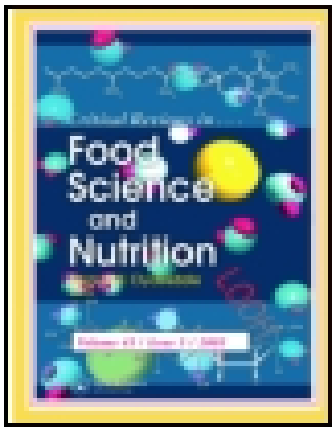

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Undesirable Enzymatic Browning in Crustaceans: Causative Effects and Its Inhibition by Phenolic Compounds

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Undesirable Enzymatic Browning in Crustaceans: Causative Effects and Its Inhibition by Phenolic Compounds

To be submitted to Critical Reviews in Food Science and Nutrition

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Abstract:

Undesirable enzymatic browning mediated by polyphenol oxidase (E.C. 1.14.18.1) on the surface of seafood from crustaceans have been a great concern to food processors, causing quality losses of seafood products. Seafoods especially from crustaceans are worldwide consumed due to their delicacy and nutritional value. However, black spot formation (melanosis) is the major problem occurring in crustaceans during post mortem handling and refrigerated storage induce deleterious changes in organoleptic properties and therefore decreases commercial value. Polyphenoloxidase (PPO), the copper-containing metalloprotein involved in oxidation of phenol to quinone is the major biochemical reaction of melanosis formation. This enzymatic mechanism causes unappealing blackening in post harvest crustaceans. To alleviate the melanosis formation in crustaceans, use of phenolic compounds from plant extract can serve as antimelanotics and appears to be a good alternative to the conventional sulfites which are associated with health-related disorders. In this review, we focuses on the unique features about the structure, distribution and properties of PPO as well as mechanism of melanosis formation and provide a comprehensive deeper insight on the factors affecting melanosis formation and its inhibition by various antimelanotics including newly discovered plant phenolic compounds.

Keywords: crustaceans, melanosis, polyphenoloxidase, plant extract, phenolic compounds.

1. Introduction

Crustaceans are highly perishable with the limited shelf-life, mainly associated with black spot formation during postmortem storage. Although melanosis seems to be harmless to consumers, it drastically reduces the productôs market value, consumerôs acceptability and hence causing the considerable financial loss (Montero *et al*., 2001a). Melanosis is triggered by a biochemical reaction, which oxidizes phenols to quinones by the action of an enzymatic complex called PPO (Nirmal and Benjakul, 2009a). This is followed by non-enzymatic polymerization and autoxidation of the quinones, giving rise to dark pigments of high molecular weight (Benjakul *et al.*, 2005). Melanosis is also influenced by the method of capture, molting stage, species, protease and season (Opoku-Gyamfua *et al*., 1992). Lopez-Caballero *et al* (2007) reported that melanosis and spoilage were retarded during frozen storage, but continued in defrosted shrimp. As per the shelf life of crustaceans is concerned, melanosis is a more important limiting factor than microorganisms (Martinez-Alvarez *et al*., 2005).

To extend the shelf-life and prevent the melaonosis formation in shrimps or crustaceans, melanosis inhibitors have been used. Sulfites and their derivatives are widely used as PPO inhibitor by preventing the polymerization of quinones, combining irreversibly with them, and forming colorless compounds (Montero *et al.*, 2001b). However, sulfiting agents are known to produce allergic reactions and serious disturbances in asthmatic subjects (DeWitt, 1998). Therefore, safe compounds from natural origin such as ficin (Taoukis *et al*., 1990), kojic acid (Chen *et al*, 1991), oxalic acid (Son *et al.*, 2000), enokitake extract (Jang *et al.*, 2003), dodecyl gallate (Kubo *et al.*, 2003) and grape seed extract (Gokoglu and Yerlikaya, 2008) have been reported as the melanosis inhibitors.

Plant polyphenols are other promising agents possessing antimelanotic properties, antimicrobial and antioxidant activities (Gokoglu and Yerlikaya, 2008; Chanthachum and Beuchat, 1997; Souza *et al.*, 2008). Plant polyphenols such as flavonoid compounds, tocopherols, coumarins and cinnamic acid derivatives have an antioxidative effect (Jayaprakash *et al.*, 2001). Chiari *et al*. (2010) have reported that different variety of native aerial plants extracts from central Argentina exhibited more than 90% inhibition of mushroom tyrosinase monophenolase activity at 1000 µg/mL. Recently, it has been reported that the ferulic acid (Nirmal and Benjakul, 2009a) and catechin (Nirmal and benjakul, 2009b), green tea extract (Nirmal and Benjakul, 2011a) and lead (*Leucaena leucocaphala*) seed extract (Nirmal and Benjakul, 2011b) could inhibit PPO and lower the melanosis formation in Pacific white shrimp (*Litopenaeus vannamei*) during iced storage. Additionally, several plant extracts can be used as the natural additive, which is able to prevent melanosis as well as to lower the chemical and microbial changes of crustacean during iced storage. The present paper presents a review about the melanosis inhibition by various antimelanotics including newly discovered plant phenolic compounds. The information gained could provide the important and potential approach for seafood processor to keep the prime quality of crustaceans during handling and storage by using natural and safe additives. As a consequence, the market value of crustaceans can be maintained.

2. Polyphenoloxidase in crustaceans

PPO plays an essential role in physiological functions, particularly for the sclerotization of the cuticle of crustaceans such as shrimp and lobsters. Sclerotization is the hardening of the chitin shell after molting, which is part of the growing phase for the organism (Terwilliger and

Ryan, 2006). The highly reactive *o*-quinones produce by oxidation of phenolic compounds particularly tyrosine in crustacean, cross-link with histidyl residues of cuticular proteins and chitin, resulting in hardening of the exoskeleton (Xu *et al*., 1997). Secondly, PPO is an important component of the immune system and self-recognition (Huang *et al*., 2010). The enzyme located in the cuticle and hemolymph of crustaceanôs blood as zymogen form. The enzyme activity was greatly enhanced by components of microorganism cell walls, such as lipopolysacchrides and ß-1,3- glucans (Perazzolo and Barracco,1997). Zotos and Taylor (1997) reported that three different proteases are involved in PPO activation namely protease I (possibly Zn-thiol protease), protease II (thiol protease) and protease III (Zn-serine protease).

