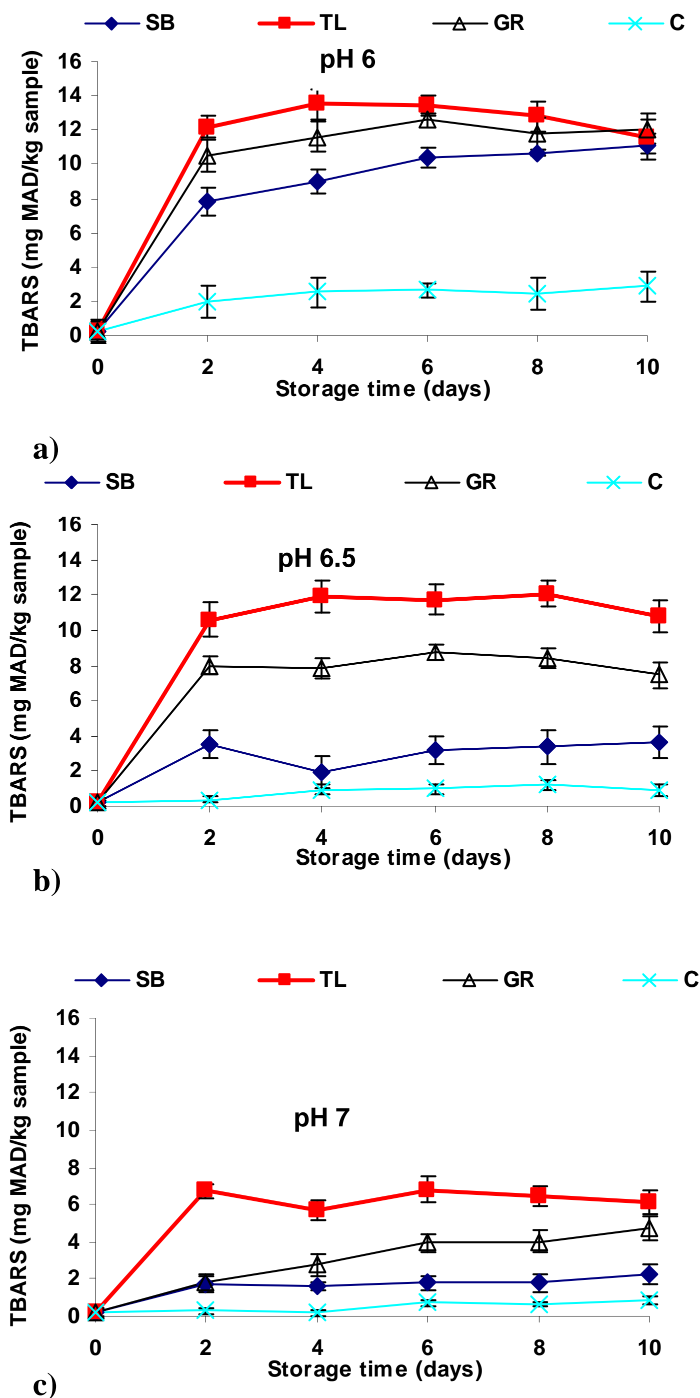


Figure 25. Changes in thiobarbituric acid-reactive substances (TBARS) of washed mince at pH 6 (a), 6.5 (b) and 7 (c) and added without and with 6 µM haemoglobin from Asian seabass, tilapia or grouper during 10 days of iced storage. Bars represent the standard deviation (n=3). Key: see Figure 24 caption.

Wilson and Knowles (1987) found that oxyhaemoglobin from bottom dwelling fish was more autoxidised than those from species living in shallow waters. It was also reported that haemoglobins are more susceptible to oxidation at low temperature than at high temperature (Undeland *et al.,* 2004). Tilapia is known to inhabit still or flowing waters in rocky or mud-bottom areas (Allen *et al.,* 2002). Tilapia is a bottom dwelling fish, where the temperature is low when compared to surface water. Groupers tend to reside on the bottom of tropical and subtropical waters and most of the species live on coral reefs (Matthew *et al.,* 1999). Thus, the haemoglobins from tilapia and grouper were more likely unstable and more prone to autoxidation. On the other hand, Asian seabass grows to maturity in the upper reaches of freshwater rivers and streams. Adults generally move downstream especially during tidal or flooding, to estuaries and coastal waters for spawning (Keenan, 1994). Seabass has been reported to live near shore and is considered as a pelagic fish (FAO, 1989), thereby exposing to higher surface water temperature than the demersal fish like tilapia and grouper. It is also evident that pelagic migratory fish may not necessarily have more lipid pro-oxidative haemoglobins because of their need for oxygen to carry out long distance migratory swimming (Undeland *et al.,* 2004). As a result, the higher stability was found for Asian seabass haemoglobin, when compared with tilapia and grouper haemoglobins. Furthermore, it was suggested that haemoglobins from tilapia and grouper were more pro-oxidative in washed mince, compared to Asian seabass haemoglobin.

5.4.3 Effect of haemoglobin from different fish species on fishy odour development in washed mince at different pH

Fishy odour intensity of washed mince added with haemoglobin from different fish species at pH 6, 6.5 and 7 during the iced storage is depicted in Figure 26. Fishy odour intensity of all washed mince samples added with haemoglobin from different fish species at all pH values increased continuously throughout the storage of 10 days ($P<0.05$). At the same pH used, washed mince added with tilapia or grouper haemoglobin showed the higher fishy odour intensity, compared with those added with Asian seabass haemoglobins, especially at pH 6 ($P<0.05$). Nevertheless, there was no difference in fishy odour intensity between washed mince added with haemoglobin

from tilapia and grouper throughout the storage of 10 days (P>0.05). For the samples added with haemoglobin from different fish species, it was noted that the higher fishy odour intensity was obtained in the samples with pH 6, followed by pH 6.5 and 7.0, respectively. The results were in accordance with the pronounced formation of TBARS in the washed mince added with tilapia or grouper haemoglobin at pH 6, compared with pH 6.5 and 7 (Fig. 25). Undeland *et al.* (2004) also reported that haemoglobin from mackerel, menhaden and flounder accelerated the development of painty odour in washed cod mince more rapidly at pH 6, compared with pH 7.2. Trout haemoglobin also induced lipid oxidation and rancidity development more rapidly at pH 6, compared with pH 7.2 in washed cod mince (Richards and Hultin, 2000). Increasing pH to 7.6 delayed the rancidity to a greater extent than pH 6 and 7.2 (Richards and Hultin, 2000). Lipid oxidation and fishy odour development catalysed by tilapia and grouper haemoglobin were intense at pH 6. This was more likely due to the lower relative oxygenation and the greater autoxidation of tilapia and grouper haemoglobin to met form at pH 6 (Fig. 22 and 23). Control samples displayed very less fishy odour development at all pH tested throughout the storage period. The off-odour developed in the fish muscle due to lipid oxidation was considered as fishy (Fu *et al.,* 2009). Thus, haemoglobin from tilapia and grouper were very active in fishy odour development in the washed mince at all pH values studied, whilst Asian seabass haemoglobin was less pro-oxidative.

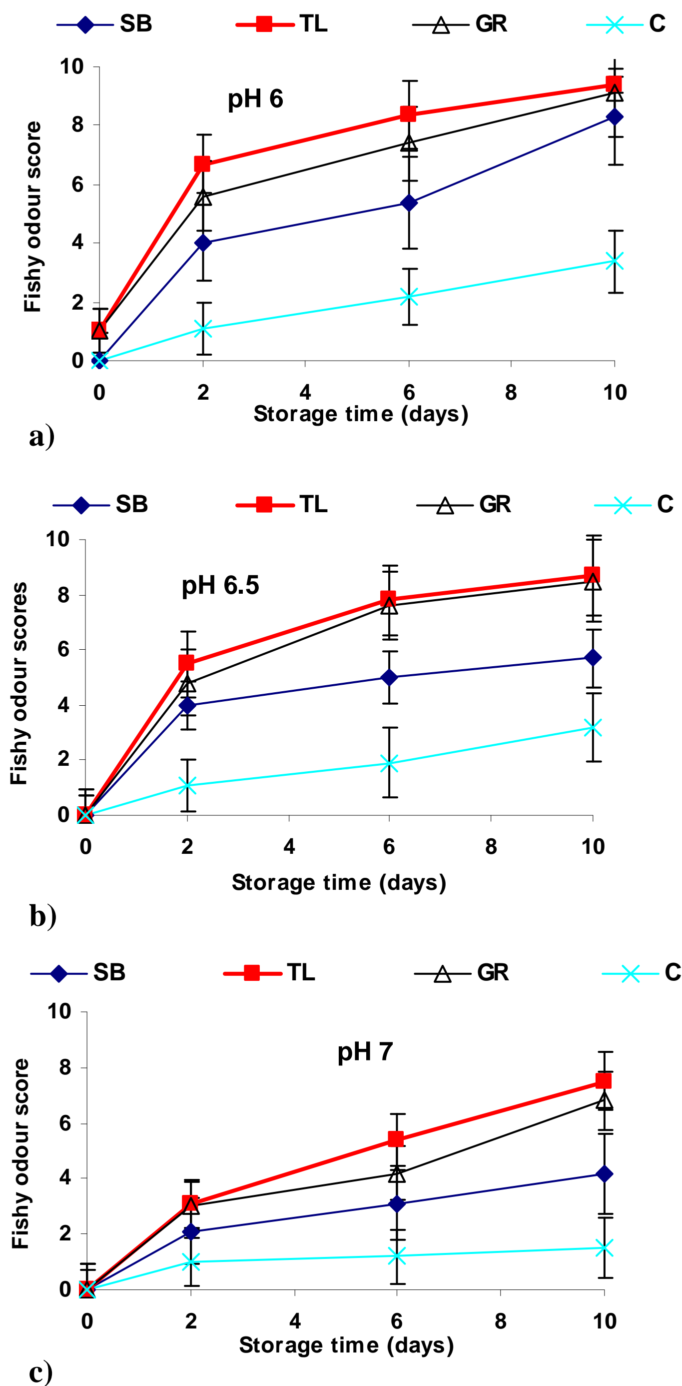


Figure 26. Changes in fishy odour development in washed mince at different pH of 6 (a), 6.5 (b) and 7 (c) and added without and with 6 μM haemoglobin from Asian seabass, tilapia, or grouper during 10 days of iced storage. Bars represent the standard deviation (n=8). Key: see Figure 24 caption.

5.4.4 Effect of haemoglobin from different fish species on the formation of volatile lipid oxidation products in washed mince at pH 6

Volatile compounds in washed mince associated with lipid oxidation catalysed by haemoglobin from different fish species at pH 6 at day 10 of iced storage are shown in Figure 27. Lipid oxidation of fish muscle is known to produce volatile aldehydic compounds including haxanal, heptanal, octanal, nonanal, etc (Yasuhara and Shibamoto, 1995). Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to follow lipid oxidation in a number of foods, including muscle foods (Ross and Smith, 2006). Among all aldehydic compounds, hexanal, heptanal, octanal and nonanal were found as the major aldehydes formed in washed mince. 2-ethylfuran, pentenylfuran, 2-pentyl furan, 1-octen-3-ol, 2-octenal, 2-hexenal and 2,3-octanedione were also found in all samples (Fig. 27). The control sample contained the lower amounts of volatile lipid oxidation products, when compared with the samples added with haemoglobin from different fish species. Among all samples added with haemoglobin, those containing tilapia or grouper haemoglobins displayed the higher formation of volatile oxidation products except octanal and 2,3-octanedione, compared with that added with Asian seabass haemoglobin. Higher formation of volatile lipid oxidation products in all haemoglobin added samples correlated well with the higher formation of TBARS in those samples (Fig. 25). The results suggested that lipid oxidation and decomposition of hydroperoxides to the secondary volatile oxidation products were more pronounced in the haemoglobin added samples, compared with the control sample. The formation of aldehydes and other volatile oxidation products, thus can produce off-odours (Boyd *et al.,* 1992). Aldehydes have been used as the indicators of lipid oxidation because they possess low threshold values and are the major contributors to the development of off-flavour and odour (Ross and Smith, 2006; Boyd *et al.,* 1992). Propanal and heptanal can serve as a reliable indicator of flavour deterioration for fish products, whilst hexanal contributes to the rancidity in meats (Augustin *et al.,* 2006; Ross and Smith, 2006).

The peak area of 2-ethylfuran, pentenylfuran and 2-pentyl furan was greater in all samples added with haemoglobin, especially in that added with tilapia haemoglobin, compared with the control sample. Furan and its related compounds are formed by the decomposition of 12-hydroperoxide of linolenate (18:3n-3), the 14-hydroperoxide of eicosapentaenoate (20:5n-3) and the 16-hydroperoxide of docosahexaenoate (22:6n-3). Those hydroperoxide can undergo ß-cleavage to produce a conjugated diene radical, which can react with oxygen to produce a vinyl hydroperoxide. Cleavage of the vinyl hydroperoxide by loss of a hydroxyl radical, forms an alkoxyl radical, that undergoes cyclisation, thereby producing furans (Medina *et al.,* 1999).

High peak area of 1-octen-3-ol was found in washed mince added with tilapia or grouper haemoglobins, compared with that of the control samples and the samples containing Asian seabass haemoglobin. Alcohols are known as the secondary products produced by the decomposition of hydroperoxide (Girand and Durance, 2000). 8-Carbon alcohols are known to be present in all species of fish (Josephson *et al.,* 1984). Thus, the haemoglobins from different fish species were able to catalyse the lipid oxidation in washed mince intensively, but their pro-oxidative acitivity varied with fish species. The subsequent decomposition of primary lipid oxidation products led to the formation of a wide range of volatile compounds, which more likely contributed to unacceptable offensive odours or rancidity in the muscle of fish without prior bleeding.

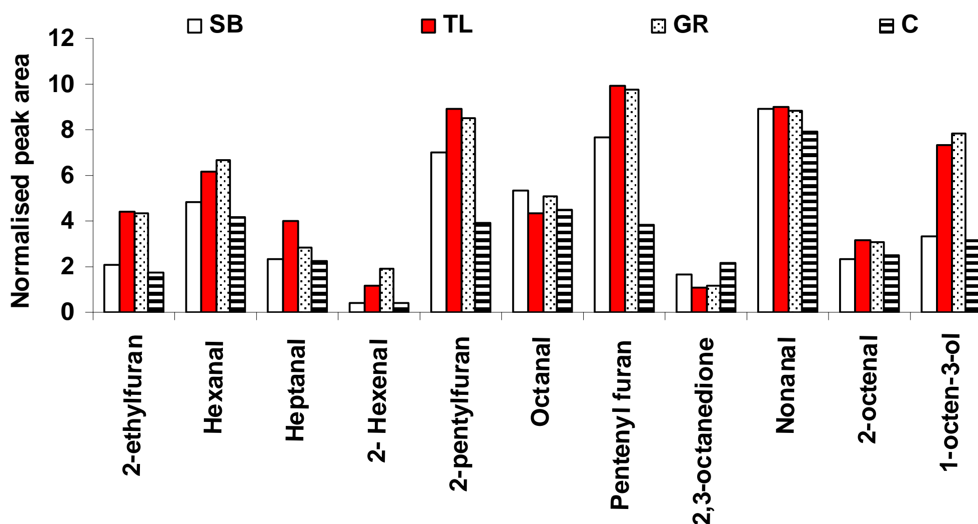


Figure 27. Normalised peak area of the secondary lipid oxidation compounds identified by SPME-GCMS technique in washed mince added with 6 μM haemoglobin from Asian seabass, tilapia or grouper at pH 6 stored in ice for 10 days. Key: see Figure 24 caption.

5.5 Conclusion

Stability to oxygenation, autoxidation and pro-oxidative activity of haemoglobins from different tropical fish species in the washed mince was highly influenced by pH. Among all haemoglobins tested, those from tilapia and grouper were more active in promoting lipid oxidation and fishy odour development in washed mince, especially at acidic pH condition. Thus, the haemoglobins in the blood could be involved in lipid oxidation and fishy odour development of fish muscle during the extended postmortem storage. Those changes were species specific, mainly governed by the pro-oxidative activity of haemoglobins.

CHAPTER 6

RETARDATION OF HAEMOGLOBIN-MEDIATED LIPID OXIDATION OF ASIAN SEABASS MUSCLE BY TANNIC ACID DURING ICED STORAGE

6.1 Abstract

Lipid oxidation mediated by haemoglobin from tilapia was monitored in washed Asian seabass mince added without and with tannic acid (200 and 400 ppm) during 10 days of iced storage. Control samples (without tannic acid) had the highest peroxide value (PV) within the first 2 days and possessed the greater amount of thiobarbituric acid-reactive substances (TBARS) throughout the storage of 10 days ($P < 0.05$). With addition of tannic acid, the lipid oxidation of washed mince was retarded, especially when the higher level (400 ppm) was used, as evidenced by lowered PV and TBARS. The retarded formation of volatile lipid oxidation products in the samples added with 400 ppm tannic acid was found. Sensory analysis revealed that samples added with 400 ppm tannic acid showed the lower fishy odour score, compared with the control sample and that added with 200 ppm tannic acid ($P < 0.05$).

6.2 Introduction

Quality deterioration in muscle foods caused by lipid oxidation is a major concern in the food industry. Odour, flavour, texture, colour and nutritional value are negatively affected by lipid oxidation (Kanner, 1994). Haem proteins (haemoglobin and myoglobin), haem-derived transition metals and enzymes in muscle foods are associated with the accelerated lipid oxidation (Decker and Hultin, 1992). In fish, haemoglobin has been identified as one of the most potent pro-oxidants and can initiate oxidation following several mechanisms (Richards and Hultin, 2002). Haemoglobin can be a source of activated oxygen due to haemoglobin autoxidation. Haem or iron can be released from the protein to promote lipid oxidation (Richards

and Hultin, 2002). The iron atom in the haem ring of the haem proteins is primarily in the ferrous (+2) state. Conversion of ferrous haem protein to met (+3) haem protein (metHP) is a process known as autoxidation (Richards and Dettmann, 2003). MetHP reacts with peroxides, leading to the stimulated formation of compounds capable of initiating and propagating lipid oxidation (Everse and Hsia, 1997).

Whole fish are commonly supplied in the market due to the ease of distribution or transportation. For fillet production, whole fish without bleeding have been used widely in fish processing industry, retailed shops or restaurants. Blood, which is an excellent source of haem protein, plays a role in acceleration of lipid oxidation of whole fish, fillets as well as other fish muscle products. In postmortem fish, haemoglobin can react with the muscle lipids and enhance lipid oxidation (Richards *et al.,* 1998). Therefore, the inhibition of haemoglobin mediated lipid oxidation in fish muscle by antioxidant, especially natural phenolic compound, can be a promising means to prevent the onset of lipid oxidation. Recently, Maqsood and Benjakul (2010 a, 2010 b) reported that tannic acid exhibited the superior radical scavenging activities as well as reducing power and effectively inhibited the lipid oxidation in fish mince, fish oil-in-water emulsion and fish slices. Tannic acid is also affirmed as Generally Recognised As Safe (GRAS) by the Food and Drug Administration (FDA) at a level of 10 - 400 ppm for the use as an ingredient in some food products including meat products (US Code of Federal Regulation, 2006; Chung *et al.,* 1993). Nevertheless, the inhibitory activity of tannic acid towards lipid oxidation mediated by haemoglobin in fish muscle has not been elucidated. Thus, the objective of this study was to investigate the impact of tannic acid on the retardation of haemoglobin mediated lipid oxidation in washed Asian seabass mince during iced storage.

6.3 Materials and methods

6.3.1 Chemicals

Tannic acid, cumene hydroperoxide, streptomycin sulphate, sodium heparin, pentanal, hexanal, heptanal, octanal and nonanal were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, potassium iodide, tris [hydroxy-methyl] aminomethane (Tris) and trichloroacetic acid were obtained from Merck (Damstadt, Germany). Disodium hydrogen phosphate, 2-thiobarbituric acid, ammonium thiocyanate and ferrous chloride were purchased from Fluka Chemical Co. (Buchs, Switzerland). All chemicals used were of analytical grade.

