ANTHOCYANIN COLOR ENHANCEMENT BY USING CATECHIN AS COPIGMENTS AND STABILITY DURING STORAGE

THESIS

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ABSTRACT

Anthocyanins are phenolics responsible for the red, blue, and purple colors of many plants and foods. However, anthocyanins have limited stability and are susceptible to degradation. The color stability of anthocyanins is influenced by pH, temperature, presence of enzymes, light, structure, concentration of the anthocyanins, and the presence of complexing compounds such as other flavonoids, phenolic acid, and metals. Anthocyanins can associate with other phenols - copigments – resulting in a color stabilizing mechanism. The purpose of this study was to investigate the effect of catechins, the major polyphenol and antioxidant in tea (*Camellia sinensis*), as copigment on 5 different anthocyanin sources; also, to observe the color and stability over 70 days. The impact of type of anthocyanins and ratio of anthocyanins to catechins were evaluated. Anthocyanins extracted from strawberry, blackberry, American eggplants, and red cabbage were combined with green tea catechins at different ratios: 1:1, 1:5, 1:10, 1:20, 1:50 (anthocyanins:catechins). All samples and control contained 30 mg anthocyanin/L. Samples were stored at 4°C in the dark for 70 days. Color (HunterCIE Lab L*a*b*, chroma, and hue angle), monomeric anthocyanins (pH differential), total phenolic (Folin method) and pH were monitored at 0, 1, 5, 10, 20, 30, 45, 70 days. Anthocyanins, catechins, gallic acid and caffeine were simultaneously determined by HPLC at days 0 and 70.

Copigmentation with catechin enhanced the color strength in all of the anthocyanin sources. The color of all samples was clearly affected by the addition of
the colorless catechins, in a dose dependent manner. The color intensity increased up to 40% when the anthocyanin:catechin ratio was 1:50. Chroma and hue increased, while the L* value decreased (the color became darker) in all treatment samples. The chroma values of treatment samples slightly decreased over the 70 days storage time in the non-acylated anthocyanin. Total phenolic content increased in a fairly linear fashion (R>0.9) with increasing the concentration of catechin in the treatment samples, as expected. Results show that it is possible to supply color and phenolics using anthocyanin-rich materials combined with other polyphenols to obtain a brighter red/purple color with high phenolic and antioxidant content in food product.
Dedicated to:

My parents, Yao-Bing Siu and Pi-Sui Siu-Hsu
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CHAPTER 1

INTRODUCTION

Functional foods are becoming more popular each year as the consumer recognizes that a healthy diet is becoming more a part of life in order to control the weight or prevent the diseases. Functional foods have been referred to as foods that can provide health benefits beyond the basic nutritional value (e.g., vitamins and minerals), or the foods that have been modified by eliminating a component known to cause diseases or by adding a component that shows beneficial effects for human (Hasler, 2002; Henry, 2010). Today, functional foods represent one of the most intensively investigated and widely promoted areas in the food and nutrition science. Supplement stores such as GNC and Vitamin Shoppe, and natural grocery stores such as Whole Foods and Wild Oats have grown rapidly over the past 10 years, in part due to the new concept of healthy living.

Fruits and vegetables are the most common health foods that can obtain in the grocery store or any convenient shop. There have been numerous studies showing that high consumption of fruits and vegetables is associated with lower risk of chronic disease, such as cancer, cardiovascular disease, cataracts, and hypertension (Giusti and Jing, 2007). Fruits and vegetables contain phenolic and flavonoids that act as antioxidants (Wang and Lin, 2000). Anthocyanins are phenolic molecules that are
responsible for the red, blue, and purple colors in more than 100 common fruits and vegetables. Many in vitro and in vivo studies show that anthocyanins are related to a number of biological activities, such as protection against cardiovascular disease, metabolic syndrome, type II diabetes, various cancers including in the gastrointestinal tract, vision problems, neurodegenerative disease, aging skin and inflammation (Nunez and Magnuson, 2013).

Anthocyanins are natural colorants that are found in fruits (particularly in berries), vegetables, and flowers. Natural colorants are gaining popularity in the marketplace and among consumers. Scientists point out that the use of natural colors is a powerful selling tool in the marketplace (Korolishin, 2004). The most widespread areas for natural colorants are in beverages and confection products. Anthocyanins are probably the best known of natural food colorants, because they provide the attractive red, blue and orange colors in many fruits and vegetables. However, like most natural pigments, anthocyanins are unstable and highly degradation. They are influenced by pH, temperature, light, and the presence of complexing compounds such as other phenolic acid and flavonoids (Markakis, 1982). Recent studies (Mazza and Brouillard, 1990) show that the molecular complexation of anthocyanins with other phenols, also named as copigments, is the major color stabilizing mechanism in plants.

Tea and coffee are the most prevalent beverages consumed in the world. Green tea contains high amounts of catechins such as epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), gallocatechin gallate (GCG), and epigallocatechin gallate (EGCG). Catechins are the major polyphenol and antioxidant in tea, which show beneficial effects such as reduction in the risk of cardiovascular disease and some forms of cancer, as well as anti-hypertensive effect, body weight control (Cabrera et al., 2006).
Our overall goal was to design a food enriched with phytochemicals that may enhance the health benefits and maintain the color stability of anthocyanin as natural colorants. These products could be in the market for specific population. Our hypothesis was that the catechin can acts as copigment and could possibly enhance the color stability of certain types of anthocyanin during the storage period. In this study, we measured the color characteristic, total monomeric anthocyanin, and total phenolic content of five different types of anthocyanin with different concentrations of catechin. A major challenge for the use of anthocyanins as natural colorants is their instability during long storage. Thus, we wanted to determine if it is possible that anthocyanin and catechin can be incorporated into a beverage product. We also evaluated the effect of copigment addition on color and pigment stability. Recent studies (Mazza and Brouillard, 1990) show that the molecular complexation of anthocyanins with other phenols, also known as copigments is the major color stabilizing mechanism in plants.
CHAPTER 2

LITERATURE REVIEW

2.1 Functional Foods

All foods can provide aroma, taste, and nutritive value; however, the new food category recognized as “Functional Foods”, which identified as if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional affects (Dipiocl et al., 1999) or foods from both plants and animals (known as phytochemicals and zoochemicals, respectively) that potentially could reduce risk for a variety of chronic diseases (Hasler, 2010). Typically, the foods provide not only the nutritional value but also positive influence for the human body such as disease prevention.