2.1. Molecular structure of polyphenoloxidase:

The active site of PPO (EC 1.14.18.1) consists of two copper atoms and three states, namely oxy-PPO [Cu (II) Cu (II) O2], met-PPO [Cu (II) Cu (II)O] and deoxy-PPO [Cu (I) Cu (I)] as shown in Figure 1. Oxy-PPO has two tetragonal Cu (II) atoms. Each Cu atom is coordinated by three N-His ligands, consisting of a weak axial and two strong equatorial bonding. The exogenous oxygen molecule (O_2) is bound to this site as peroxide and bridges the two Cu ions. Met-PPO has a tetragonal bi-cupric structure with endogenous oxygen bridge (Solomon *et al.,* 1996). Deoxy-PPO has a bi-cuprous structure Cu (I)ï Cu (I). No oxygen bridge is present in this structure. Two-electron reduction to the de-oxy site followed by binding of molecular oxygen regenerates oxy-tyrosinase (Sanchez-Ferrer *et al.*, 1995).

2.2. Biocatalysis by polyphenoloxidase:

PPO is a bi-functional, copper-containing enzyme which catalyzes two basic reaction, in the presence of molecular oxygen, the *o*-hydroxylation of monophenols to give *o*-diphenols (Monophenol oxidase, EC 1.14.18.1) and the further oxidation of *o*-diphenols to *o*-quinones (Diphenoloxidase, EC 1.10.3.1) (Garcia-Molina *et al.*, 2005). PPOs have broad substrates specificities and are able to oxidize a variety of mono, di or polyphenols.

2.2.1. Monophenol oxidase:

It catalyzes the hydroxylation of monophenols to diphenols. As shown in Figure 2, the monophenolase activity begins with the binding of the substrate monophenol to one of the Cu atoms of the oxygenated form (oxy-PPO) to generate oxy-PPO (Rodriguez-Lopez *et al*., 1992). Then, *o*-hydroxylation of the monophenol by the bound peroxide occurs, and an enzyme-coordinated *o*-diphenol structure (Met-D) is formed. It should be noted that monophenol can react with oxy-PPO, but not with met-PPO, to form the product *o*-quinone. Monophenolase activity shows a characteristic lag period. This may be due to the fact that PPO in the resting form contains 15 % oxy sites, which is the only form that can react with monophenol substrates (Likhitwitayawuid, 2008).

2.2.2. Diphenol oxidase:

It catalyzes the oxidation of the di-phenol to quinones. The reaction of oxidation of diphenol to quinone has shown in Figure 2. This reaction received more attention because of its faster rate than the monophenol oxidase and its association with the formation of quinones, which polymerize with amino acids / protein or self polymerize, non-enzymetically to form

melanin (Figure 3) (Garcia-Molina *et al.*, 2005). The *o*-diphenol can react with both the oxy and the met forms to produce *o*-quinone The reaction of diphenol with met-PPO converts the enzyme to the de-oxy form, bringing it into the monophenolase cycle (Solomon *et al.*, 1996). When quinones are formed and undergo polymerization with protein or amino acids or self polymerized, they form a melanoid compound (dark brown color) (Satoh *et al.*, 1999).

3. PPO activity and distribution in crustaceans

PPO is localized in the carapace of the cephalothorax, in the caudal zone and in the cuticle of the abdomen, mainly where the cuticle segments are joined and where the cuticle is connected to the pleopods (Benjakul *et al*., 2005). PPO from different species, body location, sex, molting stage and tissue might have different activity levels as well as varying properties (Ferrer *et al*., 1989). PPO is distributed in many parts of shrimps with different levels of activity (Montero *et al*., 2001a; Zamorano *et al*., 2009). PPO also localizes in the surface membrane covering the muscle and in the hemolymph (Haung *et al*., 2010). When the activity of partly purified PPO from different body portions of deep water pink shrimp was determined, the greatest PPO activity was found in the carapace, followed by the abdomen exoskeleton, cephalothorax and pleopods and telson (Zamorano *et al*., 2009). The muscle and the pereopods and maxillipeds showed no detectable PPO activity.

PPO from different shrimps comprised the different iso-forms with varying molecular weights (Chen *et al*., 1991). Also, the molecular weight of PPO varied with the molting stage (Ferrer *et al*., 1989). PPO from the kuruma prawn cephalothorax had the molecular weight of 160 kDa (Benjakul *et al*., 2005). For PPO from shrimp (*P. setiferus*), its molecular weight was

30 kDa (Simpson *et al*., 1987). The molecular weights of pink shrimp PPO were 30 and 35 kDa, while those of white shrimp were 20 and 25 kDa (Chen *et al*., 1997). Zamorano *et al*. (2009) reported that PPO with the molecular weight of 500 kDa and 200 kDa were found in deep water pink shrimp (*Parapenaeus longirostris*). PPO from cephalothorax of Pacific white shrimp (*Litopenaeus vannamei*) had molecular weight of 210 kDa (Nirmal and Benjakul, 2009a).

3.1. Hemocyanin derived PPO:

Hemocyanin is the main component of hemolymph and shows strong structural resemblance with PPO (Martinez-Alvarez *et al*., 2008). It is normally found in an inactive state (proPPO), but if required it is activated after limited proteolysis with serine proteases (Decker and Jaenicke, 2004). The pro-PPO activating system present in the hemolymph of crustaceans and other arthropods is regarded as an important component of the immune system and plays an important role in defense system (Huang *et al*., 2010). The potential contribution of hemocyanin to the PPO activity have been reported in some crustacean such as kuruma prawn (*Penaeus japonicus*, Adachi *et al*., 2001) deep water pink shrimp (*Parapenaeus longirostris*, Martinez-Alvarez *et al*., 2008), Norway lobster (*Nephrops norvegicus*, Gimenez *et al*., 2010) and crab (*Chionoecetes japonicus*, Encarnacion *et al*., 2011).

4. Melanosis in crustaceans

The host defense mechanism, in which quinones are formed and get polymerized with protein or amino acids or self polymerized. They form a melanoid compound (dark brown color) which is possesses active antibacterial or antifungal activities. Unfortunately, PPO can cause

browning of the shell post harvest, which affects the quality of these products and consumers acceptability. Melanosis or browning in sea foods occurs primarily in crustaceans (Figure 4).

The spread of melanosis in pink shrimp (*Penaeus duorarum*) is faster than in white shrimp (*Penaeus setiferus*). This could be related to the difference in levels of substrate or levels of enzyme concentration or enzymatic activity in each species (Simpson *et al*., 1987). Severe melanosis on these products can cause tremendous economic losses in the market (Nirmal and Benjakul, 2009b).