6.3.2 Fish supply and bleeding

Live tilapia (*Oreochromis niloticus*) was purchased from a fish farm, Songkhla, Thailand. Fish were transported in live condition to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, the fishes were bled from the caudal vein after the tail of the fish was cut off. Blood was collected in a 20 mL-glass test tube rinsed with 150 mM NaCl solution containing sodium heparin (30 U/mL). The collected blood was stirred continuously in order to avoid the coagulation.

6.3.3 Preparation of haemolysate

Haemolysate from tilapia, which showed the highest pro-oxidative activity, compared with other species including Asian seabass and grouper (Maqsood and Benjakul, 2011a), was prepared according to the method of Richards and Hultin (2000). Four volumes of cold 1 mM Tris buffer (pH 8.0) containing 1.7 % NaCl were added to heparinised blood. Thereafter, the mixture was centrifuged at 700 x *g* for 10 min at 4 °C using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA). Plasma was then removed. Red blood cells were washed by suspending three times in 10 volumes of the above buffer and centrifuging at 700 x *g*. Cells were lysed in 3 volumes of 1 mM Tris buffer (pH 8.0) for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before centrifugation at 28000 x *g* for 15 min at 4 °C. Prepared haemolysate was stored at -40 °C and was thawed just before used.

6.3.4 Quantification of haemoglobin

Haemoglobin content was determined following the method described by Richards and Hultin (2000) with a slight modification. The haemolysate was diluted in 50 mM Tris buffer (pH 8.0) to obtain the absorbance of 0.5-0.6 at 415 nm. Approximately 1 mg of sodium dithionite was added to the sample (2 ml) and mixed thoroughly. The sample was bubbled with oxygen gas with a purity of 99.5 % to 100 % (TTS Gas Agency, Hat Yai, Songkhla, Thailand) for 10 min. Prepared sample (2 ml) was transferred to a 3 ml disposable cuvette. The absorbance of the sample was then measured at 415 nm against a blank, which contained only buffer, using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The haemoglobin concentration was calculated by Lambert-Beer's law using a millimolar extinction coefficient of 125 for oxyhaemoglobin at pH 8 (Antoni and Brunoni, 1971).

6.3.5 Preparation of washed Asian seabass mince

Washed mince was prepared as per the method of Richards and Hultin (2000). Live fresh Asian seabass (*Lates calcarifer*), a lean fish, from a fish farm in Songkhla, Thailand, with an average weight of 1-1.2 kg were caught and ice-shocked. The fish were kept in ice and then transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, fish were washed with chilled tap water, descaled, filleted, de-skinned and minced using a mincer with a hole diameter of 5 mm. The mince was washed twice in cold distilled water at a mince-to-water ratio of 1:3 (w/v). The mixture was stirred with a plastic rod for 2 min. Thereafter, the mixture was allowed to stand for 15 min in a cold room (4 ºC), followed by dewatering on a fiberglass screen. Resulting washed mince was mixed with 50 mM sodium phosphate buffer (pH 6.0) at a ratio of 1:3 (w/v) and homogenised using an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany) at a speed of 13,500 rpm for 1 min. The final pH was monitored by using a pH-meter (Sartorious North America, Edgewood, NY, USA) and was adjusted to 6 using 6 M HCl. The mixture was allowed to stand for 15 min at 4 ºC and then centrifuged at 15000 x *g* for 20 min at 4 °C. The resulting pellet was referred to as "washed mince"

and used as the composite sample. The final moisture content of the washed mince was 88 %.

6.3.6 Preparation of washed Asian seabass mince containing haemoglobin and tannic acid

Washed Asian seabass mince (pH 6) was divided into four portions. Streptomycin sulphate, as an antimicrobial agent, was added in washed Asian seabass mince to obtain a final concentration of 200 ppm. Haemolysate (8.35 mL) was added to three portions of washed Asian seabass mince (225 g each) to obtain a final concentration of 6 µM of Hb/kg washed mince. For the last portion, the same volume of distilled water was added instead of haemolysate and used as the control (C). The mixtures were then mixed manually until the homogeneity was obtained.

Tannic acid was dissolved in 25 mL of sterilized distilled water and was adjusted to pH 7 by 2 M NaOH. Neutralised tannic acid solution (25 mL) was added separately to two portions of washed Asian seabass mince (225 g each) to obtain the final concentration of 200 and 400 ppm, designated as TA200 and TA400, respectively. The sample added with haemolysate without addition of tannic acid was referred to as the "the control containing haemolysate (CH)". All prepared samples (C, CH, TA200 and TA400) were packed in the separate polyethylene bags and sealed. The samples were stored in dark inside the insulated polystyrene box filled with ice at a sample/ice ratio of 1:2 (w/w). Molten ice was removed every day and the same quantity of ice was replaced. For each samples, three sample bags were randomly taken at each sampling day. Samples were taken for peroxide value (PV) and thiobarbituric acid-reactive substances (TBARS) determination every 2 days for totally 10 days, whilst the evaluation of fishy odour was carried out at Day 0, 2, 6 and 10. Additionally, volatile lipid oxidation compounds in the samples were analysed at the end of storage (day 10).

6.3.7 Analyses

6.3.7.1 Determination of peroxide value

Peroxide value (PV) was determined as per the method of Richards and Hultin (2002) with a slight modification. Analysis was performed in triplicate. Washed mince (1 g) was mixed with 11 mL of chloroform/methanol (2:1, v/v). The mixture was homogenised at a speed of 13,500 rpm for 2 min. Homogenate was then filtered using Whatman No. 1 filter paper (Whatman International, Ltd, Maidstone, England). Two millilitre of 0.5 % NaCl was then added to 7 mL of the filtrate. The mixture was vortexed at a moderate speed for 30 s using a Vortex-Genie2 mixer 4 (Bohemia, NY, USA) and then centrifuged at 3000*g* for 3 min to separate the sample into two phases. Two millilitre of cold chloroform/methanol (2:1, v/v), was added to 3 mL of the lower phase. Twenty-five microlitre of 30 % ammonium thiocyanate and 25 µL of 20 mM iron (II) chloride were added to the mixture (Shantha and Decker, 1994). Reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A standard curve was prepared using cumene hydroperoxide at the concentration range of 0.5-2 ppm.

6.3.7.2 Determination of thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Analysis was performed in triplicate. Washed mince (0.5 g) was mixed with 2.5 mL of a TBA solution containing 0.375 % thiobarbituric acid, 15 % trichloroacetic acid and 0.25 N HCl. The mixture was heated in a boiling water for 10 min to develop a pink colour, cooled with running tap water and then sonicated for 30 min using an Elma (S 30 H) sonicator (Kolpingstr, Singen, Germany). The mixture was then centrifuged at 5000*g* at 25 °C for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (MAD) at the concentration ranging from 0 to 10 ppm and TBARS were expressed as mg of MAD equivalents/kg sample.

6.3.7.3 Determination of volatile lipid oxidation compounds

Volatile lipid oxidation compounds in washed Asian seabass mince containing haemolysate without (CH) and with 400 ppm tannic acid (TA400) stored in ice for 10 days were determined by solid phase micro extraction/gas chromatography-mass spectrometry (SPME/GC-MS) (Iglesias and Medina, 2008). The analysis was run in triplicate.

6.3.7.3.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 3 g of sample were homogenised at a speed of 13,500 rpm for 2 min with 8 mL of ultra-pure water. The mixture was centrifuged at 2000 x g for 10 min at 4 ºC. The supernatant (6 mL) was heated at 60 ºC with equilibrium time of 10 h in a 20 mL-headspace vial. Finally, the SPME fibre (50/30 µm DVB/Carboxen^{TM}/PDMS StableFlex ^{TM}) (Supelco, Bellefonte, PA, USA) was exposed to the head space of the vial containing the sample extract and the volatile compounds were allowed to absorb in the SPME fibre for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC. Extraction was carried out in triplicate.

6.3.7.3.2 GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatography coupled with HP 5972 mass selective detectors equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m x 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC and finally an increase of 15 ºC/min to a final temperature of 250 ºC and hold for 10 min. Helium was employed as a carrier gas, with a constant flow of 1.0 mL/min. Injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 250 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 145

ºC. Initially, a full scan mode data was acquired to determine appropriate masses for the later acquisition in selected ion monitoring (SIM) mode under the following conditions: mass range: 25-500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionisation energy of 70 eV, filament emission current at 150 µA and the electron multiplier voltage at 500 V. Analysis was run in triplicate.

6.3.7.3.3 Analyses of volatile compounds

Identification of the volatile compounds in the samples was based on the retention times of individual aldehydic standards including pentanal, hexanal, heptanal, octanal and nonanal. Identification of the compounds was also done by consulting ChemStation Library Search (Wiley 275.L) using Probability-Based Matching (PBM) algorithm developed by Prof. Fred McLafferty and co-workers at Cornell University. Quantitative determination was carried out by using an internal calibration curve that was built using stock solutions of the compounds in ultra-pure water saturated in salt and analysing them by the optimised HS-SPME method. Quantification limits were calculated to a single-to-noise (S/N) ratio of 10. The identified volatile compounds related with lipid oxidation were presented in the form of abundance of each identified compound.

6.3.4 Sensory analysis

The sensory evaluation was performed by eight trained panelists, who were the graduate students in Food Science and Technology programme with the age of 25-33 years and were familiar with fish consumption. Panelists were trained in two sessions using a 10 point scale, where 0 represented no fishy odour and 10 represented the strongest fishy odour. Reference samples were prepared by storing Asian seabass slices packed in polythene bags in ice for 0, 5, 10 and 15 days representing the score of 0, 3, 7 and 10, respectively. To test the samples, panellists were asked to open the sealable polyethylene bags containing the test samples and sniff the headspace above the samples. The samples were then scored.

6.3.5 Statistical analysis

All experiments were run in triplicate. Completely randomized design (CRD) was used for this study. The experimental data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's New Multiple Range Test. For pair comparison, *t*-test was used (Steel and Torrie, 1980). Data analysis was performed using a SPSS package (SPSS 14.0 for Windows, SPSS Inc, Chicago, IL, USA).

6.4 Results and discussions

6.4.1 Effect of tannic acid on the lipid oxidation mediated by haemoglobin in washed Asian seabass mince

Changes in PV and TBARS in washed Asian seabass mince added without haemoglobin (C) and those containing haemoglobin and treated without (CH) and with tannic acid (200 and 400 ppm) (TA200 and TA400) during 10 days of iced storage are shown in Figure 28 and 29, respectively. Gradual increase in PV was observed in all samples up to 6 days of iced storage ($P<0.05$). Thereafter, PV remained unchanged up to 10 days ($P>0.05$). The CH sample had the highest increase in PV within the first 2 days of storage and the continuous decrease was observed thereafter ($P<0.05$). The decrease in PV of CH sample with increasing storage time was most likely caused by decomposition of hydroperoxide formed into the secondary oxidation products (Boselli *et al.,* 2005). When comparing PV of all samples containing haemoglobin, it was found that CH sample contained the highest PV up to day 4 of storage, while TA400 sample contained the lowest PV up to day 8 of storage ($P<0.05$). The results showed that tannic acid at a level of 400 ppm was very effective in retarding the propagation stage of lipid oxidation. Tannic acid showed the radical scavenging activity via hydrogen donating and reducing power, thereby terminating the propagation (Maqsood and Benjakul, 2010a). At day 10, hydroperoxide formed in CH sample more likely underwent decomposition to a higher rate, than the rate of formation. As a result, the lowest PV was obtained in CH sample. Maqsood and Benjakul (2010b) reported that higher level (200 ppm) of tannic acid was more

effective than the lower level (100 ppm) on the retardation of lipid oxidation in striped catfish slices stored under modified atmosphere packaging (MAP, 60%N_2 / 35%CO_2 / 5%O_2). For the C sample, PV gradually increased during the storage. Although washing could remove most of the haem protein and lipids, there was small percentage of membrane lipids such as phospholipids that were difficult to remove by washing (Lanier *et al.,* 2005). Those lipids contain high level of polyunsaturated fatty acids which could be autoxidised, particularly when storage time increased (Lanier *et al.,* 2005). The white muscle of a typical lean fish contains less than 1 % lipids. Of this, the phospholipids make up about 90 % (Ackman, 1980). Some haem protein might be bound with muscle protein and could not be leached out during washing. Those proteins might exhibit pro-oxidative activity in washed mince.

TBARS values of all samples added with haemoglobin increased gradually up to 8 days of storage (P<0.05). No change in TBARS of TA200 and TA400 samples were found during 8-10 days of storage (P>0.05). TBARS values of CH sample increased abruptly at day 2 of storage (P<0.05). Thereafter, the slight decrease in TBARS values was noticed. The decrease in TBARS was probably due to their reaction with free amino acids, proteins and peptides present in the washed Asian seabass mince system to form Schiff's base (Dillard and Tappel, 1973). Furthermore, volatile oxidation products with low molecular weight could be lost during extended storage.

When tannic acid was incorporated in washed mince added with haemoglobin, tannic acid, especially at a level of 400 ppm displayed the higher efficacy in prevention of lipid oxidation as indicated by the lowered TBARS formation, when compared with the samples added with 200 ppm tannic acid (P<0.05). Apart from acting as a radical scavenger, tannic acid, especially at a level of 400 ppm could chelate iron, which might be released from the haemoglobin during the storage.

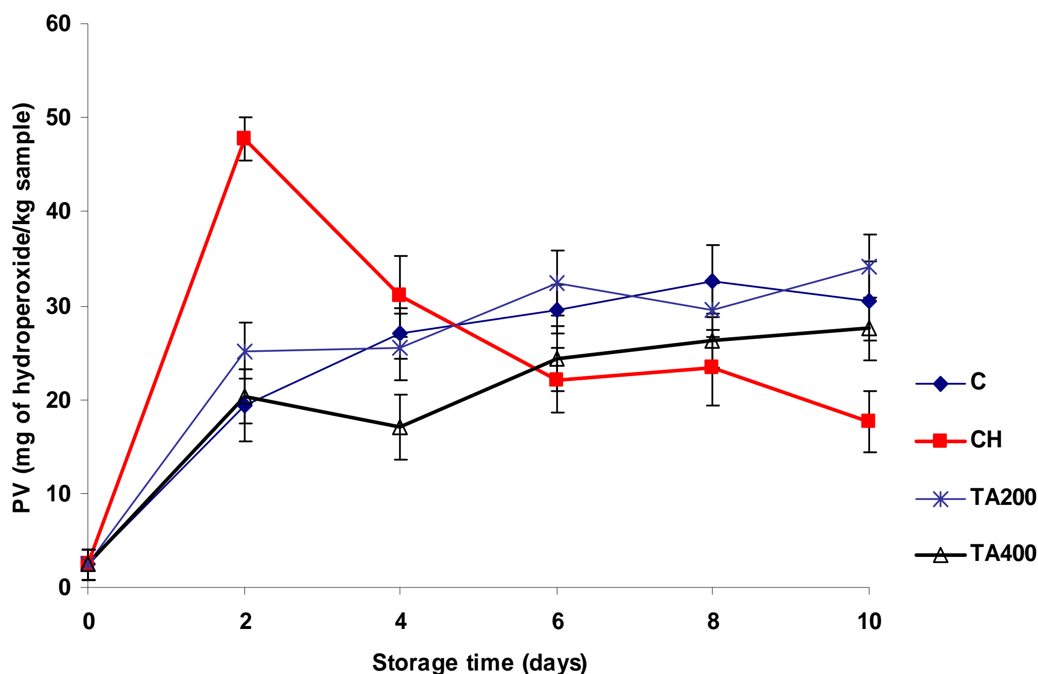


Figure 28. Changes in peroxide value (PV) in washed Asian seabass mince and those containing haemoglobin from tilapia and treated without and with tannic acid (200 and 400 ppm) during iced storage. Bars represents the standard deviation (n=3). C: control washed mince without haemoglobin and tannic acid; CH: washed mince added with haemoglobin (6 µM/kg); TA200: washed mince added with haemoglobin (6 µM/kg) and 200 ppm tannic acid; TA400: washed mince added with haemoglobin (6 µM/kg) and 400 ppm tannic acid.

Tannic acid has the ability to chelate iron, particularly in the free form (Lopes *et al.,* 1999). Tannic acid chelates iron due to its ten galloyl groups and might also be able to inhibit iron-mediated oxyradical formation like other iron chelators, such as desferrioxamine (DFO), 1,10-phenanthroline and pyridoxal isonicotinoyl hydrazone (PIH) (Lopes *et al.,* 1999). Lipid oxidation in the washed mince could be pronounced at acidic pH (pH 6) because of the enhanced autoxidation of haemoglobin at reduced pH (Tsuruga *et al.,* 1998). Haemoglobin autoxidation results in the formation of oxygen radicals ($O_2^{\bullet -}$, $OH^{\bullet}$) (Shikama, 1998), protein radicals (e.g., hypervalent ferryl-haemoglobin (Fe^{4+}==O)) and lipid radicals ($LOO^{\bullet}$ and $LO^{\bullet}$) (Ryter

and Tyrrell, 2000). Methaemoglobin formed can release its haem group 60 times greater than oxy- and deoxyhaemoglobin (Hardgrove *et al.,* 1997). Recently, Maqsood and Benjakul (2010a) reported that tannic acid showed the higher ferric reducing antioxidant power (FRAP), indicating that tannic acid could easily donate the electron to Fe (III), thus reducing it to Fe (II). Lopes *et al.* (1999) and Andrade Jr *et al.* (2006) also reported that tannic acid was able to reduce Fe (III) to Fe (II). Thus, the addition of tannic acid could retard the formation of methaemoglobin. As a result, the release of free haem group was impeded. Additionally, the ability in donating electrons to the radical was another mode of action of tannic acid, termed as "reducing power". Reducing capacity of phenolic antioxidants was realised as a key function for retarding and inhibiting lipid oxidation of fish tissues (Medina *et al.,* 2007). Large-molecular weight polyphenols, such as tannic acid are strong radical scavengers (Yoshida *et al.,* 1989).

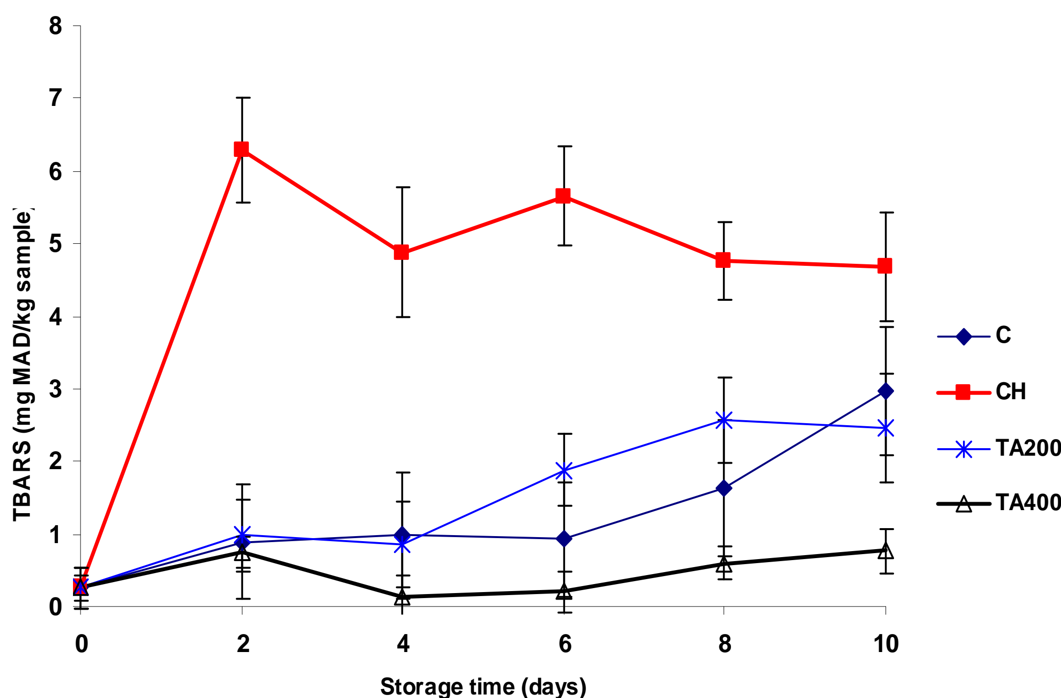


Figure 29. Changes in thiobarbituric acid reactive substance (TBARS) in washed Asian seabass mince and those containing haemoglobin from tilapia and treated without and with tannic acid (200 and 400 ppm) during iced storage. Bars represents the standard deviation (n=3). Key: see Figure 28 caption.