The concept of “functional food” was first described in ancient India and in Chinese traditional medicine, which defined “Medicine and food have a common origin” (Henry, 2010); however, there is no universally accepted definition for functional food. The Ministry of Health and Welfare was the first regulatory system to approve certain foods with documented health benefits in Japan in the 1980s (Arai, 1996). In the United States, functional foods have no specific regulatory identity. However, the U.S. Food and Drug Administration (FDA) established a number of regulatory procedures for such claims for dietary supplement labels. According to the Dietary Supplement Health and Education Act of 1994 (DSHEA), functional claims
describe the role of a nutrient or dietary ingredient intended to affect normal structure or function in humans, for example, “antioxidants maintain cell integrity”. Moreover, the FDA established the term “health claims”, which means any claims on the label or in labeling of a food present a relationship exists between the level of a substance in the food and a disease or health-relate condition (Code of Federal Regulations Title 21 101.14), for example, “Diets in low saturated fat and cholesterol may reduce the risk of heart disease”.

According to the article by Henry (2010), there are five different approaches to producing a functional food product. The first is by eliminating a component, which is known to cause or identified as causing a harmful effect when consumed (i.e. an allergenic protein). The second way is by increasing the concentration of a component that is naturally present in food to a significant level, which can induce health benefits or predicted effects. The third way is to add a component that is not normally present in most foods, and is not necessarily a macronutrient or a micronutrient, but it shows beneficial effects (i.e. anthocyanins and catechins). The fourth is to replace a component which is normally harmful to a consumer’s health (i.e. sugars and fats) with a component which has more beneficial effects (i.e. modified starch). The last approach is to increase bioavailability or stability of a component in food (i.e. increase self-life or reduce degradation).

Today, people of all ages are concerned about health, and the most important and direct factor is foods in their diet. Nowadays, more and more people prefer healthier foods in their life. The development of functional foods is a significant challenge between academic and industry scientists (Roberfroid, 1999). The food industry has special opportunities to produce products that not only provide traditional nutrient, but also improve health and reduce the risk of disease (Diplock, 1999).
Research and development are important for functional foods because the safety, stability, functionally, bioavailability and processing capabilities are all factors which scientists need to consider. Overall, the development of functional foods is a scientific challenge prior to the challenge of marketing. It requires a new way of looking at current food science practices.

2.2 Food Colorants

Color plays an important role in the food product. Whether it is an actual food or the package, consumers first judge the quality of a food product by its color, also, the food industry uses the colorants to enhance the original appearance of foods (Giusti and Wrolstad, 2003). Supermarket shelves are dynamic in color, especially in the beverage and fresh food aisle, where bright orange and yellow sodas, vibrant red juices, and green and red fresh vegetables attract consumer’s attention (Korolishin, 2004). Consumers consider a product as being healthy, tasty and fresh when the product has a bright natural color, which increases the chances of their purchasing the food. On the other hand, if a product has a brown, dull or dark color, the consumer may perceive it as being bad or decayed, which would decrease their desire to buy the product.

Both natural and synthetic colorants are widely used in food products from sodas to energy drinks. A number of factors influence the decision to use natural or artificial colors. According to the U.S. FDA, the definition of food color is: a dye, pigment or other substance, which is capable of imparting color when added or applied to a food, drug, cosmetic, or to the human body. The FDA is responsible for regulating all color additives, to ensure that foods containing color additives are safe
to consume, containing only approved ingredients, and are accurately labeled (FDA, 2005). There are currently seven approved synthetic color additives for general use in human food: FD&C Red No.3 and 40, FD&C Blue No.1 and 2, FD&C Yellow No.5 and 6, and FD&C Green No.3. In addition, two other synthetic colorants are approved for restricted use in food: Citrus Red No.2 and Orange B.

Synthetic colorants have been banned because of their adverse effects in laboratory animals. Numerous researches show that artificial food dyes could induce allergic reaction and contain carcinogenic. In addition, many of the currently approved dyes raise health concerns (Kobylewski and Jacobson, 2010). For example, Blue No.1 and Yellow No.5 have been shown that might causes hypersensitivity reaction in a small number of people and might trigger hyperactivity and other behavioral effects in children. Blue No.2 and Green No.3 have been reported that significant increases in kidney, bladder and testes tumors in rats.

Because of that concern, synthetic colors are not fully accepted around the world, for example, many European countries have banned artificial and/or synthetic colors in their food supply (Bunce, 2007). In addition, British government advised companies to stop using most food dyes by 2009. Synthetic colors may be easily used a variety of foods and beverages; however, the safety of artificial colorants has been questioned in the past years, which has lead to a decrease in their usage in food application (Giusti and Wrolstad, 2003). Today, consumers prefer and have an interest in natural colorants instead of synthetic colorants in food products.

The FDA does not recognize the term “natural colorant” (Korolishin, 2004), instead, if the colorants are from fruits and vegetables without added chemicals, consumers usually perceive it as natural colorants. However, Mazza (2008) defined “natural colorants” as materials extracted, isolated, or derived from plants, animals, or
minerals that are capable of imparting a distinguishing color when added to food. The most important colorants include anthocyanins (flavonoids), carotenoids, chlorophylls (porphyrins), curcuminoids (turmeric), betalaines (beetroot), and quinonoids (carmines).

The use of natural colors is a powerful selling tool in the marketplace (Korolishin, 2004). Due to the color being from a natural source, food retailers can claim that the product is “all natural”, “no synthetic added” and “healthier”; however, there are still several challenges for natural colorants. First, natural colors are typically more expensive to use than synthetic colors. In fact, they have been accepted by more natural-consumers and placed at natural food retailer such as Whole Foods and Wild Oats. Food and beverage industries must use only natural colors used in their product in order to sell to those chains and survive in the marketplace (Korolishin, 2004). The second challenge is the stability of natural colorants. It has been discussed for years how to increase the stability of natural colorants and decrease their degradation. There is; however, a large amount of evidence indicating that some natural pigments can be considered as crucial nutritious antioxidants and may decrease the risk of diseases such as cancer, cardiovascular disease, and other diseases related to aging (Mazza, 2000).

2.3 Anthocyanins

2.3.1 Definition and Chemical Structure

Anthocyanins are a class of natural water-soluble pigment widely distributed in plants and fruits. The word “anthocyanins” is originated from two Greek words “anthos”, which means flower, and “kyanos”, which means dark blue, represents its
significant role as natural colorants (Mazza and Miniati, 1993; Eder, 2000; Delgado-Vargas et al., 2000). The pigments are responsible for the red to purple to blue colors of many fruits, vegetables, and cereal grains (Wrolstad, 2004). It not only is found in many fruits such as blueberries, blackberries, strawberries, raspberries, and grapes, but also in plants such as red radish, red potato, purple corn, and eggplants. In plants, anthocyanins act as attractants for pollination, especially for insect species which lead to seed dispersal (Wrolstad, 2004). Moreover, these pigments help to provide natural protection against the harmful effect of UV irradiation, as well as providing anti-viral and anti-microbial activities (Wrolstad, 2004). Due to the significant importance to the color quality of fresh and processed fruits and vegetables, anthocyanins have been extensively investigated by research in horticulture and food science (Wrolstad, 2004). The interest in anthocyanins pigments has increased dramatically in recent years due to their possible health benefits, such as reducing the risk of heart disease, cancer, and stroke (Wrolstad, 2004).