4.1. Factors affecting melanosis

4.1.1. Species and Sex:

PPO activity varies with species. PPO from pink shrimp was more active than that from white shrimp (Simpson *et al*., 1987). In chilled shrimp, the rate of spread of melanosis differs among the various species. This could be related to differences in levels of substrate or levels of enzyme concentration or enzymatic activity in each species (Simpson *et al*., 1987; Montero *et al*., 2001a). Melanosis develops very rapidly in deepwater pink shrimp (Martinez-Alvarez *et al*., 2008). Radhika *et al*. (1998) reported that the phenoloxidase activity in the hemolymph of male fairy shrimp *Streptocephalus dichotomus* is only 1/3rd of that of females. In general, crustaceans do not possess immunoglobulin and they defend themselves against invading parasites or microorganisms by other defense mechanisms (Ashida & Yamazaki, 1990). PPO activity in cuticles of juvenile and adult male deep water rose shrimp (*Parapenaeus longirostris*) captured in Italy from August 2006 to June 2007 was found to be double than those of female shrimp (Bono *et al*., 2010).

4.1.2. Molting stage:

Synthesis and hardening of a new exoskeleton are essential parts of arthropod growth and molting. In both crustaceans and insects, the hardening of the newly formed exoskeleton or cuticle is referred to as sclerotization (Terwilliger and Ryan, 2006). Several essential components are involved like cuticle protein, phenols or catechols and important one is PPO. The phenols become enzymatically oxidized to highly reactive intermediates that interact with the cuticular proteins and chitin to harden and cross-link them. Ali *et al*. (1994) reported that the molting fluid was the source of the natural activators of pro-PPO. Ogawa *et al*. (1983) reported the relationship between the appearance of black discoloration and molting cycle in the integumentary tissue of the tails of *Panulirus laevicauda*. They suggested that the physiological factors in the live state may contribute to melanin formation. The preparation for molting is accompanied by an increase in oxygen consumption by the entire animal, resulting in increased metabolism in some or all tissues. In addition, Brookhart and Kramer (1990) reported that the molting fluid contained more than 10 proteolytic enzymes, possibly a complex and unique mixture of endo- and exo- cleaving proteolytic enzymes, which related to the activation of pro-PPO to PPO.

4.1.3. Proteases and protein:

In crustaceans, PPO is localized in the cuticle and hemolymph as a zymogen or proPPO form, which can be activated by protease, fatty acids and lipids, laminarin (β 1,3- glucan) acetone, alcohol and sodium dodecyl sulfate (Ferrer *et al*., 1989). The activation causes the formation of melanins, which possess antimicrobial properties. However, Trypsin had no effect

on PPO activity from kuruma prawn cephalothorax (Benjakul *et al*., 2005) and shrimp (*Penaeus setiferus*) (Simpson *et al*., 1987), suggesting that the activated PPO might be present in the extract. However, Norway lobster prophenolase was activated by the addition of trypsin (Yan and Taylor, 1991). In addition, Zn-serine protease, Zn-thiol protease and thiol protease were found to activate prophenolase from Norway lobster (Zotos and Taylor, 1997). Gollas-Galvan *et al*. (1999) reported that proPPO purified from blood cell of brown shrimp (*Penaeus californiensis*) is a 114-kDa monomeric protein, which can be hydrolyzed by proteinases, producing a 107-kDa active PPO. During post-mortem storage of crustaceans, proPPO can be also activated into PPO by the action of proteolytic enzymes leaching from the digestive tract. Moreover; protein hydrolysis by these proteases originates substrates for active PPO (Ali *et al.*, 1994). Lee *et al*. (2000) showed that the 36-kDa LGBP (lipopolysaccharide- and ß-1,3-glucan-binding protein) plays a role in the activation of the proPPO activating system in freshwater crayfish (*Pacifastacus leniusculus*).

4.1.4. Method of capture and season:

Capture, rough handling of the catch and other ótraumaticô events seem to trigger a defense mechanism in shellfish involving the activation of PPO, resulting ultimately in increased black spot. Lobster and shrimp can be induced to form melanin by injuring them while alive (Ogawa, 1987). Danish Norway lobster processors have reported an annually recurring rise in black spot-related problems around September each year. Annual drop in catch quality was truly related to changes in PPO activity (Bartolo and Birk, 1998). PPO activity in cuticles of juvenile and adult males and females deep water rose shrimp (*Parapenaeus longirostris*) captured in Italy

was found to be highest during late summer (August and September) and was lowest between February and March (Bono *et al.*, 2010).

5. Prevention of melanosis

Many studies have focused on various techniques and mechanisms to control these undesirable enzyme activities. These techniques attempt to eliminate one or more of the essential components (oxygen, enzyme, copper, or substrate) from the reaction (Gokoglu and Yerlikaya, 2008). Enzymatic browning can be inhibited by targeting the enzyme, the substrates (oxygen and polyphenols) or the products of the reaction. Melanosis inhibitors can be classified into different groups according to their mode of action

5.1. Acidulants:

pH of the chemical has the tendency to affect ionizable groups of protein by reduction or oxidation; it can affect enzyme and substrate by changing ionization state and breakdown of structural conformation, respectively. These groups must be in the appropriate ionic form in order to maintain the conformation of the active site, to bind substrates, or to catalyze the enzymatic reaction. Generally acidulants maintain the pH below that required for optimum catalytic activity of an enzyme. Acidulants are often used in combination with other antibrowning agents. Acidulants such as citric, malic, and ascorbic acids are capable of lowering the pH (å 3) of a system, thus rendering PPO inactive (Montero *et al*., 2001a). Citric acid is the one of the most widely used acid in the food industry. Citric acid exerts inhibition on PPO by reducing the pH as well as by chelating the copper at the enzyme-active site. Benner *et al*. (1994)

reported that brown shrimp *(Penaeus aztecus*) treated with L-lactic acid in combination with 4-hexylresorcinol (0.0025 %) had the decrease in melanosis.

5.2. Chelating agents:

PPO possess metal ion at their active site for the functional activity. These metal ions are participated in enzyme reaction. Removal of these metal ion by chelating agents make enzyme inactive. Chelating agents are able to form complex with PPO activators such as copper and iron ions, through an unshared pair of electrons in their molecular structures (Kim *et al*., 2000). The well known chelating agent is EDTA (ethylene diamine tetra acetic acid). Chelators used in food industry are polycarboxylic acid, polyphosphate and EDTA. Kojic acid has potential applicability in the prevention of melanosis in both plant and seafood products (Chen *et al*., 1991). The phenolic derivatives of benzoic acid appear to act as chelating agents of copper (Montero *et al.,* 2001a).