The tannins were 15-30 times more effective in quenching peroxyl radicals than simple phenolics or Trolox, when tested in deoxyribose assay and metmyoglobin assay (Hagerman *et al.,* 1998). The samples treated with tannic acid especially at 400 ppm level showed the lower formation of PV and TBARS, suggesting its capacity to scavange radicals formed in the propagation stage. For C sample, autoxidation took place gradually during the storage of 10 days. This was in agreement with the increase in PV (Fig. 28). Thus, tannic acid at a level of 400 ppm was very effective in retardation of lipid oxidation catalysed by haemoglobin from tilapia in washed Asian seabass mince during iced storage.

6.4.2 Effect of tannic acid on fishy odour development in washed Asian seabass mince added with haemoglobin

Fishy odour intensity in the control washed Asian seabass mince (C) and those containing haemoglobin from tilapia and treated without (CH) and with tannic acid (200 and 400 ppm) (TA200 and TA400) during iced storage is shown in Figure 30. Within the first 2 days of storage, fishy odour intensity was increased markedly in CH samples, whereas a slight increase was obtained in C, TA200 and TA400 samples. During 2-10 days of storage, fishy odour intensity increased in all samples, except CH sample, which showed no change in fishy odour intensity during 6-10 days of storage ($P>0.05$). CH sample had the higher intensity of fishy odour than other samples, indicating the role of haemoglobin in development of fishy odour, mainly via induction of lipid oxidation. The highest fishy odour intensity in CH sample was related with the highest rate of lipid oxidation (Fig. 28 and 29). Thus, tannic acid at a level of 400 ppm was very effective in retarding the formation of secondary lipid oxidation products (Fig. 29) as well as development of fishy odour in washed Asian seabass mince. The off-odour developed in the fish muscle due to lipid oxidation was considered as fishy (Fu *et al.,* 2008).

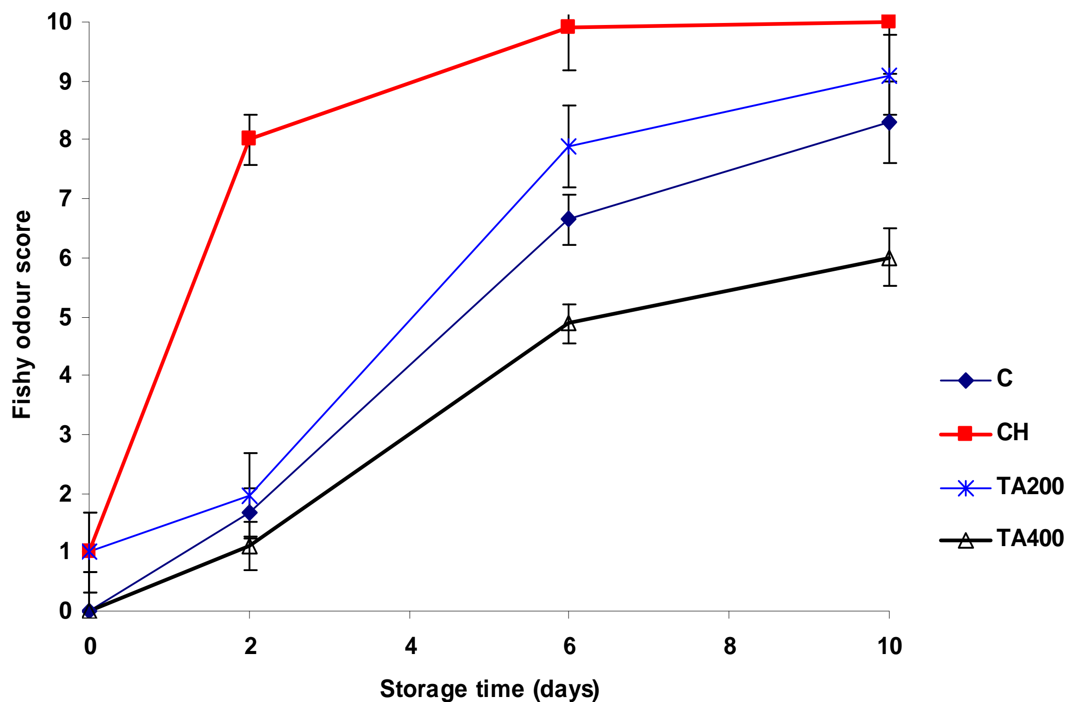


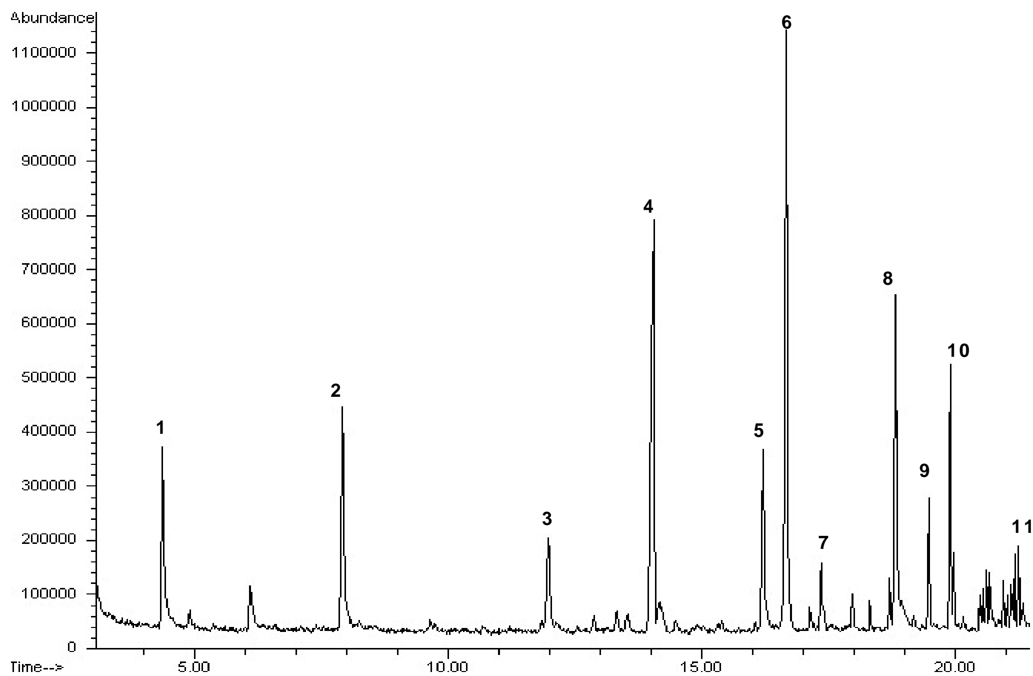
Figure 30. Changes in fishy odour development in washed Asian seabass mince and those containing haemoglobin from tilapia and treated without and with tannic acid (200 and 400 ppm) during iced storage. Bars represents the standard deviation (n=8). Key: see Figure 28 caption.

Tannic acid at a level of 200 ppm was effective in retarding lipid oxidation and off-odour development in ground and cooked fish (*Scomberomorus commersoni*) stored at 4 ºC (Ramanathan and Das, 1992). Recently, Maqsood and Benjakul (2010a; 2010b and 2010c) reported that tannic acid at a level of 200 ppm retarded the lipid oxidation and development of off-odour in the striped catfish slice and ground beef stored under modified atmospheric packaging during refrigerated storage. Therefore, tannic acid was proved to be effective in retarding the development of fishy odour in washed Asian seabass mince added with haemoglobin, simulating the whole muscle from unbled fish during iced storage.

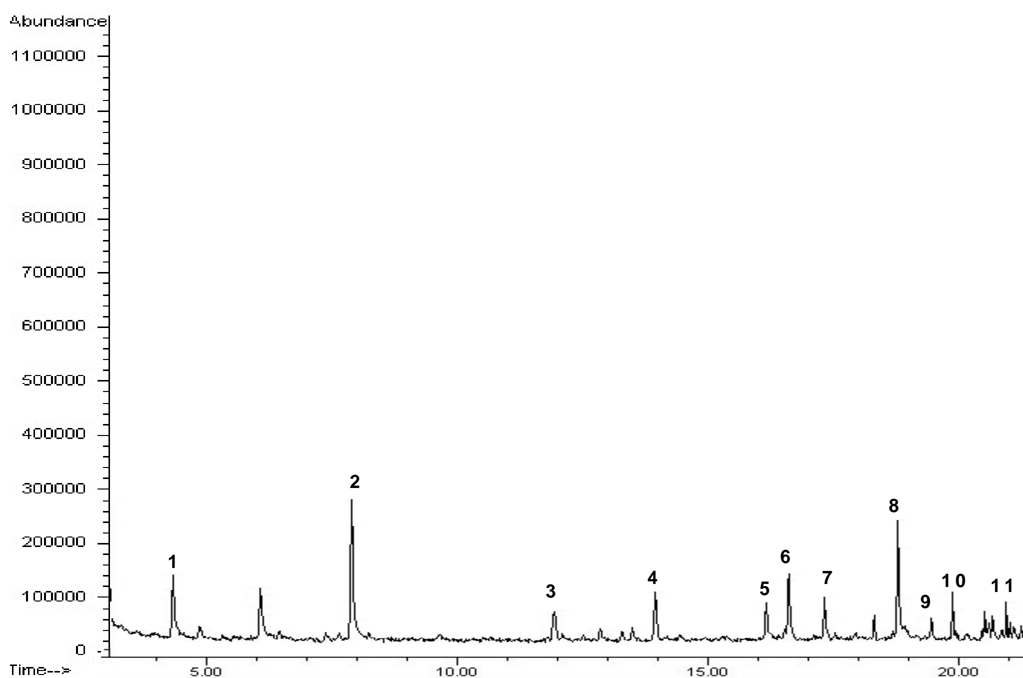
6.4.3 Effect of tannic acid on development of volatile lipid oxidation compounds in washed Asian seabass mince added with haemoglobin

Chromatograms and abundance of different volatile lipid oxidation compounds in the control washed Asian seabass mince added with haemoglobin (CH) and sample added with haemoglobin and treated with 400 ppm tannic acid (TA400) after 10 days of iced storage are shown in Figure 31 and 32, respectively. Lipid oxidation of fish muscle is known to produce volatile aldehydic compounds including haxanal, heptanal, octanal, nonanal, etc (Yasuhara and Shibamoto, 1995). Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to monitor lipid oxidation in a number of foods, including muscle foods (Ross and Smith, 2006). The CH sample contained the higher abundance of all volatile compounds except for 2,3-octanedione, compared with TA400 sample after 10 days of storage in ice. Among aldehydes, heptanal, hexanal, octanal, nonanal and 2-octenal were found at higher amounts in CH sample, compared with TA400 sample. Heptanal can serve as a reliable indicator of flavour deterioration for fish products, while hexanal contributes to the rancidity in meats (Augustin *et al.,* 2006; Ross and Smith, 2006). Higher formation of volatile lipid oxidation products in the CH sample correlated well with the higher formation of TBARS (Fig. 29). Aldehydes generated from the oxidation of fatty acids are frequently related with the off-odours (McGill *et al.,* 1974). The results suggested that lipid oxidation and decomposition of hydroperoxides to the secondary volatile lipid oxidation products were more pronounced in CH sample, compared with TA400 sample.

Furans and its related compounds were also found at high amounts in CH sample, compared with those of TA400 sample. Those compounds are formed by the decomposition of 12-hydroperoxide of linolenate (18:3n-3), the 14-hydroperoxide of eicosapentaenoate (20:5n-3) and the 16-hydroperoxide of docosahexaenoate (22:6n-3). Hydroperoxide can undergo ß-cleavage to produce a conjugated diene radical, which can react with oxygen to produce a vinyl hydroperoxide. Cleavage of the vinyl hydroperoxide by loss of a hydroxyl radical, forms an alkoxyl radical, that undergoes cyclisation, thereby producing furans (Medina *et al.,* 1999).



a)



b)

Figure 31. Chromatograms obtained from GCMS-SPME of different volatile lipid oxidation compounds in the control washed Asian seabass mince added with haemoglobin (CH) (a) and sample added with haemoglobin and treated with 400 ppm tannic acid (TA400) (b) after 10 days of iced storage.

Among alcohols, 1-octen-3-ol was detected in both samples. The higher amount was found in CH sample, compared with TA400 sample. Alcohols are the secondary products produced by the decomposition of hydroperoxide (Girand and Durance, 2000). 8-Carbon alcohols are present in all species of fish (Josephson *et al.,* 1986). Ketone is another secondary lipid oxidation product generated from the decomposition of hydroperoxide in the chilled fish muscle (Iglesias and Medina, 2008). Among ketones, 2,3-octanedione, pentachloro-2-propanone and methyl nonyl ketone were found in both samples with the higher abundance in CH sample. In general, the lower amount of volatile lipid oxidation compounds were detected in the TA400 sample, compared with the CH sample. The result confirmed that tannic acid at a level of 400 ppm was very effective in retarding the lipid oxidation, thereby preventing the formation of volatile lipid oxidation compounds, which contribute to the offensive fishy odours in fish muscle.

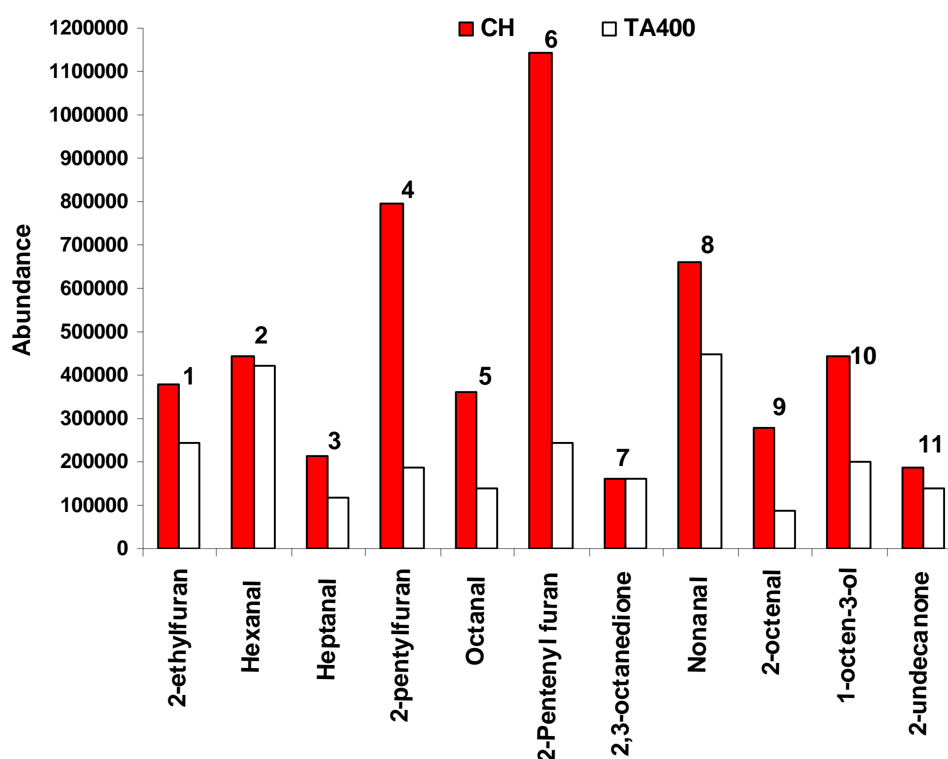


Figure 32. Abundance of different volatile lipid oxidation compounds in the control washed Asian seabass mince added with haemoglobin (CH) and sample added with haemoglobin and treated with 400 ppm tannic acid (TA400) after 10 days of iced storage.

6.5 Conclusion

Haemoglobin from tilapia was effective in catalysing lipid oxidation in washed Asian seabass mince contributing to the development of fishy odour. When tannic acid especially at a level of 400 ppm was incorporated in washed mince added with haemoglobin, it displayed the higher efficacy in prevention of lipid oxidation as indicated by the lower TBARS formation as well as retarded development of fishy odour in washed Asian seabass mince, when compared with the samples added with 200 ppm tannic acid. Additionally, preventive effect of tannic acid at a level of 400 ppm was also reflected by the lower abundance of volatile lipid oxidation products formed in washed Asian seabass mince added with haemoglobin. Thus, application of tannic acid could be a promising means to retard the lipid oxidation and fishy odour development in the fish muscle without prior bleeding.

CHAPTER 7

EFFECT OF KIAM (*COTYLELOBIUM LANCEOTATUM CRAIH*) WOOD EXTRACT ON THE HAEMOGLOBIN MEDIATED LIPID OXIDATION OF ASIAN SEABASS MUSCLE

7.1 Abstract

Effect of ethanolic kiam wood extract (EKWE) on the retardation of haemoglobin mediated lipid oxidation of washed Asian seabass mince added without and with menhaden oil stored in ice up to 10 days was investigated. Samples containing haemoglobin have the highest peroxide value (PV) within the first 8 days and possessed the greater amount of thiobarbituric acid-reactive substances (TBARS) throughout the storage of 10 days, compared with those containing no haemoglobin ($P<0.05$), regardless of 5% menhaden oil addition. Incorporation of 5% menhaden oil to the washed mince had no impact on the formation of PV and TBARS, compared with oil-free samples during the storage ($P>0.05$). With addition of EKWE (0.05 and 0.1%), the lipid oxidation of washed mince added with haemoglobin and menhaden oil was retarded, especially when the higher level (0.1%) was used, as evidenced by lowered PV and TBARS. Formation of volatile lipid oxidation compounds determined by gas chromatography–mass spectrometry (GC-MS) was retarded in the sample containing oil and haemoglobin and treated with 0.1% EKWE, compared with that without the addition of EKWE after 10 day of storage in ice ($P<0.05$). Sensory analysis revealed that samples containing haemoglobin without and with oil added had the highest intensity of fishy odour, compared to those treated with EKWE (0.05% and 0.1%) and the control sample (washed mince) ($P<0.05$). Thus, EKWE, especially at a level of 0.1% could serve as potential natural antioxidant in prevention of lipid oxidation and retardation of development of fishy odour and volatile lipid oxidation compounds in washed mince during iced storage.

7.2 Introduction

Lipid oxidation negatively affects flavour, odour, colour, texture, and the nutritional value of fish muscle seafood during post-mortem storage. Due to the presence of highly unsaturated fatty acids, fish is more susceptible to lipid oxidation than other muscle foods such as poultry, pork, beef and lamb (Tichivangana and Morrissey, 1985). Incorporation of antioxidants into fish muscle can effectively retard lipid oxidation. Although synthetic antioxidants have been widely used (Ahmad, 1996), nowadays there is a growing interest to replace such compounds by naturally occurring antioxidants (Shi *et al.,* 2000). Several natural phenolic compounds of green tea, rosemary, and extra virgin olive oil have been proven to be effective in preventing rancidity in many lipid-containing systems including fish muscle (Ikawa, 1998; Medina *et al.,* 1999; Medina *et al.,* 2003).

Haemoglobin has been identified as one of the most potent pro-oxidants and can initiate oxidation in fish following several mechanisms (Richards and Hultin, 2002). Haemoglobin can be a source of activated oxygen due to haemoglobin autoxidation. Haem or iron can be released from the protein to promote lipid oxidation (Richards and Hultin, 2002). The iron atom in haem proteins is primarily in the ferrous (2+) state. Conversion of ferrous haem protein to met (3+) haem protein (metHP) is a process known as autoxidation (Richards and Dettmann, 2003). MetHP reacts with peroxides, leading to the stimulated formation of compounds capable of initiating and propagating lipid oxidation (Everse and Hsia, 1997). In post-mortem fish, haemoglobin can react with the muscle lipids and enhance lipid oxidation (Richards *et al.,* 1998). Therefore, the inhibition of haemoglobin mediated lipid oxidation in fish muscle by antioxidant, especially natural phenolic compound, can be a promising means to prevent the onset of lipid oxidation.