Anthocyanins belong to the group of flavonoid compounds, which are widely distributed plant polyphenols (Brouillard, 1982; Jing, 2008). There are close to 700 anthocyanin structures have been discovered and identified (Anderson, 2012). The basic structure of aglycone (anthocyanidin) is shown in Figure 2.1. The R group substitution on the B ring and the present of color are shown in Table 2.1.
The major part of the anthocyanin is the aglycones (anthocyanidins), which are hydroxylated and methoxylated derivate from 2-phenylbenzopyrylium or flavylium salts: C6-C3-C6 carbon skeleton (Eder, 2000). The basic anthocyanin compound is made of 2 six-carbon rings and 1 three-carbon ring structure. The first ring (A ring) has two double bonds and two hydroxyl groups; the second ring (C ring) has three double bonds and one oxygen atom. The third ring (B ring) has three double
bonds followed by one hydroxyl group and two R groups. Substitution of B ring with 
H, OH, and OCH$_3$ produces six different common anthocyanidins: pelargonidin, 
cyanidin, peonidin, delphinidin, petunidin, and malvidin (Eder, 2000).

In addition, the mono, di, or tri-saccharides may be glycosylated or acylated 
at the A and C rings at the 3 and/or 5 positions (Wrolsrud, 2004). Glucose is the most 
common sugar that is glycosylated. Also, anthocyanins can be glycosylated with other 
sugars, for example, rhamnose, xylose, galactose, arabinose, fructose, rutinose, and 
sophorose (Delgado-Vargas et al., 2000). Moreover, research shows that many 
glycosylated anthocyanins can be acylated with organic acids. In nature, the common 
acids for anthocyanin acylation are aromatic acids such as p-coumaric, caffeic, 
ferulic, gallic, as well as aliphatic acids such as acetic, malic, malonic, oxalic and 
succinic acid (Francis, 1989; Delgado-Vargas et al., 2000; Robbins, 2003). One 
example of an acylated anthocyanin is shown in Figure 2.2. There are a wide variety 
of chemical structures that can determine anthocyanins: different glycosylating 
patterns, different acylating groups, and plenty of hydroxyl groups, and the presence 
of cinnamic acids in different stereo isomeric forms (Giusti et al., 1998). Therefore, 
there are more than 700 structurally distinct anthocyanins, that have been found in 
Figure 2.2 Chemical structure of red cabbage (*Brassica oleracea* L.): cyanidin-3-O-diglucoside-5-O-glucoside, which can be acylated with p-coumaric, caffeic, ferulic and sinapic acids (Tanchev and Timberlake, 1969; McDougall et al., 2007; Scalzo et al., 2008).

### 2.3.2 Anthocyanin Rich Sources

Anthocyanins are water-soluble pigments that can be found in many varieties of plants, flowers, fruits, and vegetables. Over 100 common foods were found to contain anthocyanins, and some sources have higher amounts of anthocyanins than others. Also, the specific type of anthocyanins present in foods are quite different (Wu et al., 2006).

Strawberries (*Fragaria x ananassa* Duch., family Rosaceae), consumed both as fresh fruit and processed, may be an important source of health promoting compounds for many people especially in North America (Aaby et al., 2012). Not only consumed fresh, it is also frozen or canned and used in making jam, jelly, and
wine. Strawberries contain high levels of vitamin C, folate, and phenolic compounds (Giampieri et al., 2012). The most common phenolic compounds in strawberries are anthocyanin, which represents the bright red color of the berries. Pelargonidin-3-glucoside is the major anthocyanin in all of the strawberry cultivars, contributing 60–95% of the total anthocyanin content. Pelargonidin-3-malonylglucoside was the second most abundant anthocyanin in the samples, varying from 0% to 33.5% (mean 17%) contribution (Aaby, 2012). Although the most common substituting sugars in strawberry anthocyanins are glucose, rutinose, and arabinose. Rhamnose conjugates have been discovered in some strawberry cultivars (da Silva et al., 2007; Giampieri et al., 2012;). Research shows that strawberries have a total content range of 8-64 mg/100g fresh weight (Wu et al., 2006; Aaby et al., 2012; Anderson and Jordheim, 2013).

Blackberries (Rubus fruticosus) are in the family of Rubus genus. They are widespread and a well-know group of over 375 species, many of which are common throughout Europe, northwestern Africa, North and South America. Blackberries are popular for use in desserts, jams, and some wines. In general, five anthocyanins pigments were isolated and characterized in blackberries, which are cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin-3-xyloside, cyanindin-3-(malonyl)glucoside, and cyanidin- 3-dioxyglucoside (Cho et al., 2004; Jordheim et al., 2011). As common as many fruits, cyanidin 3-glucoside is the predominant anthocyanin in blackberries. Reports show that the monomeric anthocyanin pigment content of blackberries ranged from 70 - 332 mg/100 g fresh weight (Cho et al., 2004; Siriwoharn et al., 2004; Fan-Chiang and Wrolstad, 2005; Wu et al., 2006).
Eggplant (*Solanum melongena* L.), originating from Asia is one of the most popular and widespread vegetables though the world. The color of eggplant peels is noted for its beautiful dark purple, which is known as “Nasu blue” in Japan (Noda et al., 2000). Different varieties of eggplant produce different sizes, shapes, and colors. The most common cultivated varieties in Europe and North America are elongated ovoid, broad, and deep purple skin; however, the narrower and slightly pendulous in shape and light purple, which are called Japanese eggplant in North America, are much more common in Asia. The color of eggplant peel is due to anthocyanins. Nasunin (delphinidin-3-(p-coumaroyl)rutinoside)-5-glucoside) is the major anthocyanins in eggplant peels (Noda et al., 2000). Nasunin occurs as cis- and trans-isomers, and the trans- form was reported to be more stable (Sadilova et al., 2006). In a recent paper, Wu and Prior (2005) reported that delphinidin3-rutinoside is the major anthocyanin in eggplant in the US market. The evidence shows that the concentration of anthocyanins in eggplant is 86 mg/ 100g fresh weight (Wu et al. 2006). Moreover, Noda et al. (2000) found that Nasunin shows much more potent lipid peroxidation effects in vitro than other anthocyanins.