5.3. Reducing agents:

The most widely used chemicals in preventing enzymatic browning are reducing agents such as sulfiting agent, ascorbic acid, cysteine and glutathione. Reducing agents prevent enzymatic browning either by reducing *o*-quinones to colorless diphenols, or by reacting irreversibly with *o*-quinones to form stable colorless products (Ferrer *et al*., 1989). Ascorbic acid is highly water-soluble, which is acidic and moderately strong reducing compound. Ascorbic acid also acts as an oxygen scavenger for the removal of molecular oxygen in PPO reactions. Lopez-caballero *et al*. (2007) reported that PPO inhibition by ascorbic acid has been attributed to

the reduction of enzymatically formed *o*-quinones to their precursor diphenols. The inhibition of melanosis by sulfhydryl compounds, such as cysteine and glutathione is thought to be due to the formation of colorless thiol-conjugated *o*-quinones (Benjakul *et al*., 2006). Cysteine-quinone adducts were proved to be the competitive inhibitors of PPO (Richard-Forget *et al*., 1992).

Sulfiting agents (sulfur dioxide, SO_2; sulfite, SO_3; hydrogen sulfite, HSO_3; metabisulfite, S_2O_5) are the most widely applied reagents for the control of browning in the food industry (Gokoglu and Yerlikaya, 2008). Bisulfite (HSO_3^-) is a competitive inhibitor of PPO by binding a sulfhydryl group on the PPO active site (Madero and Finne, 1982). Inhibition on the PPO catalyzed melanosis in lobster was accomplished by bisulfite via its reaction with intermediate quinones forming sulfoquinones, and via its complete inactivation of PPO (Ferrer *et al*., 1989). Martinez-Alvarez *et al*. (2005a) reported that prawns (*Marsupenaeus japonicus*) treated with sulfite-based solution had the lowest melanosis score up to 8 days. Gomez-Guillen *et al*. (2005) used sodium metabisulfite (6.2 to 50 gKg^{-1}) to prevent melanosis in fresh deepwater pink shrimp (*Parapenaeus longirostris*) by immersion method for 1 h. The FDA has proposed the maximum residual sulfur dioxide levels for certain foods. Shrimp products having residual sulfite levels above 100 ppm are considered adulterated, since these levels are considered unsafe. It has been necessary to search for alternatives that show effective inhibitory effect on melanosis but are devoid of health concerns to consumers (Chen *et al*., 1991).

5.4. Enzyme inhibitors:

4-Hexylresorcinol (4-HR) has several advantages over sulfites in food, including its specific mode of inhibitory action, its lower level required for effectiveness, its inability to

bleach preformed pigments, and its chemical stability (McEvily *et al*., 1992). 4-HR acts as an enzyme-competitive inhibitor due to structural resemblance to phenolic substrates (McEvily *et al.,* 1991). McEvily *et al*. (1991) reported that dipping shrimps, brown shrimp (*Penaeus aztecus*) and pink shrimp (*Penaeus duorarum*), into 50 ppm 4-HR in sea water with subsequent storage on crushed ice inhibited black spot formation up to 14 days. Lambrecht (1995) reported that the headless brown shrimp (*Penaeus aztecus*) dipped in 4-HR for 1 min controlled black spot formation for a longer period of time than the control or those treated with 1.25 % sodium metabisulfite. 4-HR alone or in combination with ascorbic or citric acid, was effective as inhibitors of melanosis and microbial spoilage in prawns (*Penaeus japonicus*) (Montero *et al.*, 2001b). Montero *et al*. (2006) studied inhibition of melanosis in pink shrimp (*Parapenaeus longirostris*) treated by immersion and dusting method with various concentration (0.0025 to 5 g of inhibitor per 100 g of shrimp) of 4-HR during chilled storage of 12 days. Melanosis inhibition increased with inhibitor concentration.

6. Phenolic compound

Phenolic compounds are naturally occurring secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Ryan and Robards, 1998). They are present in fruits, vegetables, leaves, nuts, seeds, flowers, and barks (Sellappan *et al.*, 2002). These compounds are of considerable physiological and morphological importance in plants (Balasundram *et al.*, 2006). The functionality of phenolic compounds is expressed through their action as an inhibitor or an activator for a large variety of mammalian enzyme system and as metal chelators as well as scavengers of free radicals (Pringent, 2005; Rice-Evans *et al.*, 1997;

Shahidi *et al.*, 2007). Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables, because of their antioxidant activity (Balasundram *et al.*, 2006; Sellappan *et al.*, 2002) and could therefore be a natural source of antioxidants.

6.1. Chemistry of phenolic compound:

Chemically, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl groups, and range from simple phenolic molecules to highly polymerized compounds (Dykes and Rooney, 2007). Most naturally occurring phenolic compounds are present as conjugates with saccharides (mostly mono and disaccharides), linked to one or more of the phenolic groups by acid-labile hemiacetal bond (Ryan and Robards, 1998) and may also occur as functional derivatives such as esters and methyl esters (Balasundram *et al.*, 2006). Despite the structural diversity, the wide range of phenolic compounds are often referred to as polyphenols and basically be categorized into several classes as shown in Table.1 (Ryan and Robards, 1998; Naczk and Shahidi, 2004; Balasundram *et al.*, 2006; Dykes and Rooney, 2007).

Phenolic acids consist of two subclasses, hydroxybenzoic acids and hydroxycinnnamic acids. Hydroxybenzoic acids (C6-C1) include gallic, *p*-hydroxybenzoic, vanillic, syringic and protocatecguic acids. The hydroxycinnamic acids have C6-C3 structure and include coumaric, caffeic, ferulic, and sinapic acids (Dykes and Rooney, 2007). The flavonoids are a large class of compounds, ubiquitous in plants (Rice-Evans *et al.*, 1997). Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C_6-C_3-C_6 configuration. They contain several phenolic hydroxyl functional groups attached to ring structures, designated as A,

B and C (Figure 5) (Balasundram *et al*., 2006). The aromatic ring A is derived from the malonate pathway, while ring B is derived from phenylalanine through shikimate pathway (Balasundram *et al*., 2006).

Variation in substitution pattern to ring C result in the major flavonoid classes; Flavonols (e.g. quercetin), with the 3-hydroxy pyran-4-one C ring; Flavones (e.g. apigenin), lacking the 3-hydroxyl group; Flavanols (e.g. catechin), lacking the 2,3-double bond and the 4-one structure; Isoflavones (e.g. genistein), in which the B ring is located in the 3 position on the C ring; Anthocyanidin (e.g. cyanidin), in which 3,4 double bond is present and lacking in 4-one (Rice-Evans *et al.*, 1997). Tannins, the relatively high molecular weight compounds, which constitute as the third important group of phenolics may be subdivided into hydrolysable tannin, which is ester of gallic acid and condensed tannins, are polymers of polyhydroxy flavanol monomers (Naczk and Shahidi, 2004).