Tannins are polyphenolic compounds commonly occurring in the barks, woods and fruits of many kinds of plants. Extraction of tannins from the bark of different trees was carried out (Yazaki and Collins, 1994). Kiam (*Cotylelobium lanceotatum craih*) trees are very common in the southern part of Thailand. Pieces of wood from the kiam tree have been traditionally submerged in sugar palm sap to

prevent or retard microbial fermentation (Chanthachum and Beuchat, 1997). Additionally, kiam wood is also burned for energy production. The preparation of kiam extract containing phenolic compounds could increase the value of those wood and the novel natural additives can be applied in food industry. Balange and Benjakul (2009) reported that tannic acid (456.3 mg/kg) was found as the major component of the kiam wood extract. Tannic acid exhibited the superior radical scavenging activities as well as reducing power and effectively inhibited the lipid oxidation in fish mince and fish oil-in-water emulsion (Maqsood and Benjakul, 2010a). Haemoglobin mediated lipid oxidation in washed Asain seabass mince was also impeded by incorporation of tannic acid (Maqsood and Benjakul, 2011b). Thus, the objective of present study was to use the natural extract of kiam wood (*C. lanceotatum craih*) in preventing the haemoglobin mediated lipid oxidation and fishy odour development in Asian seabass muscle during iced storage.

7.3 Materials and methods

7.3.1 Chemicals

Menhaden fish oil, cumene hydroperoxide, streptomycin sulphate, sodium heparin, pentanal, hexanal, heptanal, octanal and nonanal were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, sodium bicarbonate, potassium iodide, tris[hydroxy-methyl] aminomethane (Tris) and trichloroacetic acid were obtained from Merck (Damstadt, Germany). Disodium hydrogen phosphate, 2-thiobarbituric acid, ammonium thiocyanate and ferrous chloride were purchased from Fluka Chemical Co. (Buchs, Switzerland). All chemicals used were of analytical grade.

7.3.2 Fish supply and bleeding

Live tilapia (*Oreochromis niloticus*) were purchased from a fish farm, Songkhla, Thailand. Fish were transported in live condition to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, the fish were bled from the caudal vein after the tail of the fish was cut off. Blood was collected in a 20

mL-glass test tube rinsed with 150 mM NaCl solution containing sodium heparin (30 U/mL). The collected blood was stirred continuously in order to avoid the coagulation.

7.3.4 Preparation of haemolysate

Haemolysate from tilapia, which showed the highest pro-oxidative activity, compared with other species including Asian seabass and grouper (Maqsood and Benjakul, 2011a), was prepared according to the method of Richards and Hultin (2000). Four volumes of cold 1 mM Tris buffer (pH 8.0) containing 1.7 % NaCl were added to heparinised blood. Thereafter, the mixture was centrifuged at 700 *g* for 10 min at 4 °C using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA). Plasma was then removed. Red blood cells were washed by suspending three times in 10 volumes of the above buffer and centrifuging at 700 *g* for 10 min at 4 °C. Cells were lysed in 3 volumes of 1 mM Tris buffer (pH 8.0) for 1 h. One-tenth volume of 1 M NaCl was then added to aid in stromal removal before centrifugation at 28000 *g* for 15 min at 4 °C. Prepared haemolysate was stored at -40 °C and was thawed just before used.

7.3.5 Quantification of haemoglobin

Haemoglobin content was determined following the method described by Richards and Hultin (2000) with a slight modification. The haemolysate was diluted in 50 mM Tris buffer (pH 8.0) to obtain the absorbance of 0.5-0.6 at 415 nm. Approximately 1 mg of sodium dithionite was added to the sample (2 mL) and mixed thoroughly. The sample was bubbled with oxygen gas with a purity of 99.5 -100 % (TTS Gas Agency, Hat Yai, Songkhla, Thailand) for 10 min. Prepared sample (2 mL) was transferred to a 3 mL disposable cuvette. The absorbance of the sample was then measured at 415 nm against a blank, which contained only buffer, using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The haemoglobin concentration was calculated by Lambert-Beer's law using a millimolar extinction coefficient of 125 for oxyhaemoglobin at pH 8 (Antoni and Brunoni, 1971).

7.3.6 Preparation of washed Asian seabass mince

Washed mince was prepared as per the method of Richards and Hultin (2000). Live fresh Asian seabass (*Lates calcarifer*), a lean fish, supplied by a fish farm in Songkla, Thailand, with an average weight of 1-1.2 kg were caught and ice-shocked. The fish were kept in ice and then transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, fish were washed with chilled tap water, descaled, filleted, de-skinned and minced using a mincer with a hole diameter of 5 mm. The mince was washed twice in cold distilled water at a mince-to-water ratio of 1:3 (w/v). The mixture was stirred with a plastic rod for 2 min. Thereafter, the mixture was allowed to stand for 15 min in a cold room (4 ºC), followed by dewatering on a fiberglass screen. Resulting washed mince was mixed with 50 mM sodium phosphate buffer (pH 6.0) at a ratio of 1:3 (w/v) and homogenised using an Ultra-Turrax T25 homogeniser (Janke and Kunkel, Staufen, Germany) at a speed of 13,500 rpm for 1 min. The final pH was measured by using a pH-meter (Sartorious North America, Edgewood, NY, USA) and was adjusted to 6 using 6 M HCl. The mixture was allowed to stand for 15 min at 4 ºC and then centrifuged at 15000 x *g* for 20 min at 4 °C. The resulting pellet was referred to as “washed Asian seabass mince” and used as the composite sample. The final moisture content of the washed mince was 88 %.

7.3.7 Preparation of kiam wood extract

7.3.7.1 Collection and preparation of kiam wood

The kiam wood was obtained from the forest of the Phattalung province in the Southern Thailand. The tree was about 15–20 years old and harvested in May, 2010. The tree was cut by using a sawing machine; the leaves and branches were separated manually by cutting and the trunk was kept for sun drying for 3 months. The trunk was chopped into smaller flakes of wood and then dried in an oven at 70 °C for 8 h and cut into pieces with an average size of 1.5 x 1.5 cm^2. Those pieces were ground using a portable grinding machine (Spong-90, Leeds, UK) with a sieve size of 6 mm and was then subjected to size reduction using a blender (National Model MKK77, Tokyo, Japan) and finally sieved using a stainless steel sieve of 80 mesh size. The

obtained powder was placed in a polythene bag, sealed and kept at room temperature until used.

7.3.7.2 Extraction of phenolic compound from kiam wood

Kiam wood powder was subjected to extraction according to the method of Santoso *et al.* (2004) with slight modifications. The powder (10 g) was mixed with 150 mL of absolute ethanol. The mixture was stirred at room temperature (28–30 °C) using a magnetic stirrer (IKA-Werke, Staufen, Germany) for 6 h. The mixture was then centrifuged at 5000 x g for 10 min at room temperature using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA). The supernatant was filtered using Whatman filter paper No. 1 paper (Whatman International, Ltd, Maidstone, England). The filtrate was then evaporated at 40 °C using Eyela rotary evaporator (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan). To remove the residual ethanol, the extract was purged with nitrogen gas. The extract was then dried using a freeze dryer to obtain the dry extract. Dried extract was powdered using a mortar and pestle and was kept in an amber bottle and stored in a dessicator until use. The dried powderised extract was referred to as “ethanolic kiam wood extract; EKWE”.

7.3.7.3 Determination of total phenolic content in EKWE

Quantification of total phenolic content in EKWE was carried out according to the method of Slinkard and Singleton (1977). EKWE was dissolved in 25% ethanol to obtain concentration of 0.5% (w/v). The solution (0.5 mL) was added with 100 µL of Folin–Ciocalteu reagent (two-fold diluted with de-ionized water) and mixed thoroughly. After 3 min, 1.5 mL of 2% sodium carbonate solution was added. The reaction mixture was mixed thoroughly and placed in dark for 40 min and the absorbance was read at 760 nm. The total phenolic content was calculated from the standard curve of tannic acid (0–0.1 mg/mL) and expressed as mg tannic acid per gram dry EKWE after blank substraction. Blank was prepared in the same manner, except that distilled water was used instead of Folin–Ciocalteu reagent.

7.3.7.4 Determination of tannic acid in EKWE

Qualitative analysis of EKWE was performed using an HPLC equipped with VWD detector following the method of Tian *et al.* (2009) with slight modifications. The HPLC system consisted of an Agilent 1100 series HPLC (Alginet, Wilmington, DE, USA), quaternary pump with seal wash option, degasser, solvent, cabinet and preparative autosampler with thermostat equipped with a diode array detector. The separation was performed on a column (Hypersil ODS C18 4.0 - 250 mm, 5 lm, Cole-Parmer, Hanwell, London, UK). HPLC conditions were as follows: mobile phase: 0.4% Formic acid: Acetonitrile (85:15); flow rate: 0.8 mL min^{-1}; temperature: 25 °C. The detection was carried out at 280 nm. The concentration of extracts was 25 mg mL^{-1} and each injection volume was 20 µL. Standard tannic acid was used for peak identification.

7.3.8 Study on the impact of EKWE on lipid oxidation and sensory property of washed Asian seabass mince containing haemoglobin

Washed Asian seabass mince (pH 6) was added with streptomycin sulphate as an antimicrobial agent to obtain a final concentration of 200 ppm. The resulting mince was divided into 2 portions; one portion was added with 5% (w/v) menhaden oil, whilst another portion was added with 5% distilled water. Those portions were referred to as “SB+oil” and “SB”, respectively, representing medium fatty fish and lean fish, respectively. Each portion was further divided into four portions with different treatments. Three portions (77.5 g each) were added with haemolysate (26.8 mL) to obtain a final haemoglobin concentration of 6 µM of Hb/kg sample. One portion without addition of haemolysate was used as control.

To study the effect of EKWE on lipid oxidation of “SB” or “SB+oil” samples containing haemoglobin, EKWE with total phenolic content of 602.6 mg tannic acid equivalent per g and tannic acid content of 545.57 mg/g (Fig. 33) was used. EKWE (0.039 and 0.078 g) was dissolved separately in 24 mL of sterlised distilled water and pH was first adjusted to 11 by 6 M NaOH in order to get completely solubilised solution. Thereafter, the pH was adjusted to 6 by 2 M HCl and volume was

made up to 25mL. Completely dissolved solution of EKWE (25 mL) was added separately to four portions of washed mince (77.5 g each) to obtain the final concentration of 0.05% and 0.1%. Samples without the addition of EKWE were added with 25 mL of distilled water adjusted to pH 6. For washed mince, “SB”, those added with haemoglobin was referred to as “SB+Hb”; those added with haemoglobin and treated with 0.05% and 0.1% EKWE were referred as “SB+Hb+0.05% EKWE” and “SB+Hb+0.1% EKWE”, respectively. For washed mince containing oil, “SB+oil”, those added with haemoglobin were referred to as “SB+Hb+oil” and those treated with 0.05% and 0.1% EKWE were referred to as “SB+Hb+oil+0.05% EKWE” and “SB+Hb+oil+0.1% EKWE”, respectively.

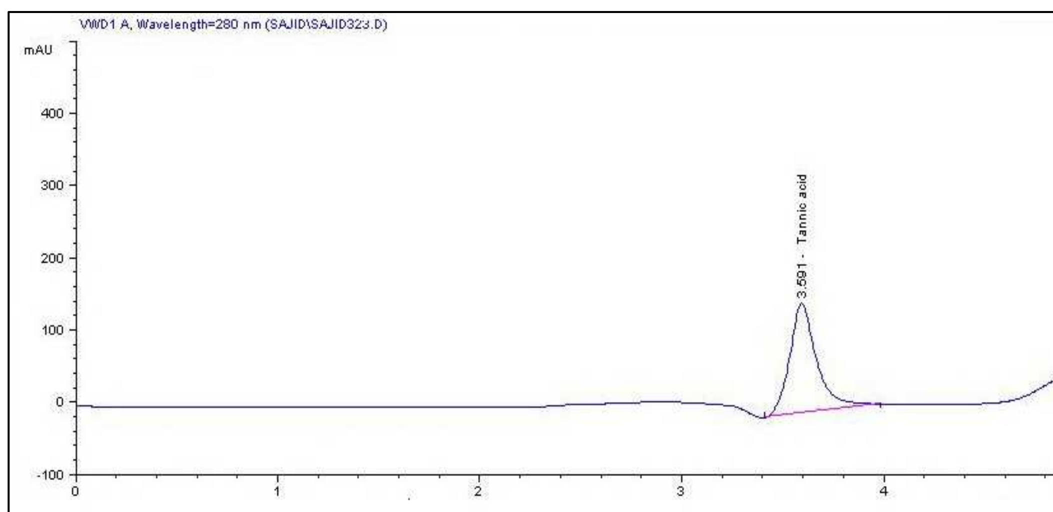


Figure 33. HPLC-DAD chromatogram of ethanolic kiam wood extract (EKWE).

All portions of washed mince were then mixed manually until the homogeneity was obtained. All prepared samples were packed in the separate polyethylene bags and sealed. The samples were stored in dark inside the insulated polystyrene box filled with ice at a sample-to-ice ratio of 1:2 (w/w). Molten ice was removed every day and the same quantity of ice was replaced. For each samples, three sample bags were randomly taken at each sampling day. Samples were taken for peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) determination every 2 days for totally 10 days, whilst the evaluation of fishy odour was carried out at day 0, 2, 6 and 10. Additionally, volatile lipid oxidation compounds in the samples were analysed at the end of storage (day 10).

7.3.9 Analyses

7.3.9.1 Peroxide value

Peroxide value (PV) was determined as per the method of Richards and Hultin (2002) with a slight modification. Analysis was performed in triplicate. Samples (1 g) were mixed with 11 mL of chloroform/methanol (2:1, v/v). The mixtures were homogenised at a speed of 13,500 rpm for 2 min. Homogenates were then filtered using Whatman No. 1 filter. Two millilitres of 0.5 % NaCl were then added to 7 mL of the filtrate. The mixtures were vortexed at a moderate speed for 30 s using a Vortex-Genie2 mixer 4 (Bohemia, NY, USA) and then centrifuged at 3,000 x g for 3 min to separate the sample into two phases. Two millilitres of cold chloroform/methanol (2:1, v/v), were added to 3 mL of the lower phase. Twenty-five microlitres of 30 % ammonium thiocyanate and 25 μL of 20 mM iron (II) chloride were added to the mixture (Shantha and Decker, 1994). Reaction mixtures were allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A standard curve was prepared using cumene hydroperoxide at the concentration range of 0.5-2 ppm.

7.3.9.2 Thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Analysis was performed in triplicate. Washed mince (0.5 g) was mixed with 2.5 mL of a TBA solution containing 0.375 % thiobarbituric acid, 15 % trichloroacetic acid and 0.25 N HCl. The mixture was heated in a boiling water for 10 min to develop a pink colour, cooled with running tap water and then sonicated for 30 min using an Elma (S 30 H) sonicator (Kolpingstr, Singen, Germany). The mixture was then centrifuged at 5,000 × g at 25 ºC for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (MAD) at the concentration ranging from 0 to 10 ppm and TBARS were expressed as mg of MAD equivalents/kg sample.

7.3.9.3 Determination of volatile lipid oxidation compounds

Volatile lipid oxidation compounds in washed Asian seabass mince containing haemoglobin and menhaden oil ("SB+oil+Hb") and that treated with 0.1% EKWE stored in ice for 10 days were determined by solid phase micro extraction-gas chromatography mass spectrometry (SPME-GCMS) (Iglesias and Medina, 2008). The analysis was run in triplicate.

7.3.9.3.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 3 g of sample were homogenised at a speed of 13,500 rpm for 2 min with 8 mL of ultra pure water. The mixtures were centrifuged at 2000 x g for 10 min at 4 ºC. The supernatants (6 mL) were heated at 60 ºC with equilibrium time of 10 h in a 20 mL-headspace vial. Finally, the SPME fibre (50/30 µm DVB/Carboxen^{TM}/PDMS StableFlex ^{TM}) (Supelco, Bellefonte, PA, USA) was exposed to the head space of the vial containing the sample extract and the volatile compounds were allowed to absorb in the SPME fibre for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

7.3.9.3.2 GC-MS analysis

GC-MS analysis was performed in a HP 5890 series II gas chromatography coupled with HP 5972 mass selective detectors equipped with a splitless injector and coupled with a quadrupole mass detector (Hewlett Packard, Atlanta, GA, USA). Compounds were separated on HP-Innowax capillary column (Hewlett Packard, Atlanta, GA, USA) (30 m x 0.25 mm ID, with film thickness of 0.25 µm). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC and finally an increase of 15 ºC/min to a final temperature of 250 ºC and hold for 10 min. Helium was employed as a carrier gas, with a constant flow of 1.0 mL/min. Injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 250 ºC. The quadrupole mass spectrometer was operated in the electron ionisation (EI) mode and source temperature was set at 140 ºC. Initially, a full scan mode data was acquired to determine appropriate masses for

the later acquisition in selected ion monitoring (SIM) mode under the following conditions: mass range: 25-500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionisation energy of 70 eV, filament emission current at 150 μA and the electron multiplier voltage at 500 V.

Identification of the volatile compounds in the samples was based on the retention times of individual aldehydic standards including pentanal, hexanal, heptanal, octanal and nonanal. Identification of the compounds was also done by consulting ChemStation Library Search (Wiley 275.L) using Probability-Based Matching (PBM) algorithm developed by Prof. Fred McLafferty and co-workers at Cornell University. Quantitative determination was carried out by using an internal calibration curve that was built using stock solutions of the compounds in ultra-pure water saturated in salt and analysing them by the optimised HS-SPME method. Quantification limits were calculated to a single-to-noise (S/N) ratio of 10. The identified volatile compounds related with lipid oxidation were presented in the form of normalised area under peak of each identified compound.

7.3.9.4 Sensory analysis

The sensory evaluation was performed by 8 trained panelists, who were the graduate students in Food Science and Technology programme with the age of 25-33 years and were familiar with fish consumption. Panelists were trained in two sessions using a 10 point scale, where 0 represented no fishy odour and 10 represented the strongest fishy odour (Maqsood and Benjakul, 2011a). Reference samples were prepared by storing Asian seabass slices packed in polythene bags in ice for 0, 5, 10 and 15 days representing the score of 0, 3, 7 and 10, respectively. To test the samples, panelists were asked to open the sealable polyethylene bags containing the test samples and sniff the headspace above the samples. The samples were then scored.

7.3.10 Statistical analysis

All experiments were run in triplicate. Completely randomized design (CRD) was used for this study. The experimental data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's

New Multiple Range Test. For pair comparison, *T*-test was used (Steel and Torrie, 1980). Data analysis was performed using a SPSS package (SPSS 14.0 for Windows, SPSS Inc, Chicago, IL, USA).

7.4 Results and discussions

7.4.1 Effect of EKWE on the lipid oxidation mediated by haemoglobin in washed Asian seabass mince

Impact of EKWE at levels of 0.05 and 0.1% on lipid oxidation mediated by haemoglobin in washed mince added without and with menhaden during 10 days of iced storage is shown in Figure 34 and 35. A sharp increase in PV was observed in all samples up to 2 days of iced storage (P<0.05). The changes in PV after day 2 varied with samples. The continuous increase in PV was observed up to day 4 for both “SB+Hb” and “SB+Hb+oil” (P<0.05). After attaining the maximum PV at day 2, those samples had the decrease in PV until the end of storage period (P<0.05). “SB+Hb” and “SB+Hb+oil” samples displayed the highest PV up to day 8 of storage, compared with others (P<0.05). At day 10, hydroperoxide formed in the “SB+Hb” and “SB+Hb+oil” samples underwent decomposition to a higher rate than the rate of formation, resulting in the lower PV in those samples at the end of storage. For the “SB” sample, PV gradually increased during the storage, but was lower than others, especially within the first 4 day of storage (P<0.05). Although washing could remove most of the haem protein and lipids, there was small amount of membrane lipids such as phospholipids that were difficult to be removed by washing (Lanier *et al.,* 2005). Those lipids contain high level of polyunsaturated fatty acids, which could be autoxidised, particularly when storage time increased (Lanier *et al.,* 2005). The white muscle of a typical lean fish contains less than 1 % lipids. Of this, the phospholipids make up about 90 % (Ackman, 1980). Some haem protein might be bound with muscle protein and could not be leached out during washing. Those haem proteins might exhibit pro-oxidative activity in washed mince.