Red cabbage (*Brassica oleracea* L. var. capitata), also known as purple cabbage, red kraut or blue kraut, is a vegetable which originated from the Mediterranean region and southwestern Europe, and now grows in regions all over the world. Red cabbage belongs to the family of *Brassicaceae* (Apapitsas et al., 2008). The leaves are colored dark red/purple. Recently, red cabbage became popular due to increased marketing of fresh-cut products, away-from-home eating, and potential health benefits (Charron and Clevidence, 2007). Red cabbage is a useful food coloring because it is stable over a wide pH range (McDougall et al., 2007) and it is a natural colorant that can replace the blue synthetic colorants (Bridle and Timberlake,
The predominant anthocyanins in red cabbage are 3-diglucoside-5-glucoside and acylated anthocyanins. It can be non-acylated, mono-acylated or di-acylated with other organic acid such as p-coumaric, caffeic, ferulic and sinapic acids (Tanchev and Timberlake, 1969; Giusti et al., 1999). Research shows that red cabbage has a total anthocyanins content of 75-363 mg/100 g fresh weight (Wu et al., 2006; Li et al., 2012), which is higher than other vegetables such as black bean, red radish, and red onion.

2.3.3 Anthocyanin Stability

Anthocyanins are probably the best known of natural food colorants, because they provide the attractive red, blue and orange colors in many fruits and vegetables. Like most natural pigments, anthocyanins are unstable and highly susceptible to degradation. The color stability of anthocyanins is influenced by pH, temperature, presence of enzymes, light, structure, concentration of the anthocyanins, and the presence of complexing compounds such as other flavonoids, phenolic acid, and metals (Markakis, 1982).

The chemical structure is the most important factors that affect the stability. Anthocyanins are based on the flavylium and glycosides of anthocyanindins. Due to the instability of the chromophoric aglycones (anthocyanindins), which are red polyhydroxylated salts, it is rare to find the free form in plant tissue (Mazza and Brouillard, 1987). The number of hydroxyl groups, the degree of methylation of these hydroxyl groups, the number and position of the sugar attached, and the number of aliphatic or aromatic acid attached to the sugars are the factors that occur for the many different anthocyanins in natural (Mazza and Brouillard, 1987). Because of the
blocking reactive hydroxyl group by methylation, pelargonidin, cyanidin and
delphinidin are less stable than peonidin, petunidin and malvidin (Jing, 2006).
Therefore, there are some anthocyanins that are easier to find in nature. Research
shows that the anthocyanins with a 4-substitution is more stable than others. For
example, 5-carboxy pyranopelargonidin 3-O-b-glucopyranoside from strawberry
\textit{(Fragaria x ananassa} Duch.) is more stable than other anthocyanidins. (Anderson et
al., 2004).

Pigments are usually sensitive to light and temperature. The light usually has a
negative effect on foods, which contain anthocyanins (Mescher, 1953). Anthocyanins
are generally unstable when exposed to UV, visible light and other sources of ionizing
radiation (Markakis, 1982). Palamidis and Markakis (1975) reported that the exposure
to light accelerated the degradation of the pigment. For example, at the same store
conduction (20°C, 135 days), colorants extracted from grapes lose 30% in the dark
and 50% under continuous fluorescent light. Van Buren et al. (1968) claim that
acylated anthocyanins in wine were more stable than nonacylated when exposed to
light. Thus, avoiding light and/or storing under dark conditions are necessary to
preserve the pigment in foods.

There have been many of studies showing the rapid decrease in pigment at
high temperature (Mazza and Brouillard, 1987; Francis, 1989; Romero and Bakker,
2000). Temperature is the most important factor in changing the kinetic of the
degradation of color (Mescher, 1953). For example, the half-life of the color of
strawberries is 1300 hours (54 days) at room temperature (20°C); however, the half-
life increases to 6000 to 8000 hours (250 to 320 days) when stored at refrigeration
condition (4°C) (Mescher, 1953). Moreover, Giusti and Jing (2007) reported that
cranberries stored at 20°C lost 62% of their anthocyanins, but only lost 20% of
anthocyanins when stored at 7°C; also, there was no loss of anthocyanins when the fruit was stored at 0°C. Therefore, storage temperature and condition are two important factors that affect pigment degradation (Giusti and Jing, 2007).

The color of non-acylated and monoacylated anthocyanins are influenced by pH. In an aqueous environment, anthocyanins undergo reversible structure transformation with changes in pH, followed by dramatic changes in color (Figure 2.3) (Wrolstad et al., 2002). In an acidic condition (pH=1), anthocyanins exists predominately in the flavylium cation form, which present the orange or red color. When the pH is at 4.5, the form of colorless carbinol pseudobase or chalcone is present. The 2- position on the flavylium cation can be hydrolyzed by nucleophilic attack, then equilibrated to the colorless hemiketal form. At this point, the C-ring is open, and the color is lost. For example, at pH 3.01, only 50% of cyanidin-3-glucoside will be in the colored form (Wrolstad, 2004). Once the pH value increases to 8 or higher, the quinonoidal base is formed. When the pH is near 7, the unstable blue color quinonoidal base is formed, which exists in natural aqueous solutions (Giusti and Wrolstad, 2001). Thus, the red flavylium cation form is much more stable than the colorless form and quinonoidal form.
2.3.4 Potential Health Benefits of Anthocyanins

In the United States, fruits and vegetables are available in every fresh market and grocery store. There have been numerous studies showing that high consumption of fruits and vegetables is associated with lower risk of chronic diseases, such as cancer, cardiovascular disease, cataracts, and hypertension (Giusti and Jing, 2007). Moreover, consuming and digesting fruits and vegetables have been found that reduce blood pressure, reduce inflammation, and boost the immune system (Ascherio, 1992). There is no question that finding treatment and cures for diseases and preventing diseases are important in our society. Many recent studies focus on the compounds in fruits and vegetable that are responsible for disease prevention. Natural pigments not
only are food colorants but also are potent antioxidants and seem to have a number of potential health benefit (Giusti and Jing, 2007).

Anthocyanins are the major flavonoids in fruits and vegetables, and are convenient to consume from the diet. The potential health benefits of anthocyanins and anthocyanin-rich sources have been studied for more than 30 years. Many in vitro and in vivo studies show that anthocyanins are related to a number of biological activities, such as protection against cardiovascular disease, metabolic syndrome, type II diabetes, various cancers including in the gastrointestinal tract, vision problems, neurodegenerative disease, aging skin and inflammation (Nunez and Magnuson, 2013).

Antioxidation is one of most important mechanisms to prevent or delay aging disease, including cancer, heart disease, and cognitive dysfunction. The antioxidants are believed to block oxidative activity and free radicals that contribute to chronic disease (Mazza, 2000). Recently, several authors reported that anthocyanins show strong antioxidative activity (Tsuda et al., 1996; Narayan et al., 1999). Tsuda et al. (1996) investigated the anthocyanins (cyanidin 3-O-β-D-glucoside) reacted with 2,2’-azobis (2,4-dimethyl-valeronitirile) to generate alkylperoxyl radicals. The research showed that antioxidative mechanism of cyanidin 3-O-β-D-glucoside may be different from α-tocopherol; and cyanidin 3-O-β-D-glucoside would produce another radical scavenger that can break down the structure and scavenge the radicals. Narayan et al. (1999) studied the antioxidant effect of anthocyanins on enzymatic and non-enzymatic lipid peroxidation. They found that in vitro enzymatic and non-enzymatic polyunsaturated fatty acid peroxidation was significantly inhibited in a dose dependent manner by purified anthocyanin, which was from carrot cell culture. The results showed that anthocyanin is a non-competitive inhibitor of lipid
peroxidation. Anthocyanin was found to be a potent antioxidant compared to classical antioxidants, for example, butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and α-tocopherol.