6.2. Extraction of phenolic compounds

The structural diversity of phenolic compounds varies from simple to highly polymerized substances that include varying proportions of phenolics, distributed randomly in plants at the tissue, cellular and subcellular levels. They may also exist as complexes with carbohydrates, proteins and other plant components (Luthria *et al.*, 2006). Extraction of phenolic compounds in plant materials is therefore influenced by their chemical nature, the extraction method employed, sample particle size, storage time and conditions as well as the presence of interfering substances (Naczk and Shahidi, 2004). Therefore, phenolic extracts of plant materials are always mixture of different classes of phenolics that are soluble in the solvent system used. Solubility of phenolic

compounds is governed by the type of solvent (polarity) used, degree of polymerization of phenolics, as well as interaction of phenolics with other chemical constituents. Therefore, there is no uniform or completely satisfactory procedure that is suitable for extraction of all phenolics or a specific class of phenolic substances in plant materials (Luthria *et al.*, 2006). Methanol, ethanol, acetone, water, ethyl acetate and their combinations are frequently used for the extraction of phenolics. Mechanical techniques used to enhance molecular interaction between the phenolic compounds and solvent are reflux, maceration, stirring, sonication and microwave assisted extraction.

Row and Jin (2006) reported the recovery of catechin compounds from Korean tea by solvent extraction. The optimum extraction condition was 80^{o}C for 40 min in pure water. The extract was partitioned with water/chloroform (1:1 v/v), which was suited to remove caffeine impurity from the extract. The resulting extract was further partitioned with water/ethyl acetate (1:1 v/v) to deeply purify the catechin compounds. Torre *et al.* (2008) investigated the release of ferulic acid from corn cobs by alkaline hydrolysis. Optimum hydrolysis condition for hydroxy-cinnamic acids content was 0.5 N NaOH and solid/liquid ratio of 0.084 for 6 h Rusak *et al.* (2008) extracted phenolics from bagged and loose leaves of white and green tea by distilled water (80^{o}C), distilled water (80^{o}C) with 5 ml of lemon juice and aqueous ethanol (10, 40 and 70 %). Addition of lemon juice could enhance the extraction of phenolic from white tea and aqueous ethanol (40 %) was most effective in the extraction of catechins. Extracction of ferulic acid and vanillin from flax shives, wheat bran and corn bran were carried out by non-pressurised alkaline hydrolysis (0.5 M NaOH) and pressurized solvents (0.5 M NaOH, water, ethanol and ammonia) (Buranov and Mazza, 2009). However, solvent used for extraction of phenolic

compounds should be completely evaporated by means of rotary evaporator and powdered extract can be used to treat crustaceans.

6.3. Identification and characterization of phenolic compounds

The analytical technique that has dominated the separation and characterization of phenolic compounds is high performance liquid chromatography (HPLC) with reverse phase (RP) column technology. Other less common means of detection, coupled to Liquid chromatography (LC) are through electrochemical detection (EC), fluorescence (F), and mass spectrometry (MS). There is no single wavelength appropriate to monitor all phenolics since they display the absorbance maxima at different wavelengths. Most benzoic acid derivatives show the maximum absorbance close to 254 nm. However, gallic acid, salicylic and syringic acid have their maxima absorbance at 275, 310 and 280 nm, respectively. Chlorogenic, caffeic and *p*-coumaric acid have the maximum absorbance close to 325 nm, and ferulic acid close to 310 nm (Herrera and Luque de Castro, 2005).

Ma *et al*. (2009) detected seven phenolic compounds of two families including cinnamic acids and benzoic acid from citrus peel extract using C-18 reverse phase column and elution was performed with the mobile phase consisting of 4 % (v/v) acetic acid in water: 100 % methanol (80:20, v/v) at solvent flow rate of 1 mL/min. Charrouf *et al*. (2007) studied the separation and characterization of phenolic compounds in argan fruit pulp using liquid chromatography (LC) negative electrospray ionization (ESI) mass spectroscopy (MS/MS). A C-18 (50 x 2.1 mm, i.d 3.5 εm) was used for the separation and gradient elution was performed with water/0.05% acetic

acid (solvent A) and acetonitrile (solvent B) at a flow rate of 600 ε L/min. Sixteen compounds were identified, mainly flavonoid glycosides and flavonoid aglycons.

Weisz *et al*. (2009) extracted eleven phenolic compounds from defatted sunflower (*Helianthus annuus* L.) kernels and shells and characterized by HPLC with diode array and electrospray ionization (ESI) mass spectrometric detection in negative mode. The column used was 150mm x 3.0mm inner diameter, 4 ε m particle size, C18 Hydro-Synergi. The mobile phase consisted of 2 % (v/v) acetic acid in water (eluent A) and of 0.5 % acetic acid in water and acetonitrile (50:50, v/v, eluent B) with flow rate of 0.4 mL/min. UV-Visable spectra were recorded in the range of 200-600 nm. Five phenolic compounds, namely gallic acid, procyanidin B2, (-)-galllocatechin, (-)-epicatechin, and (-)-epicatechin-3-gallate were identified from 50 % ethanol extract of litchi (*Litchi sinensis* Sonn.) seeds, after application of reverse phase high performance liquid chromatography, coupled to a diode array detector and electrospray ionization mass spectra (Prasad *et al.*, 2009). The samples were eluted with gradient system consisting of solvent A (2 % acetic acid, v/v) and solvent B (acetonitrile:methanol, 10:15, v/v), with a flow rate of 1 mL/min from C-18 column (250 x 4.6 mm, 5 ε m particle size). UV-visible absorption spectra were recorded from 200 ï 600 nm during HPLC analysis.

6.4. Antioxidant activity of phenolic compounds*:*

The antioxidant activity of phenolic compounds depends on the structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings. The chemical activity of phenols in terms of their reducing properties as hydrogen or electron donating agents predicts their potential for action as antioxidants (Rice-Evans *et al.*,