For the “SB+Hb” and “SB+Hb+oil” samples treated with 0.05% EKWE, all showed the increase in PV up to day 2 of storage. Thereafter the PV

remained unchanged during 2-4 days and decreased gradually until the end of storage period ($P<0.05$). On the other hand, samples treated with 0.1% EKWE ("SB+Hb+0.1% EKWE" and "SB+Hb+oil+0.1% EKWE") had the lower PV than those added with 0.05% EKWE within the first 4 days ($P<0.05$). It was noted that the gradual increase was found until the end of storage, indicating that the samples were still in propagation stage of lipid oxidation. In general, the decrease in PV of some samples with increasing storage time was most likely caused by decomposition of hydroperoxide formed into the secondary oxidation products (Boselli *et al.,* 2005)

The results showed that EKWE, which contained tannic acid as a major component, was effective in retarding the propagation stage of lipid oxidation. Tannic acid exhibited the radical scavenging activity via hydrogen donating and reducing power, thereby terminating the propagation (Maqsood and Benjakul, 2010b). Recently, Maqsood and Benjakul (2011b) reported that tannic acid at a level of 400 ppm was very effective in retarding haemoglobin mediated lipid oxidation in washed Asain seabass mince during iced storage. Maqsood and Benjakul (2010b) also found that higher level (200 ppm) of tannic acid was more effective than the lower level (100 ppm) on the retardation of lipid oxidation in striped catfish slices stored under modified atmosphere packaging (MAP, 60% N_2 / 35% CO_2 / 5% O_2).

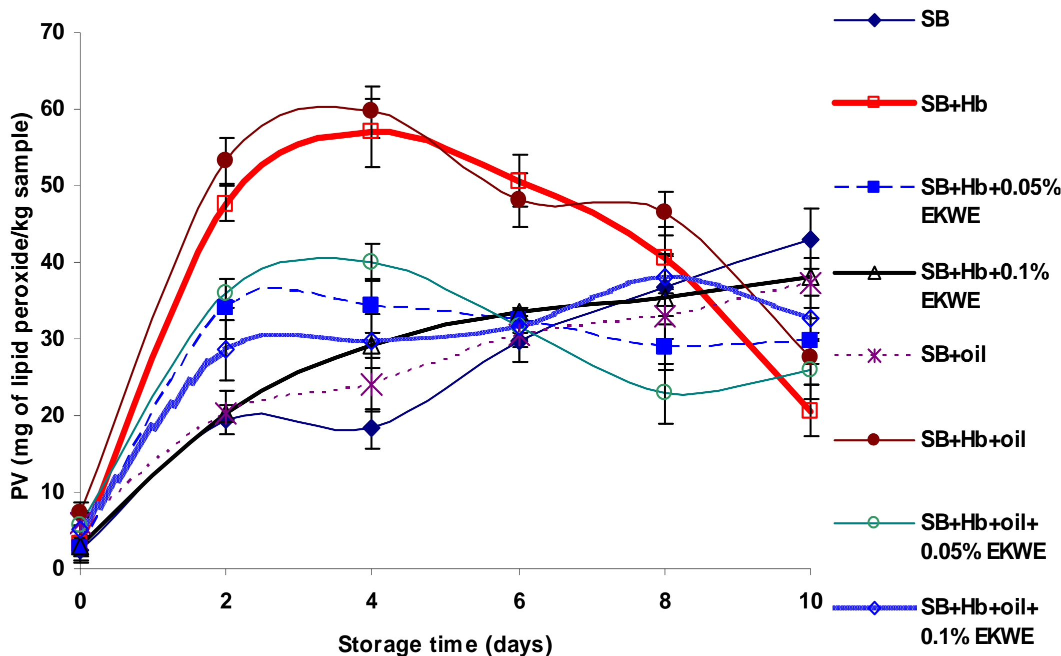


Figure 34. Effect of EKWE at different levels on changes in peroxide value (PV) in washed Asian seabass mince added without and with menhaden oil in the presence or absence of tilapia haemoglobin during iced storage. Bars represents the standard deviation (n=3). SB: Asian seabass mince; SB+Hb: washed mince added with haemoglobin; "SB+Hb+0.05% EKWE" and "SB+Hb+0.1% EKWE": washed mince added with haemoglobin and treated with 0.05% and 0.1% EKWE, respectively; "SB+oil" and "SB+Hb+oil": mince added with menhaden oil and those added with menhaden oil and haemoglobin, respectively; "SB+Hb+oil+0.05% EKWE" and "SB+Hb+oil+0.1% EKWE": mince added with haemoglobin and menhaden oil and treated with 0.05% and 0.1% EKWE, respectively.

TBARS values of "SB+Hb" and "SB+Hb+oil" increased sharply within the first 2 days of storage, followed by the slight increase up to 6 days (Fig. 35). Thereafter, the decrease in TBARS was noticed. The decrease in TBARS was probably due to their reaction with free amino acids, proteins and peptides present in the washed mince system to form Schiff's base (Dillard and Tappel, 1973). Furthermore, volatile oxidation products with low molecular weight could be lost during extended storage. Among all samples tested, "SB+Hb" and "SB+Hb+oil"

displayed the highest formation of TBARS throughout the storage time of 10 days, compared with other samples (P<0.05). TBARS were markedly formed when haemoglobin was incorporated. Thus, haemoglobin was necessary for the production of hydroperoxide breakdown products associate with fishy odour (Undeland *et al.,* 2002). For "SB" and "SB+oil" samples, only slight increase in TBARS was observed. However, the latter showed non-significantly higher TBARS value than the former. This indicated that menhaden oil added underwent oxidation to some degree. Saanchez-Alonso *et al.* (2007) reported that the development of PV and TBARS were not affected by addition of 10% herring oil in the washed cod mince system containing haemoglobin during iced storage. Furthermore, the addition of 15% triacylglycerols in the form of menhaden oil did not alter the rate or intensity of haemoglobin-catalyzed oxidation of washed cod mince containing about 0.7% membrane lipids (Undeland *et al.,* 2002). Rapid oxidation in the muscle of fatty fish species is often attributed to its high total lipid content (Aubourg *et al.,* 1999). However, washed cod muscle system with reduced lipid (~0.1%) had a strong rancid odor developed in the presence of whole blood (Richards and Hultin, 2001). Therefore, it was more likely that membrane lipid in SB played an important role in oxidation, rather than the triglycerides. Surface area of membrane lipids exposed to the aqueous phase is ~50-100 times greater than that of oil droplet (Hultin, 1994). In addition, the higher polarity of the membrane lipids as compared to that of oil may allow for better interaction with a water-soluble pro-oxidant such as haemoglobin (Borst *et al.,* 2000).

When EKWE was incorporated, the formation of TBARS was retarded and the efficacy in lowering lipid oxidation was in dose dependent manner. This was evidenced by the lower formation of TBARS in "SB+Hb+0.1% EKWE" and "SB+Hb+oil+0.1% EKWE" samples, in comparison with that found in "SB+Hb+0.05% EKWE" and "SB+Hb+oil+0.05% EKWE" samples (P<0.05). Thus, EKWE containing tannic acid was effective in retarding the formation of TBARS in washed mince added with haemoglobin, regardless of addition of menhaden oil. Apart from acting as a radical scavenger, tannic acid present in EKWE, especially at a level of 0.1%, could chelate iron, which might be released from the haemoglobin during the storage. Tannic acid has the capability of chelating iron, particularly in the free form

(Lopes *et al.,* 1999). Tannic acid chelates iron due to its ten galloyl groups and might also be able to inhibit iron-mediated oxyradical formation like other iron chelators, such as desferrioxamine (DFO), 1,10-phenanthroline and pyridoxal isonicotinoyl hydrazone (PIH) (Lopes *et al.,* 1999). Recently, Maqsood and Benjakul (2011b) reported that tannic acid at a level of 400 ppm was very effective in retarding the haemoglobin mediated lipid oxidation in washed Asian seabass mince during 10 days of iced storage. Tannic acid showed the higher ferric reducing antioxidant power (FRAP) than other phenolic compounds (catechin, ferulic acid and caffeic acid) (Maqsood and Benjakul, 2010a). Lopes *et al.* (1999) and Andrade Jr *et al.* (2006) also reported that tannic acid was able to reduce Fe (III) to Fe (II). Thus, the addition of tannic acid could retard the formation of methaemoglobin. Methaemoglobin was reported to show the higher pro-oxidative activity, compared with the other forms (Richards and Dettman, 2003). Additionally, the ability in donating electrons to the radical was another mode of action of tannic acid, termed as “reducing power”. Reducing capacity of phenolic antioxidants was realised as a key function for retarding and inhibiting lipid oxidation of fish tissues (Medina *et al.,* 2007). The tannins were 15-30 times more effective in quenching peroxyl radicals than simple phenolics or Trolox, when tested in deoxyribose assay and metmyoglobin assay (Hagerman *et al.,* 1998). The samples treated with EKWE especially at 0.1% level showed the lower formation of PV and TBARS, suggesting its capacity of scavenging the radicals formed in the propagation stage. Thus, EKWE especially at 0.1% was effective in retarding the formation of hydroperoxides and TBARS in washed mince, especially that containing haemoglobin, irrespective of menhaden oil addition during iced storage.

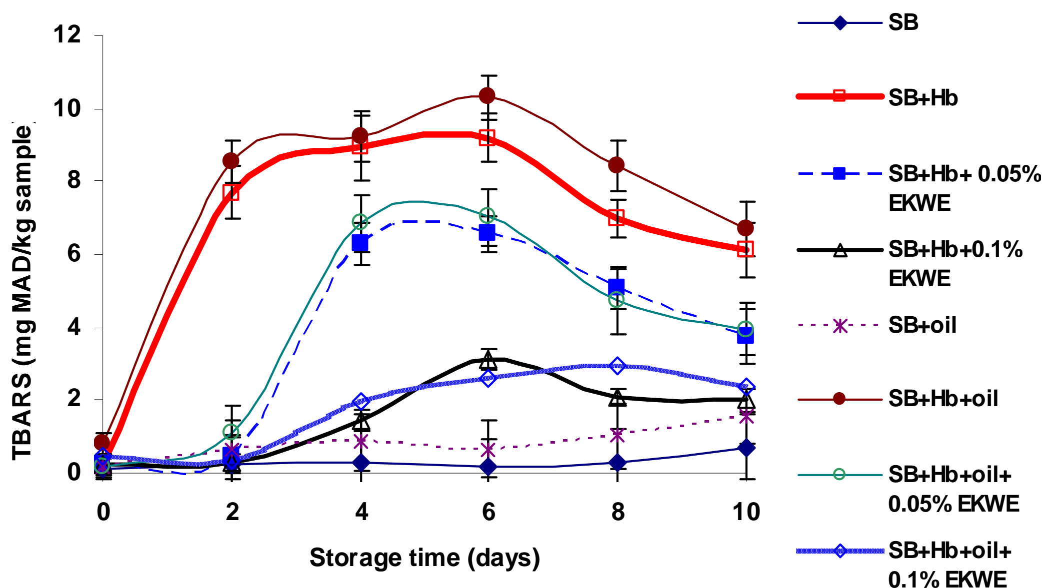


Figure 35. Effect of EKWE at different levels on changes in thiobarbituric acid-reactive substance (TBARS) in washed Asian seabass mince added without and with menhaden oil in the presence or absence of tilapia haemoglobin during iced storage. Bars represents the standard deviation (n=3). Key: see Figure 34 caption.

7.4.2 Effect of EKWE on fishy odour development in washed Asian seabass mince added with haemoglobin

Fishy odour intensity associated with haemoglobin-induced lipid oxidation in the washed mince without and with menhaden oil and treated without and with EKWE (0.05% and 0.1%) during 10 days of iced storage is shown in Figure 36. Within the first 2 days of storage, fishy odour intensity increased markedly in “SB+Hb” and “SB+Hb+oil” samples, whereas a slight increase was obtained in other samples (P<0.05). Fishy odour intensity of both “SB+Hb” and “SB+Hb+oil” samples increased continuously during 2-6 days of storage and remained constant after 6 days of storage (P>0.05). The higher fishy odour intensity in “SB+Hb” and “SB+Hb+oil” sample was related with the higher rate of lipid oxidation (Fig. 34 and 35). Fishy odour intensity of the control sample “SB” showed a steady increase throughout the storage and was lowest, compared with other samples (P<0.05). “SB+oil” samples had slightly higher fishy odour intensity, compared with “SB”. It was found that

samples containing menhaden oil received slightly higher scores on fishy odour intensity than those added without menhaden oil. This was probably due to indigenous fishy odour present in the menhaden oil, which could be detected by the panelists. When EKWE was incorporated, it was noted that fishy odour intensity was lowered, in comparison with the samples without EKWE addition ($P<0.05$). The samples treated with 0.05% EKWE developed more fishy odour intensity than those treated with 0.1% EKWE ($P<0.05$). This result correlated well with the higher formation of TBARS in "SB+Hb+oil+0.05% EKWE" than "SB+Hb+oil+0.1% EKWE" samples (Fig. 35).

Thus, EKWE especially at a level of 0.1% was effective in retarding the development of fishy odour in washed mince containing haemoglobin, irrespective of oil incorporated. The off-odour developed in the fish muscle due to lipid oxidation was considered as fishy (Fu *et al.,* 2008). Recently, Maqsood and Benjakul (2011b) reported that tannic acid at a level of 400 ppm was very effective in retarding the development of fishy odour associated with haemoglobin mediated lipid oxidation in washed Asian seabass mince during iced storage.

Tannic acid at a level of 200 ppm was also found to be effective in retarding lipid oxidation and off-odour development in ground and cooked fish (*Scomberomorus commersoni*) stored at 4 ºC (Ramanathan and Das, 1992). Maqsood and Benjakul (2010 b and c) reported that tannic acid at a level of 200 ppm retarded the lipid oxidation and development of off-odour in the striped catfish slice and ground beef stored under modified atmospheric packaging during refrigerated storage. Thus, EKWE was effective in lowering the formation of fishy odour associated with lipid oxidation mediated by haemoglobin in washed mince during iced storage.

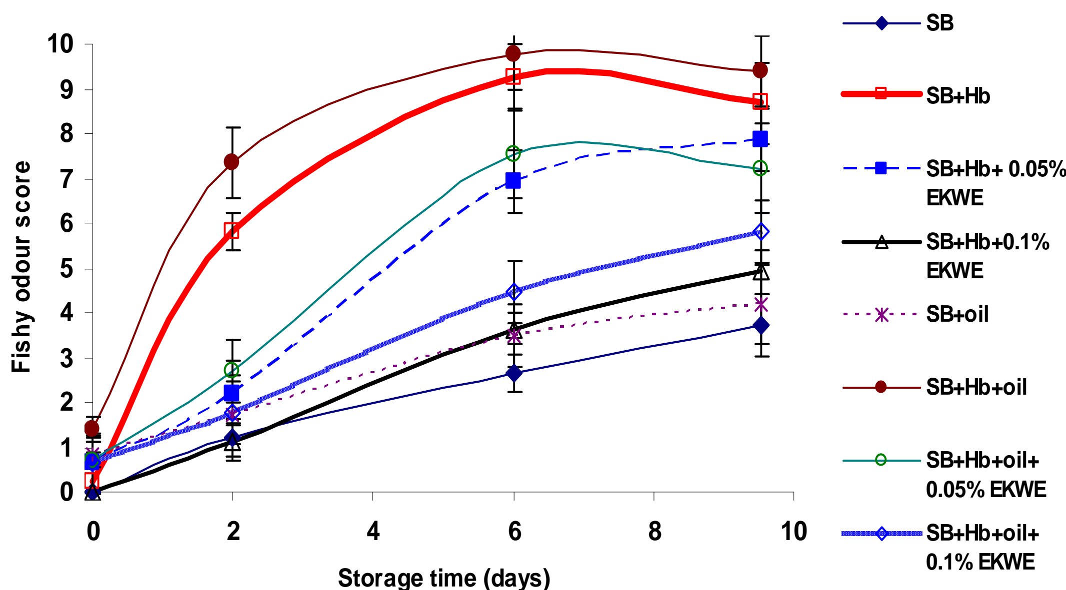


Figure 36. Effect of EKWE at different levels on changes in fishy odour development in washed Asian seabass mince added without and with menhaden oil in the presence or absence of tilapia haemoglobin during iced storage. Bars represents the standard deviation (n=3). Key: see Figure 34 caption.