Carcinogenesis is a complex process that is related to three steps: initiation, promotion, and progression (Giusti and Jing, 2007). Numerous anticarcinogenesis studies show that phytochemicals from fruits and plants play an important role in inhibiting carcinogenesis. According to recent cell culture researches, as well as animal and human studies, anthocyanins from berries have been found to significantly suppress the growth of cultured tumor cells and to have greater inhibitory effect than other flavonoids (Kamei et al., 1995). Anthocyanins have been found to have good antiproliferation or apoptosis effects. Shih et al. (2005) investigated the effect of anthocyanins on cell cycle progression and induction of apoptosis in human cultured gastric adenocarcinoma (AGS) cells. The results showed that malvidin exhibited the most potent antiproliferation effect on AGS cells on a time- and dose-dependent manner. Moreover, Hou et al. (2004) investigated the effect of six kinds of anthocyanins on tumor promotion in mouse JB6 cells, which is a model for screening cancer chemopreventive agents and elucidating the molecular mechanisms. They concluded that anthocyanidins contributes to the inhibition of tumorigenesis by blocking activation of the mitogen-activated protein kinases (MAPK) pathway.

There are numerous other health benefits of anthocyanins. Recent studies show that the presence of anthocyanins and resveratrols in red wine are associated with coronary hear disease (CHD) (Kanner et al., 1994). In addition, several epidemiological studies show that consuming moderate amounts of red wine can reduce the risk of coronary heart disease (Graziano et al., 1993; Klatsky, 1994). Moreover, Tsuda et al. (2003) proved that cyanindin 3-O-β-D-glucoside from purple
corn has the benefit of preventing obesity and diabetes. As a result, anthocyanins should be a good natural pigment for preventing cancer.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established an ADI (acceptable daily intake) of 2.5 mg/kg bw/day for anthocyanins from grape skin extracts, which is around 400g of strawberries per day for 50kg adults. However, the Scientific Committee for Food (SCF) did no limit the anthocyanin ADI. According to the toxicity report of anthocyanin, the anthocyanin LD₅₀ is 20,000 mg/kg for rats, 25,000 mg/kg for mice, and > 6,000 mg/kg for rabbits (black currant, blueberry, and elderberry anthocyanin mixtures) (Pourrat et al., 1967). Overall, anthocyanin shows their low toxicity and safety for human.

2.4 Tea

2.4.1 Background

Tea, a leaf extract of the plant *Camellia sinesnsis*, is one of the most popular beverages in the world. Evidence shows that tea has been cultivated and consumed for more than 5000 years (Balentine et al., 1997; Pettigrew, 1997). Originating in China and South-East Asia, the Dutch first introduced tea into Europe at the beginning of the 16-century, and it reached England and North America by the mid-1600s (Balentine et al., 1997). Tea has been considered a medicine and health-promoting beverage since ancient times; however, consumption of tea is part of people’s daily routine today.

According to different manufacturing processes, teas are classified into three major types: the non-fermented green tea, the semi-fermented oolong tea, and the fermented black tea (Wu and Wei, 2002; Cabrera et al., 2003; Del Rio et al., 2004). Approximately 76-78% of the tea produced and consumed all over the world is black
tea, 20-22% is green tea, and only <2% is oolong tea (Wu and Wei, 2002). Worldwide, black tea is the most popular beverage in Europe, North America, and North Africa, while green tea is drank throughout Asia; oolong tea is consumed more in China and Taiwan (Zuo et al., 2002; Cabrera et al., 2003). Due to tea being considered a healthy beverage and the health perception from consumers, the market for tea is becoming increasingly popular in the next decade. According to the new market report, global tea demand will be at about 9,200 tons by 2020, and the global market of the ingredient will hit $368 million by 2020 (Schultz, 2014).

2.4.2 Tea Processing

Depending on the varies of species, season, and horticultural conditions, each type of tea has different flavor, smell, taste, and visual appearance. However, tea processing for all types of teas is similar, which is presented in the following.

Plucking: The top two leaves and their buds are picked from *Camellia sinensis* bushes, typically twice a year, during early spring and early summer. Hand-picking is common for higher quality tea or in regions where the labor costs are low. Tea flushes and leaves can also be picked by machine; however, there will be more broken leaves and reduced quality.

Withering: The tea leaves will be spread out to dry soon after picking. The leaves can be either put under the sun or heated by air for an extended period of time. Withering is used to remove excess water from the leaves and still keep them flexible. The essential chemical changes take place during this process. The tea leaf continues to respire, so that the leaf proteins breakdown into free amino acids, and lose
carbohydrates. The caffeine content of the shoot is significantly increased during withering (Roberts, 1986).

Rolling: The damp leaves are rolled and twisted by hand or machine to break up the leaf cells, causing some of the essential oils, and juices, which relates to the taste and flavor.

Oxidation/fermentation: For the teas requiring oxidation, the leaves are left in a climate-controlled room and the leaves turn progressively darker. Oxidation is the most important step in tea processing, because this process forms colors, and much of the taste and aroma. Depending on the desired qualities in the final product and the weather condition such as heat and humidity, the degree of oxidation is different. This is the main processing condition that differentiates Green, Oolong, and Black tea. Green tea does not go through this step in order to retain the most important polyphenols and other antioxidant. The degree of oxidation is 40-70% and 100% in Oolong and Black tea, respectively.

Drying: This is the final step for the tea processing. At the desired oxidation, the leaves are gently dried at about 100°C for 8-10 minutes by various ways including panning, sunning, air drying or baking to stop the fermentation process. This step is responsible for many new flavors and aroma especially in green teas (Ito et al., 2002).

**Green Tea**

Green tea is often considered as “non-fermented” or “unfermented” tea (Pettiegrew, 1997). The major processing technique for green tea is to immediately steam the fresh tea leaves and dry them for preservation, which is likely the most ancient Chinese form of tea leaf processing. Due to lack of oxidation/fermentation, the chemical compositions of green tea is complex, including proteins, amino acids,
carbohydrates, lipids, vitamins, xanthic bases, pigments, volatile compounds, minerals and trace elements (Cabrera et al., 2006). During the oxidation step, nearly 80% of tea catechins are oxidized or condensed to other large polyphenolic molecular such as theaflavins and thearubigins (Balentine et al., 1997). Therefore, compared to oolong and black tea, green tea contains the highest amount of catechins (Zuo et al., 2002; Cabrera et al., 2003). Also, due to its unoxidized state, green teas keep their vital color, which is bright green to yellow.