1997). Radical-scavenging activity (1,1-diphenyl-2-picrylhydrazyl method) and total antioxidant activity (phosphomolybdenum method) of grape (*Vitis vinifera*) seed extracts of acetone:water:acetic acid (90:9.5:0.5) and methanol:water:acetic acid (90:9.5:0.5) were determined at 25 and 50 ppm concentration. It was found that acetone:water:acetic acid (90:9.5:0.5) extract showed the higher radical scavenger than methanol:water:acetic acid (90:9.5:0.5) extract (Jayaprakasha *et al.*, 2003). Negi *et al.* (2005) successively extracted seabuckthorn (*Hippophae rhamnoides* L.) seeds with chloroform, ethyl acetate, acetone and methanol using soxhelt extractor for 8 h each. The reducing power and antioxidant activities of natural crude methanol extract evaluated using 1,1-diphenyl-2-picrylhydrazine and liposome model system were the highest. Shahidi *et al*. (2007) evaluated the antioxidant activity in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproduct ethanol extract. Extracts of hazelnut byproducts (skin, hard shell, green leafy cover, and tree leaf) exhibited the stronger antioxidant activities than hazelnut kernel at all concentrations tested. Hazelnut extracts examined showed different antioxidative efficacies, related to the presence of phenolic compounds. Among samples, extracts of hazelnut skin, in general, showed superior antioxidative efficacy and higher phenolic content as compared to other extracts. Prasad *et al.* (2009) reported that 50 % ethanol extract of *Litchi sinensis* Sonn. seeds showed total antioxidant capacity, scavenging the 1,1-diphenyl-2-picryl hydrazyl radical and inhibitory activity against lipid peroxidation, at all concentration tasted (25- 100 ε g/mL) and it was comparable to the activity of the synthetic antioxidant, butylated hydroxyl toluene. Nirmal and Benjakul, (2011a) reported that the green tea extract had the higher reducing and DPPH radical scavenging activities, compared with mulberry tea extract ($P < 0.005$). Thus, phenolic compounds could lowered the

melanosis formation in crustacean by reduction of DOPA-chrome to DOPA, possibly via its ability to donate electrons to intermediate quinoe, DOPA-chrome (Nirmal and Benjakul, 2009a; 2009b).

6.5. Metal-chelating activity of phenolic compounds:

Phenolic compounds had capacity of chelating metal ion, which is dependent on the number of hydroxyl group in orhto position (Maqsood and Benjakul, 2009). The stability of metal-phenolic compound complex is higher in six-membered than five-membered ring complexes (Wettasinghe and Shahidi, 2002). Maqsood and Benjakul (2009) reported that catechin showed the highest metal chelating activity, followed by caffeic acid, tannic acid and ferulic acid. Green tea extract had the higher copper chelating activity, compared with mulberry tea extract ($P < 0.005$) (Nirmal and Benjakul, 2011a). Borage and evening primrose crude extracts and their fractions exhibited strong metal chelating activities in aqueous media (Wettasinghe and Shahidi, 2002). Copper ions were most effectively chelated by the constituents of the tannin fractions of hazelnuts, walnuts and almonds (Karamac, 2009). Copper chelating activity of ferulic acid, catechin and mimosine has been reported by Nirmal and Benjakul (2011b; 2011c). Thus, the efficiency in metal chelation varied with the type of phenolic compounds. PPO is a metalloprotein, which is dependent on copper for its activity. Therefore, the copper chelating capacity of phenolic compounds is one of the important inhibitory mechanisms involved in the inhibition of PPO.

6.6. Antibrowning activity of phenolic compounds:

Aromatic carboxylic acids of cinnamic acid and its analogues, *p*-coumaric, ferulic, and sinapic acids are competitive inhibitors of PPO (Kim *et al.*, 2000). Some phenolic compounds inhibit PPO activity by interacting with active site of the enzyme (Janovitz-klapp *et al.*, 1990). Furthermore, phenolic compounds could interact with protein or enzymes via hydrogen bond or hydrophobic interaction (Prigent, 2005). Chen *et al*. (1991) found that kojic acid showed a mixed-type inhibition for white shrimp, grass prawn, and lobster polyphenoloxidase. Among the various phenolic acids tested, kojic acid showed the highest inhibitory effect on browning in apple slices (Son *et al.*, 2001). Cuminaldehyde (*p*-isopropylbenzaldehyde) was identified as potent mushroom tyrosinase inhibitor (ID_{50} =7.7 εg/mL) in cumin (Kubo and Kinst-Hori, 1998). Kubo *et al*. (2003) reported that 1.55 mM dodecyl gallate exhibited the inhibitory activity towards mushroom tyrosinase, in which 50 % activity loss was obtained. Prasad *et al*. (2009) identified gallic acid, procyanidin B2, (-)-gallocatechin, (-) - epicatechin, and (-)-epicatechin-3-gallate in extract of *Litchi sinensis* Sonn. seeds. This litchi seed extract showed inhibitory activity of tyrosinase in a concentration dependent manner (25-100 εg/mL). Gokoglu and Yerlikaya (2008) found that shrimp (*Parapenaeus longirostris*) treated with ethanol extract of grape seed (*Vitis vinifera* sp.) at concentration of 1.5 % had the lowered the melanosis formation during storage at 4^{o}C for 3 days. Stilbenes are C6-C2-C6 compounds, found in nature as monomers and oligomers. They possess inhibitory activity towards mushroom tyrosinase (Likhitwitayawuid, 2008). Jang *et al*. (2003) reported that shrimp (*Trachypenaeus curvirostris*) treated with 70% acetone extract of enokitake mushroom (*Flammulina velutipes*) (2.5 g wet enokitake/mL, test sample) had the delayed darkening at 24^{o}C for 20 h as compared to control. Recently, ferulic acid (Nirmal and Benjakul, 2009a), catechin (Figure 6A; Nirmal and Benjakul,

2009b), green tea extract (Nirmal and Benjakul, 2011a), lead seed extract (Figure 6B; Nirmal and Benjakul, 2011b) and mimosine (Nirmal and Benjakul, 2011c) has been shown to inhibit PPO activity from Pacific white shrimp and lowered melanosis formation during iced storage.

Conclusion

PPO is an important enzyme in the seafood industry and its activity causes melanosis formation during post-harvest storage and lowered consumerôs acceptance leading to economical losses. Melanosis formation in crustacean was affected by species, molting stage, sex, and method of capture. The improvement of methods to control enzymatic browning is an important key to enhance product value and minimize post harvest losses. The use of phenolic compound from plant extract appears to be a natural alternative to the conventional sulfites for prevention of melanosis formation. The PPO inhibitory action of phenolic compound is owing to their metal chelating activity and structural similarity with substrate. Phenolic compounds could reduce DOPA-quinone to DOPA by electron donation. Although, there is increasing interest in plant phenolic compounds as food additive, practical aspect need to be considered are extraction method, standardization of additive and safety consideration.