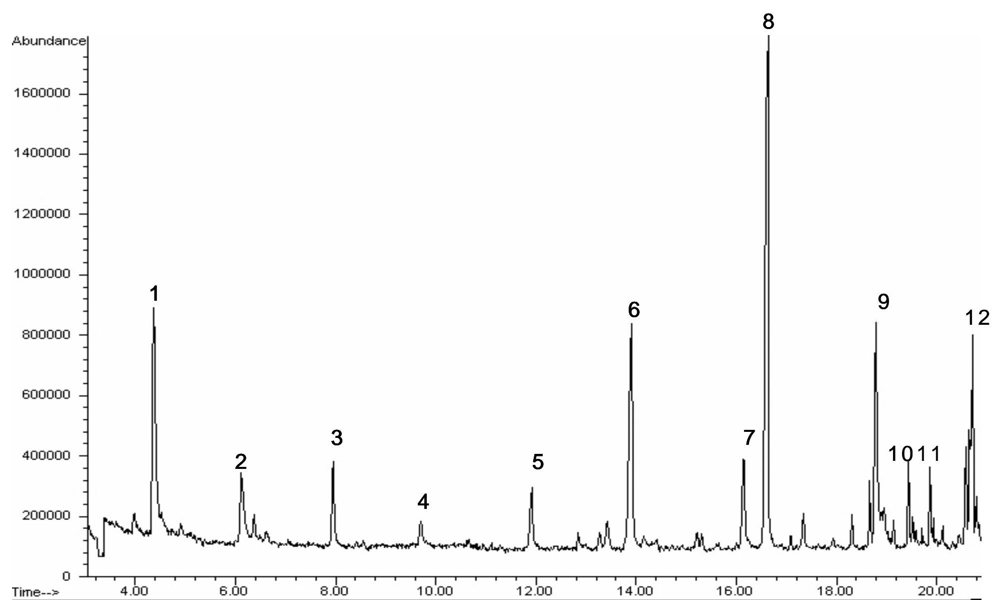
7.4.3 Effect of EKWE on development of volatile lipid oxidation compounds in washed Asian seabass mince added with haemoglobin

Chromatograms and abundance of different volatile lipid oxidation compounds in the washed mince added with haemoglobin and menhaden oil treated without 0.1% EKWE ("SB+Hb+oil") and with 0.1% EKWE ("SB+Hb+oil+0.1% EKWE") after 10 days of iced storage are shown in Figure 37 and 38, respectively. The development of volatile lipid oxidation compounds in both samples was in accordance with the sensory analysis and formation of TBARS. Lipid oxidation of fish muscle is known to produce volatile aldehydic compounds including haxanal, heptanal, octanal, nonanal, etc (Yasuhara and Shibamoto, 1995). Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to monitor lipid oxidation in a number of foods, including muscle foods (Ross and Smith, 2006). Aliphatic aldehydes are commonly known to derive from lipid oxidation which occurs in fish flesh (Serot *et al.,* 2002). The "SB+Hb+oil"

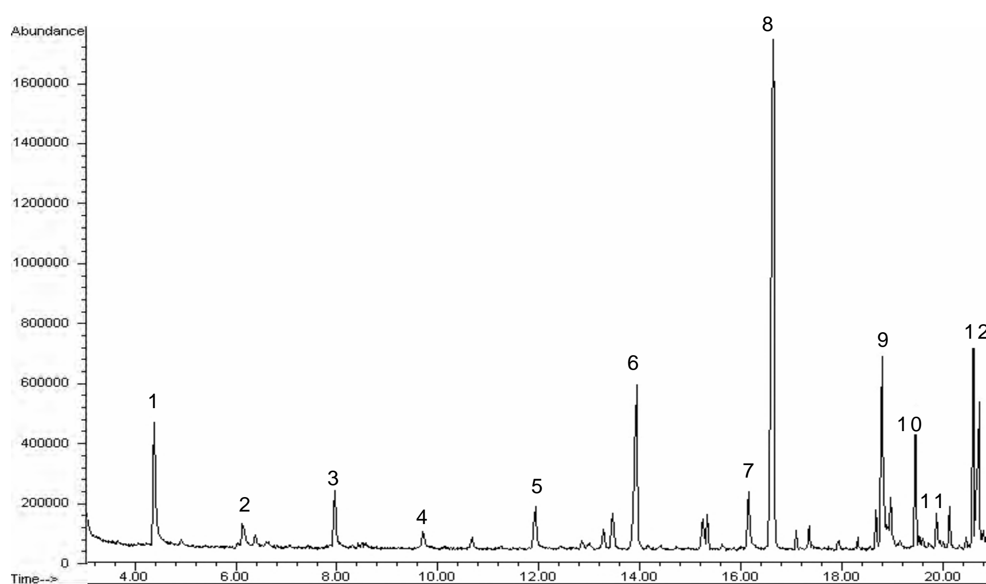
sample contained the higher abundance of all volatile compounds except for 2-dodecenal, compared with "SB+Hb+oil+0.1% EKWE" sample after 10 days of storage in ice. Among aldehydes, hexanal, heptanal, pentenal, octanal, nonanal and 2,4-heptadienal were found at higher amounts in "SB+Hb+oil" sample, compared with "SB+Hb+oil+0.1% EKWE" sample. Heptanal can serve as a reliable indicator of flavour deterioration for fish products, while hexanal contributes to the rancidity in meats (Ross and Smith, 2006). Higher formation of volatile lipid oxidation products in the "SB+Hb+oil" sample correlated well with the higher formation of TBARS (Fig. 35) and higher fishy odour score (Fig. 36). Aldehydes generated from the oxidation of fatty acids are frequently related with the off-odours (McGill *et al.,* 1974). The results suggested that lipid oxidation and decomposition of hydroperoxides to the secondary volatile lipid oxidation products were more pronounced in "SB+Hb+oil" sample, compared with "SB+Hb+oil+0.1% EKWE" sample.

Furans and its related compounds such as 2-ethylfuran, 2-pentylfuran and 2-pentenylfuran were also found at high amount in "SB+Hb+oil" sample, compared with those of "SB+Hb+oil+0.1% EKWE" sample. Those compounds are formed by the decomposition of 12-hydroperoxide of linolenate (18:3n-3), 14-hydroperoxide of eicosapentaenoate (20:5n-3) and 16-hydroperoxide of docosahexaenoate (22:6n-3). Hydroperoxide can undergo ß-cleavage to produce a conjugated diene radical, which can react with oxygen to produce a vinyl hydroperoxide. Cleavage of the vinyl hydroperoxide by loss of a hydroxyl radical forms an alkoxyl radical. This radical undergoes cyclisation, thereby producing furans (Medina *et al.,* 1999).

Among ketones, pentachloro-2-propanone was found in both samples with the higher abundance in "SB+Hb+oil" sample. Ketone is another secondary lipid oxidation product generated from the decomposition of hydroperoxide in the chilled fish muscle (Iglesias and Medina, 2008). Among alcohols, 1-octen-3-ol was detected in both samples. The higher amount was found in "SB+Hb+oil" sample, compared with "SB+Hb+oil+0.1% EKWE" sample.



a)



b)

Figure 37. Chromatograms obtained from GCMS-SPME of different volatile lipid oxidation compounds in the washed Asian seabass mince added with haemoglobin and oil ("SB+Hb+oil") and those treated with 0.1% EKWE ("SB+Hb+oil+0.1% EKWE") after 10 days of iced storage.

Alcohols are the secondary products produced by the decomposition of hydroperoxide (Girand and Durance, 2000). 8-Carbon alcohols are present in all species of fish (Josephson *et al.,* 1984). Recently, Maqsood and Benjakul (2011b) reported that the tannic acid at a level of 400 ppm prevented the formation of volatile lipid oxidation compounds, which contribute to the offensive fishy odours in fish muscle. In general, the lower abundance of volatile lipid oxidation compounds was obtained in the "SB+Hb+oil+0.1% EKWE" sample, compared with the "SB+Hb+oil" sample. The result confirmed that EKWE at a level of 0.1% was effective in retarding the lipid oxidation. As a result, the formation of volatile lipid oxidation compounds causing the fishy odours in fish muscle was retarded.

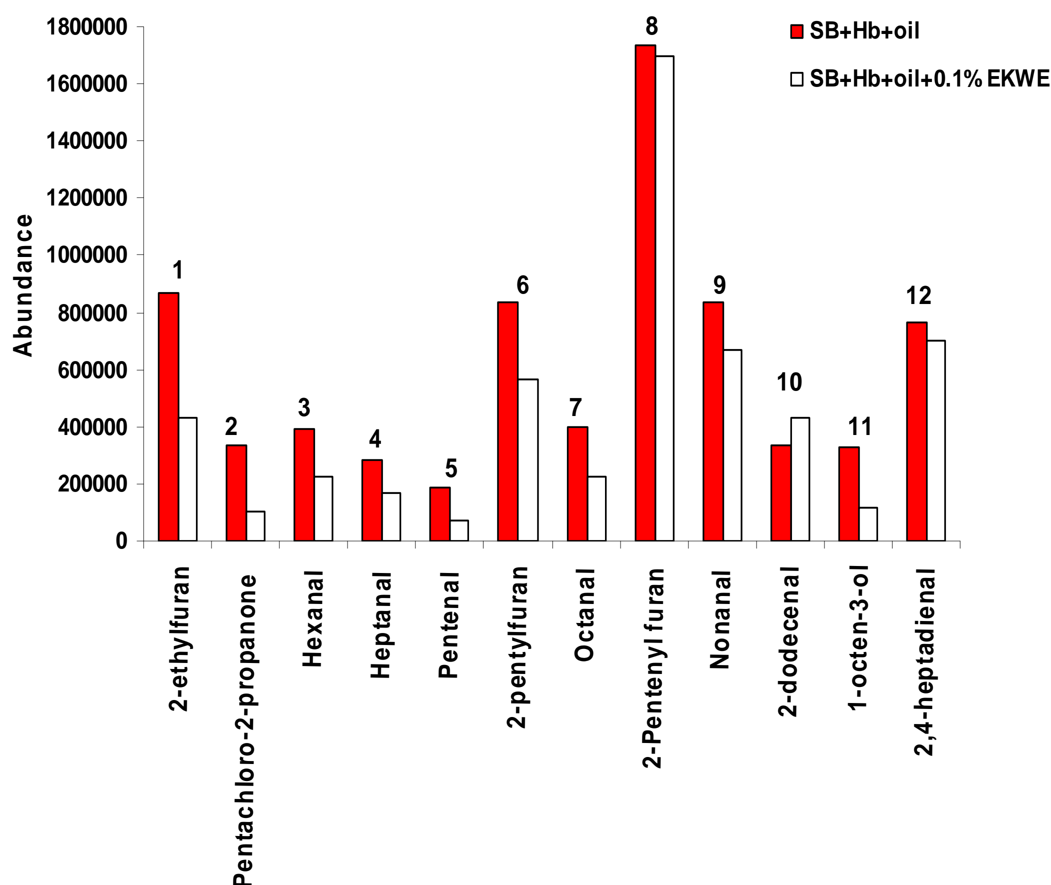


Figure 38. Abundance of different volatile lipid oxidation compounds in the washed Asian seabass mince added with haemoglobin and oil ("SB+Hb+oil") and those treated with 0.1% EKWE ("SB+Hb+oil+0.1% EKWE") after 10 days of iced storage.

7.5 Conclusion

Haemoglobin was proven to be an effective pro-oxidant in washed Asian seabass mince contributing to the development of fishy odour, regardless of oil content. When EKWE, especially at a level of 0.1%, was incorporated in washed mince containing haemoglobin and menhaden oil, the progression of lipid oxidation was retarded as indicated by the lower TBARS formation as well as lower development of fishy odour. Additionally, the preventive effect of EKWE at a level of 0.1% on lipid oxidation and fishy odour development was also reflected by the lower abundance of volatile lipid oxidation products formed. Thus, EKWE could be a promising natural antioxidant used to prevent the lipid oxidation and fishy odour development in the fish muscle.

CHAPTER 8

EFFECT OF TANNIC ACID AND KIAM WOOD EXTRACT ON LIPID OXIDATION AND TEXTURAL PROPERTIES OF FISH EMULSION SAUSAGES DURING REFRIGERATED STORAGE

8.1 Abstract

Effect of tannic acid (0.02 and 0.04%) and ethanolic kiam wood extract (EKWE) (0.04 and 0.08%) on lipid oxidation and textural properties of emulsion sausages from stripped catfish during 20 days of refrigerated storage was investigated. Control samples (C) had the highest peroxide value (PV) and thiobarbituric acid-reactive substances (TBARS) value up to day 16 and 8 of storage, respectively. With the addition of tannic acid and EKWE, PV and TBARS value in the sausages were retarded effectively, compared to the control ($P<0.05$), especially when the tannic acid and EKWE at higher level were used. At the same level, EKWE showed the lower ability in retarding the lipid oxidation, in comparison with tannic acid. Tannic acid at both levels (0.02 and 0.04%) was also effective in retarding the formation of fishy odour in the samples throughout the storage, compared to the control and EKWE treated samples ($P<0.05$). Both tannic acid and EKWE had no detrimental effect on the sensory attributes of sausages. However, EKWE treated sample had lower L^* and higher a^* and ΔE^* values, compared to the control samples ($P<0.05$). After 20 days of storage, the sample added with 0.04% tannic acid had higher hardness, gumminess and chewiness, compared with others ($P<0.05$). Samples added with 0.04% tannic acid also displayed more compact structure with no visible voids. Furthermore, oil droplets with smaller size were dispersed more uniformly, compared to others. Thus, tannic acid (0.02 and 0.04%) and EKWE (0.08%) were effective in retarding lipid oxidation and fishy odour development as well as could maintain the textural properties of fish emulsion sausages during the refrigerated storage of 20 days.

8.2 Introduction

Emulsion sausages, such as frankfurter, are widely consumed in both Western and Asian countries. A product is typically made of beef, pork, or chicken and contains the fat by 25–30%. Fish mince and surimi have recently been used as a raw material for emulsion sausage production, particularly in Asian countries (Konno, 2005). Marine fish are generally a rich source of n-3 polyunsaturated fatty acids (PUFA) containing approximately 14–30% of total fatty acids, whereas PUFA in freshwater fish was only 1–11% of total fatty acids (Rahman *et al.,* 1995; Steffens, 1997). Biological importance of n-3 PUFA, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on brain and retina development, has been realized (Simopoulos, 1997). An increase in consumption of n-3 PUFA has been reported to reduce the risk of coronary heart disease, decrease mild hypertension and prevent certain cardiac arrhythmias (Garg *et al.,* 2006). Fish oil is actually the main dietary source of n-3 PUFA. The World Health Organization (WHO Study Group, 2003) recommends regular fish consumption to provide approximately 200–500 mg per week of EPA and DHA and replacement of saturated fat by monounsaturated.

Fortification of marine fish oil rich in n-3 PUFA to the fresh water fish sausage could be an alternative means to improve its fat quality and to increase n-3 PUFA consumption. However, marine fish oil is susceptible to lipid oxidation, thereby negatively affecting flavour, odour, colour, texture, and the nutritional value of fish products (Frankel, 1998a). To retard such a quality loss, synthetic antioxidants have been used to decrease lipid oxidation during the processing and storage of fish and fish products (Boyd *et al.,* 1993). However, the use of synthetic antioxidants has raised questions regarding safety and toxicity (Chang *et al.,* 1977). The use of natural antioxidants is emerging as an effective means for controlling lipid oxidation and limiting its deleterious consequences. Recently, Maqsood and Benjakul (2010 a, b, c) reported that tannic acid exhibited the superior radical scavenging activities as well as reducing power and effectively inhibited the lipid oxidation in fish mince, fish oil-in-water emulsion, fish slices and ground beef. Haemoglobin mediated lipid oxidation in washed Asain seabass mince was also impeded by incorporation of tannic acid (Maqsood and Benjakul, 2011). Tannic acid is also affirmed as Generally Recognised

As Safe (GRAS) by the Food and Drug Administration (FDA) at a level of 10 - 400 ppm for the use as an ingredient in some food products including meat products (US Code of Federal Regulation, 2006; Chung *et al.,* 1993).

Tannic acid or tannins are polyphenolic compounds commonly occurring in the barks, woods and fruits of many kinds of plants (Yazaki and Collins, 1994). Kiam (*Cotylelobium lanceotatum craih*) trees are very common in the southern part of Thailand. Pieces of wood from the kiam tree have been traditionally submerged in sugar palm sap to prevent or retard microbial fermentation (Chanthachum and Beuchat, 1997). Balange and Benjakul (2009) reported that tannic acid (456.3 mg/kg) was found as the major component of the kiam wood extract. Therefore, the objective of the present study was to evaluate the effect of tannic acid and ethanolic kiam wood extract (EKWE) on the lipid oxidation and textural properties of emulsion sausages prepared from the meat of striped catfish, a fatty fish, during refrigerated storage.

8.3. Materials and Methods

8.3.1 Chemicals

Menhaden oil, tannic acid, cumene hydroperoxide and osmium were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium chloride, sodium bicarbonate, potassium iodide, trichloroacetic acid and glutaraldehyde were obtained from Merck (Damstadt, Germany). Disodium hydrogen phosphate, sodium tripolyphosphate, soy protein isolate, 2-thiobarbituric acid, ammonium thiocyanate and ferrous chloride were purchased from Fluka Chemical Co. (Buchs, Switzerland). All chemicals used were of analytical grade.

8.3.2 Preparation of kiam wood extract

8.3.2.1 Collection and preparation of kiam wood

The kiam wood was obtained from the forest of the Phattalung province in the Southern Thailand. The tree was about 15–20 years old and harvested in May, 2010. The tree was cut by using a sawing machine; the leaves and branches were separated manually by cutting and the trunk was kept for sun drying for 3 months. The

trunk was chopped into smaller flakes of wood and then dried in an oven at 70 °C for 8 h and cut into pieces with an average size of 1.5 x 1.5 cm^2, until the moisture content reached 4-5% (wet weight basis). Those pieces were ground using a portable grinding machine (Spong-90, Leeds, UK) with a sieve size of 6 mm and was then subjected to size reduction using a blender (National Model MKK77, Tokyo, Japan) and finally sieved using a stainless steel sieve of 80 mesh size (with the diameter of 0.177 mm). The obtained powder was placed in a polythene bag, sealed and kept at room temperature until use.

8.3.2.2 Extraction of phenolic compounds from kiam wood

Kiam wood powder was subjected to extraction according to the method of Santoso *et al.* (2004) with slight modifications. The powder (10 g) was mixed with 150 mL of absolute ethanol. The mixture was stirred at room temperature (28–30 °C) using a magnetic stirrer (IKA-Werke, Staufen, Germany) for 6 h. The mixture was then centrifuged at 5000 x g for 10 min at room temperature using a RC-5B plus centrifuge (Beckman, JE-AVANTI, Fullerton, CA, USA). The supernatant was filtered using a Whatman filter paper No. 1 (Whatman International, Ltd, Maidstone, England). The filtrate was then evaporated at 40 °C using an Eyela rotary evaporator (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan). To remove the residual ethanol, the extract was purged with nitrogen gas. The extract was then dried using a freeze dryer to obtain the dry extract. Dried extract was powdered using a mortar and pestle and was kept in an amber bottle and stored in a dessicator until use. The dried powderised extract was referred to as “ethanolic kiam wood extract; EKWE”.

8.3.2.3 Determination of total phenolic content in EKWE

Quantification of total phenolic content in EKWE was carried out according to the method of Slinkard and Singleton (1977). EKWE was dissolved in 25% ethanol (v/v) to obtain a concentration of 0.5% (w/v). The solution (0.5 mL) was added with 100 µL of Folin–Ciocalteu reagent (two-fold diluted with de-ionised water) and mixed thoroughly. After 3 min, 1.5 mL of 2% sodium carbonate solution was added. The reaction mixture was mixed thoroughly and placed in the dark for 40

min and the absorbance was read at 760 nm. The total phenolic content was calculated from the standard curve of tannic acid (0–0.1 mg/mL) and expressed as mg tannic acid / g dry EKWE after blank substraction. Blank was prepared in the same manner, except that distilled water was used instead of Folin–Ciocalteu reagent.

8.3.2.4 Determination of tannic acid in EKWE

Qualitative analysis of EKWE was performed using an HPLC equipped with VWD detector following the method of Tian *et al.* (2009) with slight modifications. The HPLC system consisted of an Agilent 1100 series HPLC (Alginet, Wilmington, DE, USA), quaternary pump with seal wash option, degasser, solvent, cabinet and preparative autosampler with thermostat equipped with a diode array detector. The separation was performed on a column (Hypersil ODS C18 4.0 - 250 mm, 5 lm, Cole-Parmer, Hanwell, London, UK). HPLC conditions were as follows: mobile phase: 0.4% Formic acid: Acetonitrile (85:15); flow rate: 0.8 mL/min; temperature: 25 °C. The detection was carried out at 280 nm. The concentration of extracts was 25 mg/mL and each injection volume was 20 µL. Standard tannic acid was used for peak identification.

8.3.3 Preparation of fish emulsion sausages containing tannic acid and EKWE

Striped catfish (*Pangasius hypophthalamus*) weighing 3-4 kg, off-loaded 24 h after capture and stored in ice, were purchased from the fish market in Hat Yai, Songkhla, Thailand. The fish were kept in ice during transportation to the Department of Food Technology, Prince of Songkla University. Upon arrival, fish were washed with tap water, filleted, deskinned and minced using a mincer with a hole diameter of 5 mm. Moisture content of the mince was adjusted to 86 %. Fish emulsion sausages were prepared following the method described by Panpipat and Yongsawatdigul (2008) with slight modifications. Fish mince (85 g) was added with sodium chloride (2 g), sodium tripolyphosphate (1.5 g) and soy protein isolate (1.5 g) and menhaden oil (10 g). The mixture was ground for 3 min using a Panasonic Food Processor (MK, 5087M, Selangor Darul Ehsan, Malaysia).

To study the effect of tannic acid or EKWE on lipid oxidation and textural properties of emulsion sausages, tannic acid and EKWE with total phenolic content of 602.6 mg tannic acid equivalent/g dry powder and tannic acid content of 545.57 mg/g dry powder was added to obtain the designated final concentration. Tannic acid (0.02 and 0.04 % w/w) or EKWE (0.04 and 0.08 % w/w) dissolved in distilled water were added to the mixture along with menhaden oil (10% v/w). Subsequently, the mixture was further ground thoroughly for 5 min in order to obtain a homogenous paste. The paste was stuffed into a cellophane casing (diameter of 22 mm) and pre-incubated at 55 °C for 40 min prior to cooking at 80 °C for 15 min (Panpipat and Yongsawatdigul, 2008) in a temperature controlled water bath (Memmert, D-91126, Schwabach, Germany). The samples added with tannic acid at a level of 0.02 and 0.04 % were referred to as 'TA-0.02' and 'TA-0.04' respectively and those added with EKWE at a level of 0.04 and 0.08 % were referred to as 'EKWE-0.04' and 'EKWE-0.08', respectively. The control samples (C) were prepared in the similar manner but distilled water was added instead of tannic acid or EKWE. Samples were cooled for about 30 min in iced water. Samples were cut into cylinders (30 mm height×20 mm diameter) and place in polythene bags and further stored at 4 °C. Samples were randomly taken at day 0, 4, 8, 12, 16 and 20 of storage for analysis of lipid oxidation products. Sensory analysis was conducted at day 0, 12 and 20, while samples were subjected to textural analysis at day 0 and 20.