_ Oolong Tea_

Oolong tea is commonly referred to as semi-fermented and it is mainly produced in China and Taiwan (Pettiegrew, 1997). The processing of oolong tea is similar to black tea. The first step is withering and rolling. However, the main difference is the degree of oxidation/fermentation. Oxidation occurs at only half of the time of black tea. Different tea manufactories have their preferred methods to produce their desirable tea product, thus, oolong tea has a wide variety of aromas and flavors.

_ Black Tea_

Black tea is the most well-known variety of tea in Western countries. Black tea is completely fermented and has a brown color. During the fermentation, tea catechins are oxidized or condensed to other large polyphenolic molecules such as theaflavins and thearubigins (Robert, 1958) and other undefined flavonoids, which provide distinctive flavor and color to black tea beverage (Wiseman et al., 1997). Originally form China, black tea is now cultivated worldwide. Some of the most famous black teas come from Indian regions such as the Assam, Darjeeling, and Sri Lanka.


2.4.3 Caffeine

Caffeine known as 1,3,7-trimethylxanthine (Figure 2.4), which is both water and oil soluble. Caffeine has been used for thousands of years and studied for more than a hundred years. It is possibly one of the most widely consumed food ingredients all over the world (Heckman et al., 2010). Caffeine is naturally found in more than 60 kinds of plants, including beans, leaves, and fruits. The kola nut (Cola acuminate), cacao bean (Theobroma cacao), yerba mate (Ilex paraguariensis), and guarana berries (Paullinia cupana) are the common sources of caffeine; however, the world’s primary sources of dietary caffeine are from roasted coffee beans (Coffea Arabica and Coffea robusta), and tea leaves (Camelia siniensis) (Barone and Roberts, 1996).

![Chemical structure of caffeine naturally found in coffee and tea.](image)

The common sources of caffeine are from coffee, tea, and soft drinks, cola-type drinks, and the products containing cocoa or chocolate (Barone and Robets, 1996). According to the statistical data, 71% of dietary caffeine comes from coffee, 16% comes from soft drinks, and nearly 16% comes from tea. In North America, the main sources of caffeine in adult diets are from coffee (60-75%) and tea (15-30%);
the major sources of caffeine in the diet of children are from soft drinks and chocolate (Nawrot et al., 2003). Generally, green tea contains 8.36mg of caffeine per cup, while oolong and black tea contain 12.55 mg and 25-110 mg of caffeine, respectively (Pittigrew, 1997). In North America, nearly 90% of adult consume caffeine on a daily basis and about 80% of the world’s population consumes a caffeinated product every day (Ogawa and Ueki, 2007).

Caffeine has been widely studied and some of research proves that there are health benefits from caffeine consumption. A large body of evidence shows that caffeine is associated with enhanced mood and alertness (Lorist et al., 2003), increased exercise performance (Doherty, 2004), and speed up the attention, reaction time, and awareness (Cysneiros et al., 2007). In addition, studies have indicated that caffeine can reduce the symptoms from Parkinson’s disease, which is a disorder of the central nervous system (Blandini et al., 2000). Parkinson’s disease is a neurodegenerative disease which causes by loss of dopamine, the metabolism of Antagonism of adenosine receptors stimulate dopamine release in the brain (Trevitt et al., 2009).

In recent years, there is some research that has investigated the relationship between caffeine intake and the risk of type 2 diabetes. It was reported that habitual caffeine intake is associated with a reduced risk of type 2 diabetes mellitus (van Dam and Hu, 2005). In addition, caffeine consumption has been reported to reduce weight for many years in consumers. Research suggests that caffeine is associated with increased metabolic rate, energy expenditure, lipid oxidation and thermogenic activities (Heckman et al., 2010). Due to these reactions in the human body, caffeine has been considered a weight management tool. Overall, positive influence of caffeine
for consumers may be associated with several diseases such as reducing diabetes, weight control, and cardiovascular disease prevention.

### 2.4.4 Catechins

The major constituents of tea polyphenols are catechins with flavan3-ols structures and their polymerized products. The principal naturally occurring catechins in most tea leaves are: catechin gallate (CG), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), gallocatechin gallate (GCG), and epigallocatechin gallate (EGCG) (Figure 2.5).
Figure 2.5 Chemical structure of six common catechins found in teas.
(Source: Zuo et al., 2002)
2.4.5 Potential Health Benefit of Tea Catechins

Teas have been considered as medicine and a healthful beverage since ancient times. Traditional Chinese medicine has recommended tea for many medical purposes such as headaches, body aches and pains, digestion, depression, detoxification, and as an energizer (Cabrera et al., 2006). Due to their potential chemoprotective properties, teas have been attracting attention from the scientific community over the past decade. The total flavonoid content varied from 21.2 to 103.2 mg/g for regular teas and from 4.6 to 39.0 mg/g for decaffeinated teas (Henning et al., 2003). Research shows that there is no observed adverse effect level (NOAEL) of green tea catechin in F344 rats was estimated to be 1.25% (763.9 mg/kg body weight/day for males and 820.1 mg/kg body weight/day for females) (Takami et al., 2008).

Oxidative damage to biomolecules have been found to impact a number of chronic diseases, including cardiovascular diseases, cancer, and neurodegenerative disease. The antioxidant properties of catechins and other polyphenolic compounds in tea, especially green tea, have been considered as potential health-promoting compounds. Numerous epidemiologic studies show that there are several positive relationships between tea consumption and the decreased incidence of cardiovascular diseases and cancer in humans (Higdon and Frei, 2003). Cabrera et al. (2006) indicated that catechins can act as antioxidants through several ways: 1) inhibition of redox-sensitive transcription factors; 2) inhibition of “pro-oxidant” enzymes such as inducible nitric oxide synthase, lipoxygenases, cyclooxygenases and xanthine oxidase; and 3) induction of antioxidant enzymes such as glutathione-S-transferases and superoxide dismutases. McKay and Blumberg (2002) reported that consuming green tea and encapsulated green tea extract repeatedly for one to four weeks has been shown to decrease biomarkers of oxidative status. Moreover, Klaunig et al. (1999)
investigated 40 male smokers in China and 27 male and female (smoker and non-smoker) in the United States, and showed that the oxidative DNA damage, lipid peroxidation, and free radical generation were reduced after consuming around 6 cups/day of green tea for seven days. Erba et al. (2005) studied the effect of the addition of two cups of green tea (containing approximately 250 mg of total catechins) in two groups, consuming green tea and not consuming green tea. The results suggested that the ability of green tea, consumed within a balanced, controlled diet, can improve overall the antioxidative status and can protect against oxidative damage in humans.