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Table 1: Classes of phenolic compounds in plants

Classes	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	C_6-C_1
Acethophenones, phenylacetic acids	C_6-C_2
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	C_6-C_3
Napthoquinones	C_6-C_4
Xanthones	C_6-C_1-C_6
Stilbenes, anthraquinones	C_6-C_2-C_6
Flavonoids, isoflavonoids	C_6-C_3-C_6
Lignans, neolignans	$(C_6$-$C_3)_2$
Biflavonoids	$(C_6$-C_3-$C_6)_2$
Lignins	$(C_6$-$C_3)_n$
Condensed tannins (proantocyanidins or flavolans)	$(C_6$-C_3-$C_6)_n$

Source: Adapted from Balasundram *et al*. (2006)

Figure Legends

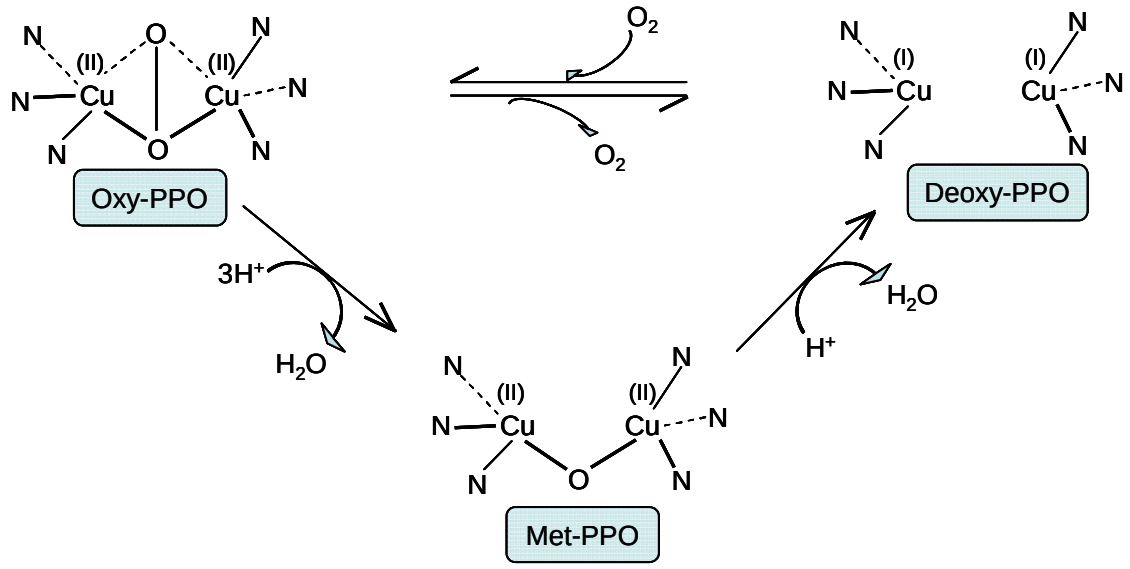

Figure 1. Three different states of PPO (Himmelwright *et al*., 1980).

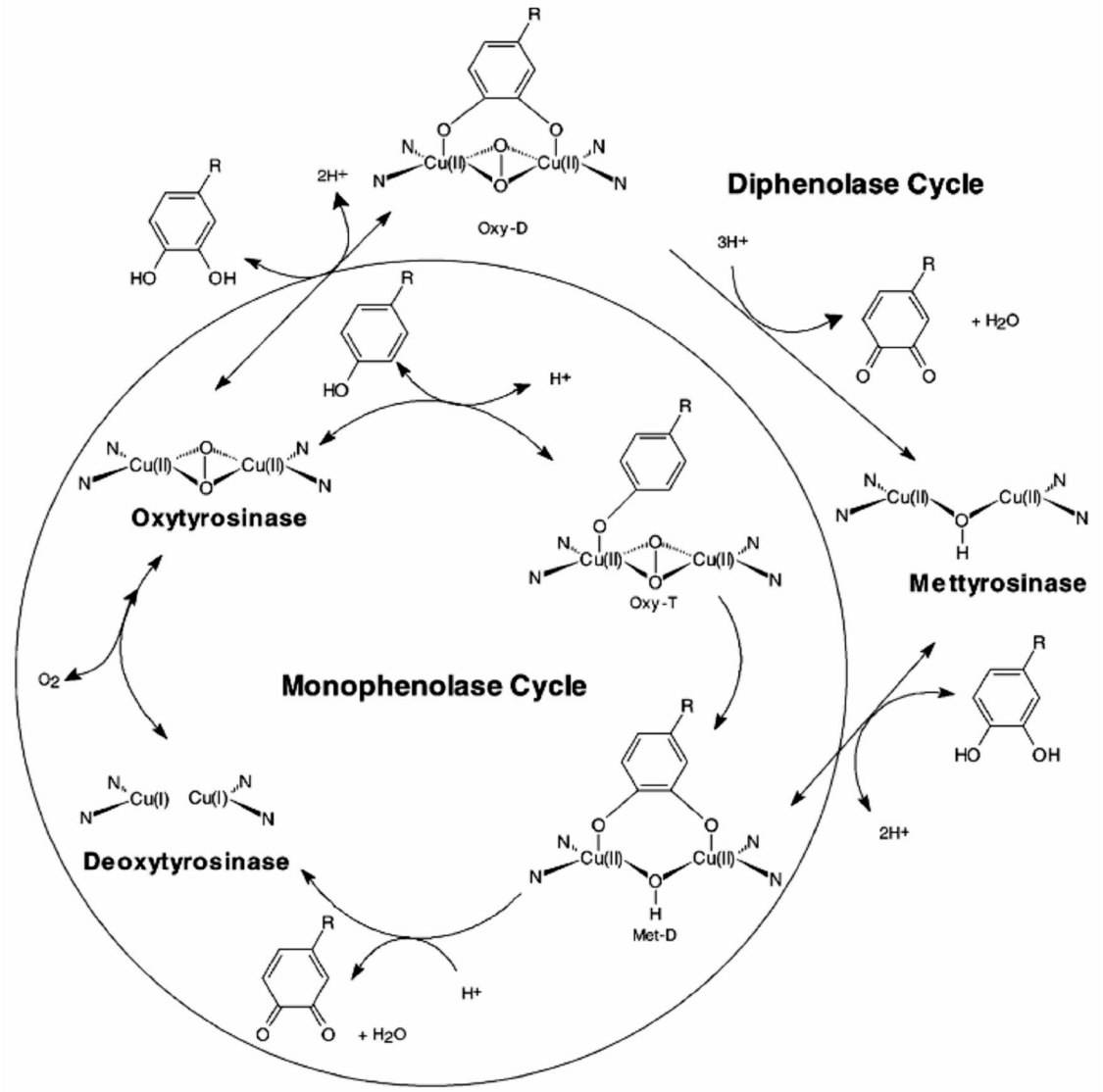

Figure 2. Mechanism for monophenolase and diphenolase activity of PPO (Likhitwitayawuid, 2008).