8.3.4 Analyses

8.3.4.1 Determination of peroxide value

Peroxide value (PV) was determined as per the method of Richards and Hultin (2002) with a slight modification. Samples (1 g) were mixed with 11 mL of chloroform/methanol (2:1, v/v). The mixtures were homogenised at a speed of 13,500 rpm for 2 min. Homogenates were then filtered using a Whatman No. 1 filter paper. Two millilitres of 0.5 % NaCl were then added to 7 mL of the filtrate. The mixtures were vortexed at a moderate speed for 30 s using a Vortex-Genie2 mixer 4 (Bohemia, NY, USA) and then centrifuged at 3,000 x g for 3 min to separate the sample into two phases. Two millilitres of cold chloroform/methanol (2:1, v/v) were added to 3 mL of the lower phase. Twenty-five microlitres of 30 % ammonium thiocyanate and 25 μL of

20 mM iron (II) chloride were added to the mixture (Shantha and Decker, 1994). Reaction mixtures were allowed to stand for 20 min at room temperature and the absorbance was read at 500 nm. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5-2 ppm.

8.3.4.2 Determination of thiobarbituric acid-reactive substances (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Samples (0.5 g) were mixed with 2.5 mL of a TBA solution containing 0.375 % thiobarbituric acid, 15 % trichloroacetic acid and 0.25 N HCl. The mixtures were heated in a boiling water for 10 min to develop a pink colour, cooled with running tap water and then sonicated for 30 min using an Elma (S 30 H) sonicator (Kolpingstr, Singen, Germany). The mixture was then centrifuged at 5,000 × g at 25 ºC for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (MAD) at the concentration ranging from 0 to 10 ppm and TBARS were expressed as mg of MAD equivalents/kg sample.

8.3.5 Sensory analysis

The sensory evaluation was performed by 30 untrained panelists, who were the graduate students in Food Science and Technology programme with the age of 25-33 years and were familiar with sausage consumption. The sausage samples were placed in the polythene bags and were dipped in the boiling water for 15 min (Masniyom *et al.*, 2002). Stick water was drained and samples were allowed to cool to room temperature (25–28 ºC) prior to evaluation. Panelists were asked to evaluate for colour, taste, texture and overall acceptability of sausage samples using a 9-point hedonic scale (Mailgaad *et al.,* 1999): 1, dislike extremely; 2, dislike very much; 3, dislike moderately; 4, dislike slightly; 5, neither like nor dislike; 6, like slightly; 7, like moderately; 8, like very much; 9, like extremely. Samples were also evaluated for fishy odour at day 0, 12 and 20 by 30 panelists using a scale 0 to 10, where 0

represented no fishy odour and 10 represented the strongest fishy odour as described by Maqsood and Benjakul (2011b).

8.3.6 Colour determination

Colour of the sausage samples was measured using a colourimeter (Hunter Lab, Model colour Flex, Reston, VIRG, USA) with the port size of 0.50 inch. The determination of colour was done on six different samples. Standardisation of the instrument was done using a black and white Minolta calibration plate. The values were reported in the CIE colour profile system as L^*-value (lightness), a^*-value (redness/greeness), and b^*value (yellowness/blueness). Total difference in colour (ΔE^*) was calculated according to the following equation (Gennadios *et al.,* 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

Where ΔL^*, $\Delta a^*$and Δb^* are the differences between the corresponding colour parameter of the sample and that of white standard ($L^*= 93.63$, $a^*= -0.92$ and $b^* =0.42$).

8.3.7 Textural profile analysis (TPA)

TPA was performed using a TA-XT2i texture analyser (Stable Micro Systems, Surrey, England) with cylindrical aluminum probe (50 mm diameter). The samples were cut into cylinders (30 mm height×20 mm diameter) and placed on the instrument's base. The tests were performed with two compression cycles. TPA textural parameters were measured at room temperature with the following testing conditions: crosshead speed 5.0 mm/s, 50% strain, surface sensing force 99.0 g, threshold 30.0 g, and time interval between the first and the second compressions was 1 s. The Texture Expert version 1.0 software (Stable Micro Systems, Surrey, England) was used to collect and process the data. Hardness, springiness, cohesiveness, gumminess, chewiness and were calculated from the force–time curves generated for each sample (Bourne, 1978).

8.3.8 Scanning electron microscopy (SEM)

Microstructure of emulsion sausages was determined using SEM. Samples were cut into a cube (4 x 4 x 4 mm) using a razor blade. The prepared samples were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2)

at room temperature for 2 h. The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50, 60, 70, 80, 90 and 100% (v/v) for 15 min at each concentration. The dehydrated samples were then fixed in 1% osmium (v/v) for 2 h and then rinsed and dehydrated in the similar manner as mentioned above. Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

8.3.8 Statistical analysis

All experiments were run in triplicate. Completely randomized design (CRD) was used for this study. The experimental data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's New Multiple Range Test. For pair comparison, *T*-test was used (Steel and Torrie, 1980). Data analysis was performed using a SPSS package (SPSS 14.0 for Windows, SPSS Inc, Chicago, IL, USA).

8.4 Results and discussion

8.4.1 Effect of tannic acid and EKWE on lipid oxidation in fish emulsion sausages

Impact of tannic acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) on lipid oxidation of fish emulsion sausages during 20 days of refrigerated storage is shown in Figure 39. A sharp increase in PV was observed in all samples up to 4 days of refrigerated storage ($P < 0.05$). The continuous increase in PV was observed up to day 8 for C and EKWE-0.04 samples ($P<0.05$). However, no changes in PV were found in EKWE-0.08, TA-0.02 and TA-0.04 during 4 and 12 days ($P>0.05$). After 12 days of storage, samples treated with TA at both levels had the increase in PV, while C and EKWE-0.04 samples had the decrease in PV up to 20 days ($P<0.05$). The increase in PV of samples added with tannic acid indicated that the samples were in propagation stage of lipid oxidation with the lower rate of decomposition of hydroperoxide formed. In general, control (C) and EKWE-0.04 samples displayed the highest PV up to day 12 of storage, compared with other samples ($P<0.05$). Thereafter, the decrease in PV was

found in those samples. The decrease in PV was most likely due to the decomposition of hydroperoxide formed into the secondary oxidation products (Boselli *et al.,* 2005). During the first 12 days, higher PV was found in samples treated with EKWE in comparison with those added with tannic acid at the same level (0.04%) ($P<0.05$). The results indicated that the EKWE was less effective in retarding the formation of hydroperoxide, compared with tannic acid. Tannic acid showed the radical scavenging activity via hydrogen donating and reducing power, thereby terminating the propagation (Maqsood and Benjakul, 2010b). Recently, Maqsood and Benjakul (2011b) reported that tannic acid at a level of 400 ppm was very effective in retarding haemoglobin mediated lipid oxidation in washed Asain seabass mince during iced storage. Maqsood and Benjakul (2010b) also found that higher level (200 ppm) of tannic acid was more effective than the lower level (100 ppm) on the retardation of lipid oxidation in striped catfish slices stored under modified atmosphere packaging (MAP, 60%N_2 / 35%CO_2 / 5%O_2).

TBARS values of all samples increased sharply up to day 12 of storage, followed by a slight decrease until the end of storage period (Fig. 39b). The decrease in TBARS was probably due to their reaction with free amino acids, proteins and peptides present in the sausages to form Schiff's base (Dillard and Tappel, 1973). Furthermore, volatile oxidation products with low molecular weight could be lost during extended storage. At day 0, TBARS values of all sample ranged from 2.7 to 4 mg MAD/kg of sample, indicating that the lipid oxidation occurred during the processing and cooking of the sausages. Control samples (C) showed the higher formation of TBARS throughout the storage of 20 days, compared with other samples ($P<0.05$), except EKWE-0.04 sample, which had the similar value during the 12-20 days of storage ($P>0.05$). The results showed that EKWE at a level of 0.04% was not effective in retarding the lipid oxidation in fish emulsion sausages. When tannic acid (0.02 and 0.04%) and EKWE (0.08%) was added in the fish emulsion sausages, the formation of TBARS was retarded effectively. Among all samples, TA-0.04 samples had the lower formation of TBARS, while EKWE-0.04 samples had the higher formation of TBARS ($P<0.05$).

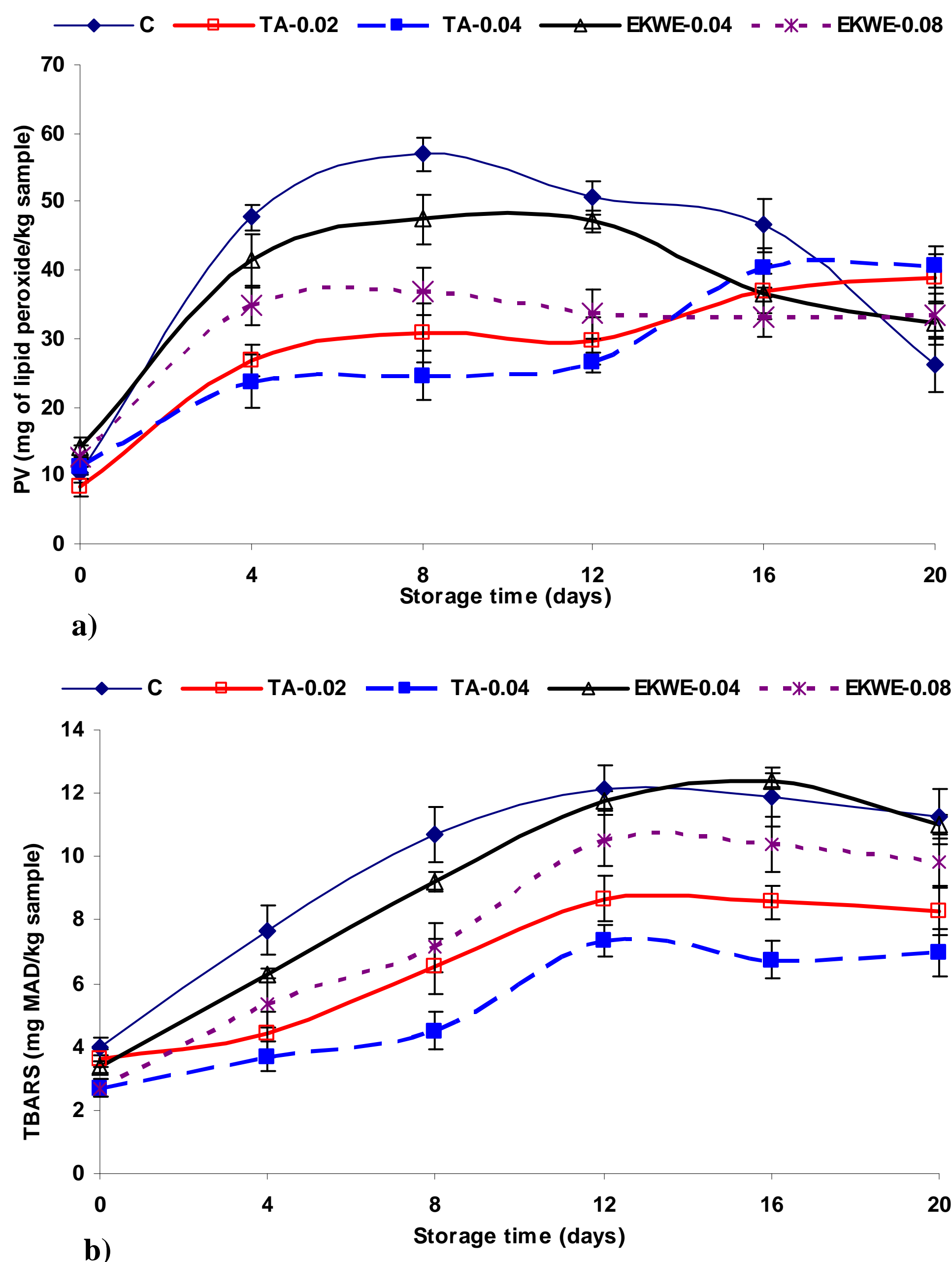


Figure 39. Effect of tannic acid acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) on (a) peroxide value (PV) and (b) thiobarbituric acid-reactive substance (TBARS) of fish emulsion sausages during 20 days of refrigerated storage. Bars represents the standard deviation (n=3). C: control samples without any treatment; TA-0.02: samples treated with 0.02% tannic acid; TA-0.04: samples treated with 0.04% tannic acid; EKWE-0.04: samples treated with 0.04% ethanolic kiam wood extract; EKWE-0.08: samples treated with 0.08% ethanolic kiam wood extract.

Tannic acid especially at higher level (0.04%) was therefore very effective in retarding the lipid oxidation. EKWE at a level of 0.08% was effective in retarding the formation of TBARS. Apart from acting as a radical scavenger, tannic acid, especially at a level of 0.04%, could chelate iron, which might be released during cooking. Tannic acid has the ability to chelate iron, particularly in the free form (Lopes *et al.,* 1999). Tannic acid chelates iron due to its ten galloyl groups and might also be able to inhibit iron-mediated oxyradical formation like other iron chelators, such as desferrioxamine (DFO), 1,10-phenanthroline and pyridoxal isonicotinoyl hydrazone (PIH) (Lopes *et al.,* 1999). Recently, tannic acid was demonstrated to show the higher ferric reducing antioxidant power (FRAP) than other phenolic compounds (catechin, ferulic acid and caffeic acid) (Maqsood and Benjakul, 2010a). Lopes *et al.* (1999) and Andrade *et al.* (2006) also reported that tannic acid was able to reduce Fe (III) to Fe (II). Thus, tannic acid (0.02 and 0.04%) and EKWE (0.08%) were effective in retarding lipid oxidation in fish emulsion sausage during the extended refrigerated storage.

8.4.3 Effect of tannic acid and EKWE on sensory properties of fish emulsion sausages

Colour, texture, taste and overall likeness of the sausages added without and with tannic acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) at day 0 of storage are shown in Table 6. There was no difference in all attributes among all samples (P>0.05), except for taste in which the samples added with EKWE at both levels received lower scores. Thus, the addition of tannic acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) to the sausages had no impact on colour and texture of all samples. The lower taste likeness scores of EKWE (0.04 and 0.08%) added sample was probably due to the presence of some compounds in the extract, which caused the off taste in the resulting sausages. However, overall likeness of samples added with tannic acid and EKWE were not different from that of control (P>0.05), indicating that both tannic acid and EKWE did not affect the overall acceptability of the sausages. Hayes *et al.* (2011) reported that overall texture, tenderness and flavour in cooked pork sausage during storage at 4 ° C were not significantly affected by the addition of lutein, ellagic acid and sesamol. Therefore, tannic acid and EKWE can be

incorporated into fish emulsion sausages without having any detrimental effect on the organoleptic quality of products.

Table 6. Effect of tannic acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) on the sensory properties of fish emulsion sausages at day 0 of refrigerated storage**.**

	Likeness score			
Samples	**Colour**	**Taste**	**Texture**	**Overall**
C	8.80±0.74[a]	7.80±1.03[a]	8.23±0.81[a]	8.31±0.24[a]
TA-0.02	8.87±0.99[a]	7.83±1.11[a]	8.13±1.11[a]	8.22±0.55[a]
TA-0.04	8.63±0.83[a]	7.83±0.95[a]	8.46±1.32[a]	8.31±0.31[a]
EKWE-0.04	8.60±0.99[a]	7.19±1.41[b]	8.28±1.41[a]	8.0±0.84[a]
EKWE-0.08	8.50±0.99[a]	7.02±1.01[b]	8.45±0.61[a]	8.02±0.41[a]

Values are mean ± SD (n=30). Different superscripts in the same column denote the significant difference (P<0.05).

Changes in fishy odour in the emulsion sausages added without and with tannic acid (0.02 and 0.04%) or EKWE (0.04 and 0.08%) during 20 days of refrigerated storage are depicted in Figure 40. Fishy odour intensity in all samples increased continuously with increasing time of storage (P<0.05). For the C, EKWE-0.04 and EKWE-0.08 samples, fishy odour scores increased abruptly within the first 12 days of storage. Nevertheless, TA-0.02 and TA-0.04 samples showed slight increase in fishy odour scores during the first 12 days of storage (P<0.05). At the end of storage, TA-0.04 sample received the lowest fishy odour scores, compared with the others (P<0.05). The retarded development of fishy odour in the TA-0.04 sample correlated well with the lower rate of lipid oxidation (Fig. 39 a and b). The off-odour developed in the fish muscle due to lipid oxidation was considered as fishy (Fu *et al.,* 2008). Recently, Maqsood and Benjakul (2011b) reported that tannic acid at a level of 400 ppm was very effective in retarding the development of fishy odour associated

with haemoglobin mediated lipid oxidation in washed Asian seabass mince during iced storage. Tannic acid at a level of 200 ppm was found to be effective in retarding lipid oxidation and off-odour development in ground and cooked fish (*Scomberomorus commersoni*) stored at 4 ºC (Ramanathan and Das, 1992). Maqsood and Benjakul (2010 b & c) also reported that tannic acid at a level of 200 ppm retarded the lipid oxidation and development of off-odour in the striped catfish slice and ground beef stored under modified atmospheric packaging during refrigerated storage. At the same level (0.04%), tannic acid exhibited the higher ability in preventing the development of fishy odour in fish emulsion sausages than EKWE. Efficacy in retardation of fishy odour by both tannic acid and EKWE was achieved in dose dependent manner.

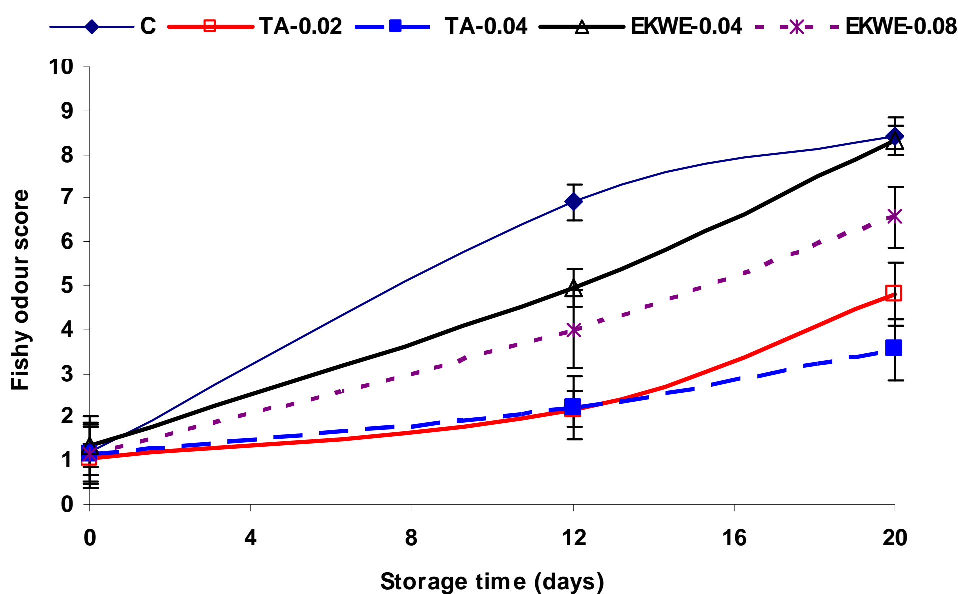


Figure 40. Effect of tannic acid acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) on fishy odour intensity of fish emulsion sausages during 20 days of refrigerated storage. Fishy odour score: 0: no fishy odour and 10: intense fishy odour. Bars represents the standard deviation (n=3). Key: see Figure 39 caption.