Cancer, also characterized as aging-related diseases, is often related to the aging process; therefore, prevention of aging-related diseases will depend on slowing the aging process and avoiding the clinical appearance of the disease (Cabrera et al., 2006). Evidence for anticarcinogenic potential of tea catechins has been studied by numerous researchers. The experimental studies described catechins action to bind directly to carcinogens, induce Phase II enzymes such as UDP-glucuronosyl transferase and inhibit heterocycle amine formation (McKay and Blumberg, 2002). Today, catechin from green tea is accepted as a cancer preventive in numerous in vitro and in vivo epidemiological studies (Cabrera et al., 2006). Fujiki et al. (1998) investigated the effect of 8,552 Japanese adults consuming ten or more cups of green tea per day. Their results showed that cancer onset of patients who had consumed over 10 cups of green tea was 8.7 years later for females and 3 years delay for males, compared with patients who only consumed under 3 cups of tea per day. The mechanisms for delaying are from the actions of EGCG inhibiting the tumor necrosis factor-α (TNF-α) release (Fujiki et al., 1998). Furthermore, a Japanese study indicated that increased consumption of green tea prior to clinical cancer onset is
significantly associated with improved prognosis of stage I and II breast cancer, on association probably related to a modifying effect of green tea on the clinical characteristics of the cancer (Nakachi et al., 1998). Catechins induce Phase I cytochrome P450 and Phase II glucuronosyl transferase; therefore, enhancing the deoxification of carcinogens (McKay and Blumberg, 2002). Moreover, EGCG induces apoptosis and cell cycle arrest in human carcinomas, and EGC inhibits the proliferative response in several different animal and human cells (McKay and Blumberg, 2002).

Numerous studies performed including have been trials and animal studies on the relationship between tea consumption and blood pressure (Cebrera et al., 2006). Recently, some epidemiological studies indicated that green tea consumption slightly reduces blood pressure. Yang et al. (2004) reported that in Chinese populations, habitual moderate strength green or oolong tea consumption, 120mL/day or more for 1 year, significantly reduced the risk of developing hypertension. Furthermore, Hodgson et al. (2003) concluded that long-term regular ingestion of tea may have beneficial effects on blood pressure for older women. Although epidemiological studies suggest that green tea consumption is related to a reduced risk of cardiovascular disease, the mechanisms for these observations still remain uncertain (Cebrera et al., 2006).

Since the increasing evidence of tea’s beneficial health effects, it could be advisable to encourage the regular consumption of this widely available, tasty and inexpensive beverage as an interesting beverage, which not only shows beneficial effects but also has energetic benefits (Cebrera et al., 2006). Even though there is no single food item that can provide a significant effect on public health, it is important to note that diet has a major impact on the cause of many diseases. Taking all of this
into account, it would be reasonable to consider consumption of green tea regularly in Western diets.

2.5 Anthocyanins Copigmentation

2.5.1 Overview

Copigmentation is a phenomenon that the molecular complex anthocyanins with other phenols, also called copigments, are the major color stabilization mechanism in plants and flowers (Davies and Mazza, 1993). When the copigmentation occurs, hyperchromic effect and bathochromic shift can be observed. Hyperchromic effect ($\Delta A$) is the fortification of color intensity and bathochromic shift ($\Delta \lambda_{\text{max}}$) is the wavelength shift of maximum absorption in the visible range toward higher wavelength (Davies and Mazza, 1993; Mazza and Miniati, 1993). Copigmentation reactions can enhance the color and stability of anthocyanins (Brouillard, 1982).

Copigmentation is a widespread phenomenon in nature and can occur in fruit-derived products such as juices and wines (Mazza and Brouillard, 1987). Moreover, this phenomenon can enhanced the color and stability of fruit and berry products, for example, in purees, jams, and syrups. In food science, copigmentation is considered an important interaction, as color is one of the quality factors that affects consumer acceptance of food and the anthocyanins color stability is still an issue in the industry. This is why more research is needed to be conducted in the field of copigmentation of anthocyanin containing foods.
2.5.2 Copigments

Copigments are naturally occurring molecules in plants that do not significantly contribute to the color. Copigments are composed of a wide range of different molecules, such as flavonoids and other polyphenols, alkaloids, amino acid, organic acid and the anthocyanins themselves (Mazza and Brouillard, 1987; Davies and Mazza, 1993). Aside from phenolic moleculars, metal complexing and condensation of anthocyanins with acetaldehyde is also related to the increase in color stability (Mazza and Brouillard, 1987). Generally, addition of a copigment will enhance the color stability during storage (Erio and Heinonen, 2002).

The most efficient copigments discovered are flavonols (quercetin and rutin), aureusidin and particularly C-glycosyl flavones such as swertisin (Mazza and Brouillard, 1987). Also, phenolic acid, such as sinapic acid and ferulic acid have been shown to greatly enhance color and produce bathochromic shift (Asen et al, 1972).

Copigmentation is affected by several factors, while pH is an important factor, it has been discovered that the copigment effect occurs from pH values close to 1 to neutrality (Asen et al., 1972; Davies and Mazza, 1993). Asen et al. (1972) reported that the formation of copigment complexes resulted in a bathochromic shift in the visible $\lambda_{\text{max}}$ of anthocyanins and a large increase in extinction at pH 3 to 5. Davies and Mazza (1993) also indicated that solutions of nonacylated and acylated pelargonidins exhibited the maximum degree of copigmentation at pH 3.2-3.5 and 3.7-4.7, respectively, with chlorogenic acid as the copigment. Other factors that dramatically affect the copigmentation phenomenon are: the type of anthocyanins and copigments present, concentration of anthocyanins and copigments, temperature and metals present (Osawa, 1982; Mazza and Brouillard, 1987).
2.5.3 Mechanisms of Copigmentation

There are several mechanisms describing the copigmentation phenomenon. The most crucial mechanisms, called intermolecular and intramolecular copigmentation, were introduced to illustrate the increased stability of acylated anthocyanins. Figure 2.6 shows the copigmentation mechanism of anthocyanins.

![Figure 2.6 Copigmentation mechanisms of anthocyanins.](Adapted from Giusti and Wrolstad, 2003)

2.5.3.1 Intermolecular Copigmentation

Intermolecular copigmentation describes the non-covalent reaction between colored anthocyanins and colorless copigments, such as flavonoids, polyphenols, amino acids and anthocyanins themselves (Brouillard, 1983; Mazza and Miniati, 1993). The main role of a copigment is controlling the extent of the hydration reaction between the flavylium cation and the colorless carbinol pseudobases (Mazza and Miniati, 1993). Both of these equilibrium forms are almost planar, with efficient
delocalization of π-electrons (Asen et al., 1972). Most non-covalent interaction are related to π-π overlap, dipole-dipole interaction, and possible hydrogen binding (Dangles and Brouillard, 1992). They result in an overlapping arrangement of the two molecules, which can prevent the nucleophilic attack on the anthocyanin molecules by water. The larger the number of hydroxyl groups in the flavonoid molecular, the stronger the complex formation (Chen and Hrazdina, 1981). Recent studies suggest that intermolecular copigmentation plays a major role in the presentation of blanching during light and heat treatment of fruit and berries (Malien-Aubert et al., 2001).