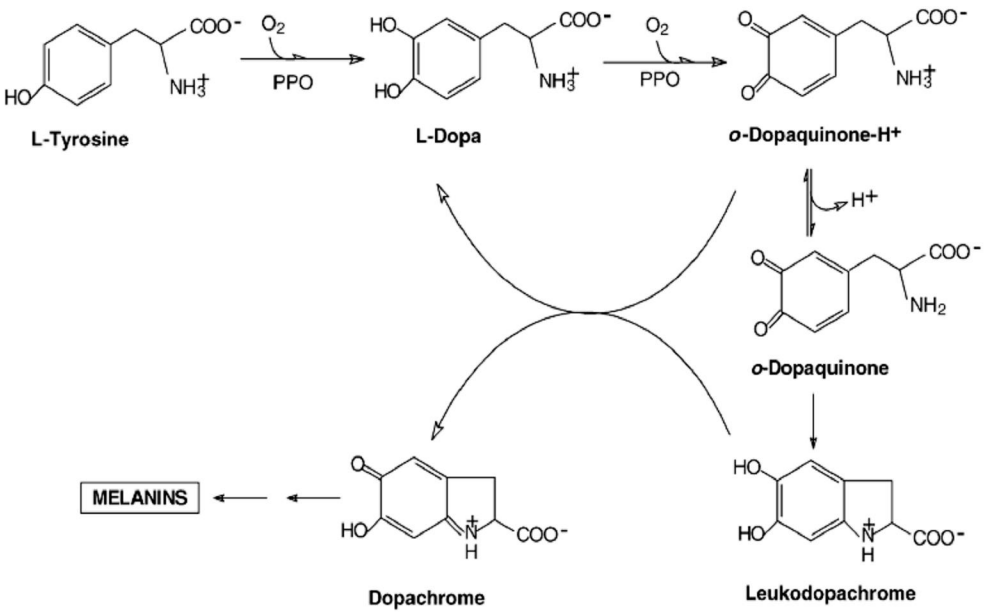

Figure 3. Melanin biosynthesis from tyrosine (Garcia-Molina *et al*., 2005).

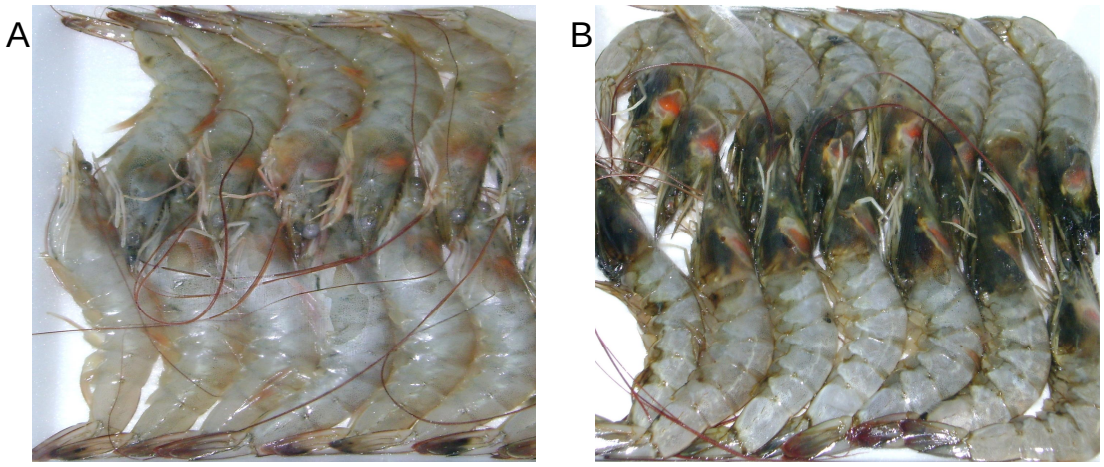

Figure 4. Photograph of fresh Pacific white shrimp (A) and blackening of Pacific white shrimp (B) (*Litopenaeus vannamei*) during iced storage.

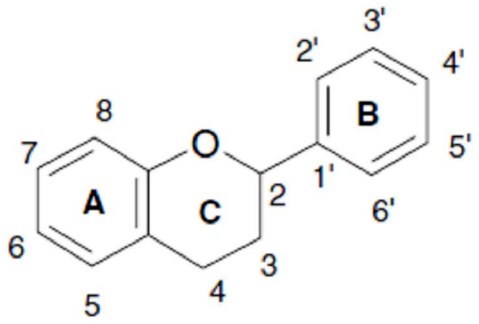

Figure 5. Generic structure of a flavonoid molecule (Balasundram *et al*., 2006).

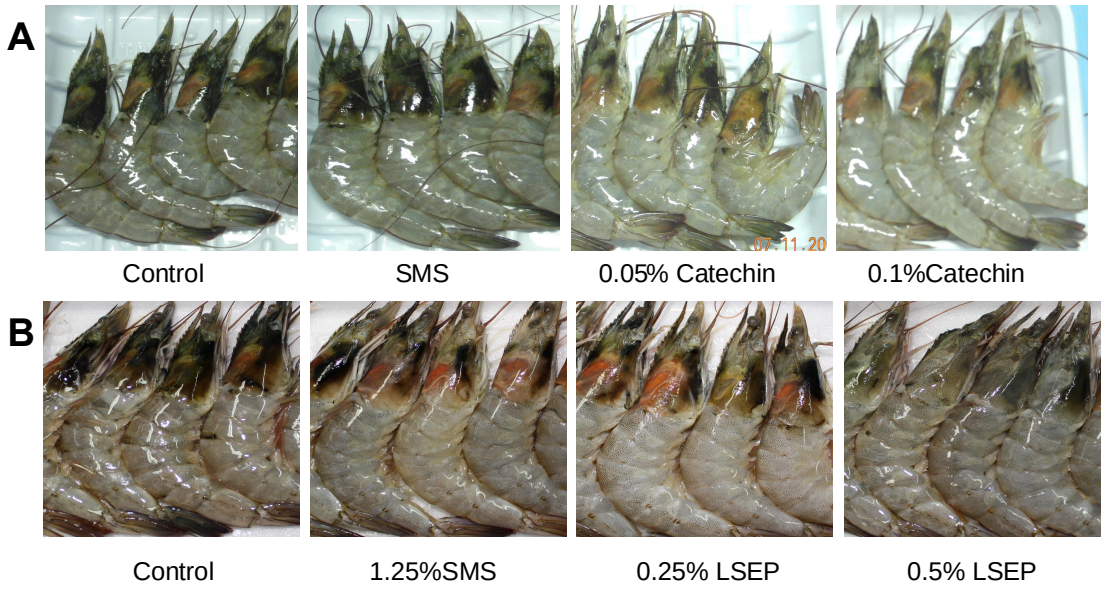

Figure 6. Photographs of Pacific white shrimp without and with treatment of catechin (A) (Nirmal and Benjakul, 2009b) and LSEP (B) (Nirmal and Benjakul, 2011b) at different concentrations after 10 and 12 days of iced storage, respectively. SMS; 1.25% sodium metabisulfite, LSEP; lead seed extract powder.