8.4.4 Effect of tannic acid and EKWE on colour of fish emulsion sausage

Colour expressed as L^*, a^*, b^* and ΔE^* of sausages added without and with tannic acid (0.02 and 0.04%) or EKWE (0.04 and 0.08%) at day 0 of refrigerated storage is shown in Table 7. After addition of tannic acid at both levels, there was no change in L^* (lightness), a^* (redness) and b^* (yellowness) values of the sausages, compared to the control (P<0.05). However, there was an increase in ΔE^* values in those samples (P<0.05). Balange *et al.* (2009) reported the lower whiteness in surimi gels from big eye snapper with the addition of 0.30% tannic acid, compared with the control gel (P < 0.05). The results correlated well with the sensory property, in which panelists did not detect any difference in the colour between the control and TA-0.02 and TA-0.04 samples (P>0.05). However, the addition of EKWE (0.04 and 0.08%) resulted in the decrease in the lightness (L^*) and an increase in the redness (a^*) and ΔE^* values (P<0.05). Nevertheless, no change in yellowness (b^*) of the sausage was observed (P>0.05). ΔE^* values of the EKWE treated samples were higher than those of tannic acid treated samples (P<0.05). EKWE was darker in colour, in comparison with tannic acid, probably due to the presence of colour compounds like lignin (Balange *et al.,* 2009). The addition of ellagic acid (300 μg/g meat) reduced ($P < 0.001$) L^* value and increased a^* value of raw pork sausages stored in MAP (80% O_2: 20% CO_2) over 21 days of storage at 4 °C (Hayes *et al.,* 2011). Moreover, Hayes *et al.* (2010a) and Hayes *et al.* (2010b) reported that the addition of lutein, natural active compounds, at concentrations of 100 and 200 μg/g sample increased a^* value of minced beef and raw pork patties. Valencia *et al.* (2008) also reported that the addition of green tea catechins (GTC) and green coffee antioxidant (GCA) at a level of 200 mg/kg in fresh pork sausage caused the increase in L^* value ($P < 0.05$), compared to the control. Therefore, the use of tannic acid up to 0.04% had no impact on colour of resulting sausages, while EKWE addition (0.04 and 0.08%) showed the detrimental effect on colour to some degree.

Table 7. Effect of tannic acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) on the *L*, a*, b** and *ΔE** values of fish emulsion sausages at day 0 of refrigerated storage.

Samples	*L**	*a**	*b**	*ΔE**
C	$77.69±0.06^{a}$	$4.54±0.01^{b}$	$21.83±0.04^{a}$	27.25^{c}
TA-0.02	$76.32±0.02^{a}$	$4.61±0.06^{b}$	$21.85±0.02^{a}$	30.03^{b}
TA-0.04	$76.35±0.09^{a}$	$4.74±0.04^{ab}$	$20.69±0.03^{a}$	29.23^{b}
EKWE-0.04	$73.61±0.04^{b}$	$4.87±0.01^{a}$	$21.81±0.01^{a}$	31.95^{a}
EKWE-0.08	$73.34±0.07^{b}$	$4.96±0.05^{a}$	$22.43±0.05^{a}$	32.58^{a}

Values are mean ± SD (n=30). Different superscripts in the same column denote the significant difference (P<0.05).

8.4.5 Effect of tannic acid and EKWE on textural properties of fish emulsion sausage

Texture profile analysis of the emulsion sausages added without and with tannic acid (0.02 and 0.04%) or EKWE (0.04 and 0.08%) at day 0 and 20 of refrigerated storage is shown in Table 8. At day 0, there was no difference in all textural parameters among all samples tested (P>0.05). Hardness, gumminess and chewiness values of all samples decreased after 20 days of storage (P<0.05), while there was no change in springiness and cohesiveness values for all samples (P>0.05). After 20 days, hardness, gumminess and chewiness of all sausages samples decreased at different degrees depending upon the treatments (P<0.05). The results indicated that softening of texture occurred after 20 days of storage, which was probably due to the proteolytic action promoted by muscle endopeptidases (calpains I and II and cathepsins B, D, H and L) (Toldra, 2006) and microbial (bacteria and yeasts) proteinases (Visser, 1993). However, at the end of storage period (day 20), samples added with tannic acid and EKWE showed the higher values on all textural parameters except springiness and cohesiveness, compared to the control (P<0.05).

Table 8. Effect of tannic acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) on texture properties of fish emulsion sausages at day 20 of refrigerated storage.

Storage time (days)	Samples	Hardness (N)	Springiness (cm)	Cohesiveness (ratio)	Gumminess (N)	Chewiness (N cm)
0						
	C	37.83±0.17^{Aab}	0.88±0.15^{Aa}	0.55±0.24^{Aa}	20.78±0.64^{Aa}	18.49±0.74^{Aa}
	TA-0.02	36.82±0.34^{Ab}	0.89±0.12^{Aa}	0.54±0.54^{Aa}	20.84±0.74^{Aa}	19.31±0.52^{Aa}
	TA-0.04	38.19±0.27^{Aa}	0.91±0.23^{Aa}	0.57±0.36^{Aa}	21.96±0.85^{Aa}	20.02±0.61^{Aa}
	EKWE-0.04	37.33±0.29^{Aab}	0.91±0.61^{Aa}	0.54±0.16^{Aa}	18.15±0.46^{Ab}	19.55±0.36^{Aa}
	EKWE-0.08	38.77±0.24^{Aa}	0.90±0.34^{Aa}	0.54±0.25^{Aa}	21.04±0.21^{Aa}	19.06±0.74^{Aa}
20						
	C	28.32±0.63^{Bc}	0.83±0.15^{Aa}	0.40±0.24^{Ba}	11.58±0.47^{Bd}	12.69±0.74^{Bd}
	TA-0.02	31.22±0.46^{Bb}	0.81±0.12^{Aa}	0.48±0.54^{Aa}	14.45±0.26^{Bbc}	14.34±0.52^{Bbc}
	TA-0.04	34.21±0.25^{Ba}	0.88±0.23^{Aa}	0.53±0.36^{Aa}	18.94±0.83^{Ba}	17.62±0.41^{Ba}
	EKWE-0.04	29.65±0.65^{Bbc}	0.83±0.61^{Aa}	0.48±0.16^{Aa}	13.54±0.54^{Bc}	14.02±0.60^{Bb}
	EKWE-0.08	31.02±0.25^{Ba}	0.87±0.34^{Aa}	0.51±0.25^{Aa}	14.64±0.93^{Bb}	14.36±0.54^{Bbc}

Values are mean ± SD (n=6). Different superscripts within the same storage time in the same column denote the significant difference (P<0.05). Different capital superscripts within the same sample in the same column denotes the significant difference (P<0.05).

This might be due to the antioxidative and antimicrobial activity of both tannic acid and EKWE in the sausage samples during storage. Tannic acid showed antimicrobial activity by lowering total viable count as well as psychrophilic bacterial count in striped catfish slices during refrigerated storage under MAP (60%N_2/35%CO_2/5%O_2) (Maqsood and Benjakul, 2010b). The protection of muscle membrane against lipid oxidation by applying antioxidants could also maintain membrane integrity of muscle fibres and reduce moisture loss, which in turn had an effect on the sausage textural properties (Mitsumoto *et al.,* 1995). It was also reported that increased oxidation with the increasing storage time caused degradation of the protein film surrounding fat globules in the emulsion system (Chaiyasit *et al.,* 2005). The retarded lipid oxidation in the samples added with tannic acid or EKWE

might prevent the negative effect caused by oxidation products to some degree. Radicals generated from lipid oxidation could induce the oxidation of protein (Shacter, 2000). Lipid and protein oxidation are closely associated with deteriorative processes occurring in meat products (Mercier *et al.,* 2004). Protein oxidation can negatively affect the sensory quality of fresh meat and meat products in terms of texture, tenderness and colour (Rowe *et al.,* 2004). Results suggested that tannic acid and EKWE could retard the softening of fish emulsion sausages stored for an extended time.

8.4.6 Effect of tannic acid and EKWE on microstructure of fish emulsion sausages

Microstructures of the emulsion sausage samples added without and with tannic acid (0.02 and 0.04%) or EKWE (0.04 and 0.08%) at day 20 of refrigerated storage are illustrated in Figure 41. Emulsion sausages containing tannic acid had the compact structure with less voids. The control and EKWE treated samples had the loosened structure with larger voids than the tannic acid added samples. After 20 days of storage, TA-0.04 sample had the highest hardness, gumminess and chewiness. This was reflected by more compact structure of the sample. Tannic acid at higher level might be involved in decreasing the growth of microorganism, which more likely degraded the protein matrix. Also, tannic acid played a role as antioxidants, in which radicals or oxidation products, capable of destruction of matrix previously formed, could be lowered.

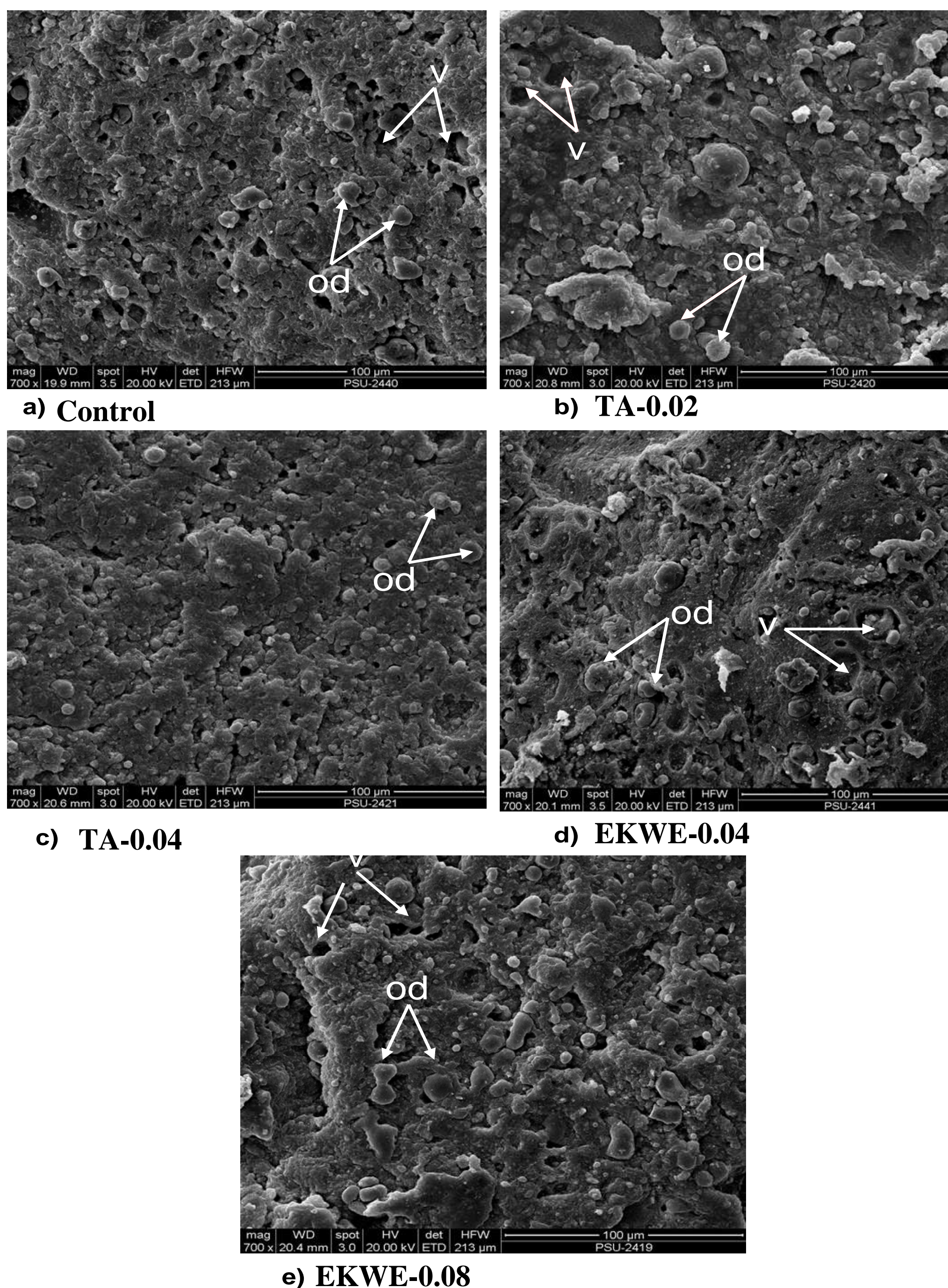


Figure 41. Effect of tannic acid acid (0.02 and 0.04%) and EKWE (0.04 and 0.08%) on microstructure of fish emulsion sausages after 20 days of refrigerated storage. Magnification: 700 x. Key: See Figure 39 captions. od: oil droplet; v: void.

For the distribution of oil droplets and formation of emulsion in the sausages, it was noticed that TA-0.04 sample contained continuously and homogenously dispersed oil droplets, which were smaller in size, compared to others (Fig. 41c). The compact matrix generally confers a greater consistency to the product and promotes hardness of the product (Caceres *et al.,* 2008). Tannic acid at a level of 0.04% might prevent the coalescence of emulsion through its protective role in retardation of the oxidative damage to the protein, which act as an emulsifier. Proteins have emulsifying properties, yielding the stable meat emulsion. The oxidative damage of proteins has an impact on protein solubility, leading to aggregation and complex formation due to cross links, thus impairing their emulsifying property (Karel *et al.,* 1975). Phytochemicals such as lutein, ellagic acid and olive leaf extract might have increased the emulsion stability in the cooked pork sausage through their protective role on proteins against oxidation (Hayes *et al.,* 2011). Thus, tannic acid at level of 0.04% was effective in maintaining the textural properties of the fish emulsion sausages stored at refrigerated temperature for 20 days.

8.5 Conclusion

Tannic acid (0.02 and 0.04%) and EKWE (0.08%) were effective in retarding the lipid oxidation as indicated by lower TBARS formation as well as lower development of fishy odour. EKWE at a level of 0.04% was not effective in preventing the lipid oxidation in the fish emulsion sausages. Addition of tannic acid at both levels (0.02 and 0.04%) in the fish emulsion sausage had no detrimental effect on the sensory properties. Samples treated with EKWE (0.04 and 0.08%) were higher in a* values, however, panelist could not detect any difference in the colour of sausages treated with EKWE, compared to the control. Additionally, tannic acid at a level of 0.04% could maintain the textural properties to the highest extent after storage for 20 days at 4° C. Thus, tannic acid, especially at the higher level (0.04%) can be used as an effective natural antioxidant in the fish emulsion sausages, while EKWE at 0.08% could be an alternative but showed the lower effectiveness.

CHAPTER 9

SUMMARY AND FUTURE WORKS

9.1 Summary

1. Selection of suitable phenolic compound as an antioxidant is of great importance to maximise the prevention of lipid oxidation in different food model systems. Antioxidative activity of phenolic compounds varied with the types and molecular structure. Tannic acid exhibited the higher antioxidative activity and could prevent lipid oxidation effectively in menhaden oil-in-water emulsion as well as in fish mince. This was most likely caused by its radical scavenging activity, LOX inhibitory activity as well as metal chelating activity, especially towards non-haem iron.

2. Shelf-life of refrigerated striped catfish slices could be extended effectively by using tannic acid (200 mg/kg) in combination with MAP (60%N_2 / 35%CO_2 / 5%O_2), which could retard the lipid oxidation and microbial growth during the extended storage. Thus, tannic acid acted synergistically with MAP to maintain the quality of refrigerated striped catfish slices.

3. Blood retained in fish could induce the lipid oxidation, fishy odour development as well as the growth of microorganism during iced storage. Oxidation of lipid mediated by haem along with microbial growth was the main cause of the development of fishy odour in the Asian seabass slices during iced storage.

4. Haemoglobins in the blood could be involved in lipid oxidation and fishy odour development of fish muscle during the extended postmortem storage. Those changes were species specific, mainly governed by the pro-oxidative activity of haemoglobins. Tannic acid displayed the efficacy in prevention of lipid oxidation and retarded development of fishy odour in fish muscle in dose-dependent manner.

5. Ethanolic kiam wood extract (EKWE) could serve as the natural antioxidant in fish muscle containing haemoglobin and oil. EKWE was able to retard the lipid oxidation and fishy odour in the fish emulsion sausages. However, its efficacy was lower than tannic acid. Thus, EKWE could be an alternative natural antioxidant in different fish and fish products and other muscle foods.

9.2 Future works

1. Different phenolic compounds from different sources should be tested for their antioxidative activities in different fish and fish products.

2. Prevention of fishy odour development in fish using the combined methods should be investigated.

3. Role of other haem proteins in lipid oxidation and fishy odour development in fish muscle should be elucidated.

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Educational Attainment

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Scholarship Awards during Enrolment

Prince of Songkla University Graduate Studies grant by Graduate School, Prince of Songkla University, Hat Yai, Thailand.

List of Publications and Proceedings

Publications

1. Balange, A.K., Benjakul, S. and Maqsood, S. 2009. Gel strengthening effect of wood extract on surimi produced from mackerel stored in ice. J. Food Sci. 74: C619-C627.
2. Maqsood, S. and Benjakul, S. 2010. Comparative studies of four different phenolic compounds on *In vitro* antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince. Food Chem. 119: 123-132.
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5. Maqsood, S., Benjakul, S. and Shahidi, F. 2010. Emerging role of phenolic compounds as natural food additives in fish and fish products. Crit. Rev. Food Sci. Nutr. (Accepted).

6. Maqsood, S. and Benjakul, S. 2011. Retardation of haemoglobin mediated lipid oxidation of Asian sea bass muscle by tannic acid during iced storage. Food Chem. 124: 1056-1062.

7. Maqsood, S. and Benjakul, S. 2011. Comparative studies on molecular changes and pro-oxidative activity of haemoglobin from different fish species as influenced by pH. Food Chem. 124: 875-883.

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11. Maqsood, S., Benjakul, S. and Balange, A.K. 2011. Effect of tannic acid and kiam wood extract on lipid oxidation and textural properties of fish emulsion sausages. Food Chem. (Submitted).

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1. Maqsood, S. and Benjakul, S. 2009. *In-vitro* antioxidative effect of different phenolic compounds and their preventive role on lipid oxidation of fish oil emulsion and fish mince. Food Innovation Asia: 2009. The International Food Conference" BITEC, Bangna, Bangkok, Thailand (Poster Presentation).

2. Maqsood, S. and Benjakul, S. 2009. Combined effect of tannic acid and modified atmospheric packaging on shelf-life extension of refrigerated striped catfish

slices *(Pangasius hypothalamus)*. International conference: "11th ASEAN Food Conference, 2009", Brunei Darussalam (Oral presentation).

3. Maqsood, S. and Benjakul, S. 2010. Effect of tannic acid in combination with modified atmospheric packaging on the lipid oxidation and discoloration of the refrigerated ground beef. Food Innovation Asia: 2010. The International Food Conference" BITEC, Bangna, Bangkok, Thailand (Poster Presentation).
4. Maqsood, S. and Benjakul, S. 2010. Use of tannic acid in retardation of haemoglobin mediated lipid oxidation in Asian seabass (*Lates calcarifer*) muscle during iced storage. International conference "BIOSCIENCE" For the Future 2010, PSU, Thailand, October 7-8, 2010 (Oral presentation).
5. Maqsood, S. and Benjakul, S. 2010. Impact of bleeding on the lipid oxidation and quality changes in the seabass (*Lates calcarifer)* slices during iced storage. 1st International Congress on Food Technology, Turkey, November 03–06, 2010 (Poster presentation).
6. Maqsood, S. and Benjakul, S. 2010. Pro-oxidative activity of haemoglobin from different fish species in Asian seabass muscle as influenced by pH. International Conference on Agriculture and Agro-Industry (ICAAI, 2010), Food, Health and Trade, Thailand, November 19-20, 2010 (Oral presentation).