2.5.3.2 Intramolecular Copigmentation

Intramolecular copigmentation represents the covalent acylation of the anthocyanin molecule, which can stabilize the pigments (Dangles et al, 1993). The covalent linkage of anthocyanin molecules can occur with an organic acid, an aromatic acyl group, or a flavonoid (Erio and Heinonen, 2002). The stability of these anthocyanins in weakly acidic or neutral solution is due to the sandwich type stacking, where the aromatic residue of acyl groups are linked with the pyrylium ring of the flavylium cation; therefore, this binding decreases the hydration at the C-2 and C-4 position (Brouillard, 1983). The extent of the hydration reaction reduces and the stability of the chromophores strongly increases, since the proton transfer reaction apparently remains unaffected by the stacking process. Generally, intramolecular copigmentation is considered to be stronger and more effective compared to intermolecular copigmentation, likely due to the covalent bonding being stronger than the non-covalent binding (Brouillard, 1983).
Color stability appears to increase with increasing content of organic acid (cinnamic and malonic acid) and increasing the substitution of the agylcone (delphinidin more stable than cyanindin or peonidin) (Saito et al, 1985). Nevertheless, the position of attachment to the sugar, the structure of the sugar, the strength and the structure of the combination are also important factors in the stacking process (Brouillard, 1983). Intramolecular copigmentation has been found to stabilize the color in many plants, such as, radishes, red potatoes, red cabbage, black carrots, and purple potatoes and other plant materials, which contain high amounts of acylated anthocyanins (Giusti and Wrolstad, 2003).

2.5.3.3 Anthocyanins interactions with Catechins

Copigmentation of anthocyanin with flavan-3-ols, such as catechin, can be induced by adding acetaldehyde (Mazza and Brouillard, 1987). The reaction produces highly pigmented new anthocyanin-catechin complexes linked by CH$_3$CH bridges (Timberlake and Bridle, 1977). Timberlake and Bridle (1977) also indicated that the nature and extent of interaction depended on the ratio of catechin:anthocyanin; the larger the ratio, the faster the interaction. According to their study, malvidin 3,5-diglucosides reacted more slowly than 3-glucosides, which gave smaller violet shifts, and their augmented color was more stable. Furthermore, Gonzalez-Manzano et al. (2009) concluded that the copigmentation effect between anthocyanins and flavanols can be determined by the quantitative and qualitative composition of both types of compounds in the medium. Their results showed that the copigmentation effect causes a decrease in lightness and increase in chroma, with the color shift toward orange tonalities; also, flavanols can induce significant modifications to the color of
anthocyanin in wine-like solutions. These findings have implications in the color of foods, as well as the augmentation of anthocyanin color; therefore, it is necessary to do more research on the copigmentation of anthocyanin and catechin to produce natural and effective copigmentation in food products.
CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Anthocyanins rich sources: strawberry (*Fragaria x ananassa* Duch.), blackberry (*Rubus fruticosus*), American eggplant (*Solanum melongena* L.), Red cabbage (*Brassica oleracea* L. var. *capitata*) and Japanese green tea were purchased from a local grocery store (Columbus, OH).

Epicatechin: EC (>98% purity), catechin gallate: CG (>98%), gallocatechin gallate: GCG (>98%), epigallocatechin gallate: EGCG (>95%) standards and caffeine powder were obtained from Sigma-Aldrich (St. Louis, MO). Epicatechin gallate: ECG (>98%) and epicatechin gallate: ECG (>98%) standards were obtained from LKT Laboratories, Inc. (St. Paul, MN). Gallic acid powder was purchased from MP Biomedicals (Aurora, OH).

3.2 Experimental Design

The anthocyanins and catechins samples were extracted from anthocyanin-rich materials (strawberry, blackberry, eggplant and red cabbage) and tea, respectively (Table 3.1). Each of control group content 30mg/L of anthocyanin; and the treatment samples content 30mg/L of anthocyanin and 30mg/L, 150mg/L, 300mg/L, 600mg/L, and 1500mg/L of catechins. The anthocyanins and catechins were combined in
different concentration ratios: 1:1, 1:5, 1:10, 1:20 and 1:50. The combination ratios were chosen based on our preliminary experiment, which indicated that the catechin concentration lower than the anthocyanin content had no significant different. All samples were stored at 4°C in dark condition for 70 days. At the specific time point, the samples were taken out to measure the pH, color, total phenolic, monomeric anthocyanins, and HPLC.

<table>
<thead>
<tr>
<th>Anthocyanin (ACN) sources</th>
<th>Catechins source</th>
<th>Storage Time (Day)</th>
<th>Analysis</th>
<th>Storage conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strawberry (Pg-3-glu)</td>
<td>Japanese Green Tea (EGC, ECG, EGCG, GCG, EC, CG, Gallic acid, Caffeine)</td>
<td>0 1 5 10 20 30 45 70</td>
<td>*Color- CIELab, hue angle, chroma *Total phenolics *Total monometric anthocyanin *HPLC- 520nm, 280nm *pH</td>
<td>4°C Stored in aluminum box in the dark</td>
</tr>
<tr>
<td>Blackberry (Cy-3-glu)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>American Eggplant (Dp-3-rut)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saponified Red Cabbage (Cy-3-soph-5-glu)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red cabbage (Acylated Cy-3-soph-5-glu)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1 Experimental design for the combinations of anthocyanins and catechins, which the samples were stored at 4°C and dark condition for 70 days of total testing.

3.3 Anthocyanins Extraction from Anthocyanins Rich Materials

Strawberry, blackberry, eggplant and red cabbage were exacted by 70% acetone and 30% 0.1% HCl acidified water for few minutes using a Waring Commercial Laboratory Blender (Warning Laboratories, Torrington, CT). The solution was vacuumed and passed through a 70mm Whatman No.4 filter paper (Whatman Inc., Florham, NJ), then collected by a Buchner Funnel (Fisher Scientific, Fair Lawn, NJ). The anthocyanins solution was transferred into a separatory funnel (Fisher Scientific, Fair Lawn, NJ) with 2 volumes of chloroform. The anthocyanin/chloroform solution was gently mixed by turning the funnel upside
down a few times then placed in the refrigerator at 4°C for overnight to allow the clear partition between the two phases is obtained. The bottom layer of chloroform and polar solvents were discarded and the top layer (anthocyanin/phenolic concentrate) was collected. Residual acetone/chloroform was evaporated by using a Buchi rotoary evaporator (Fisher Scientific, Fair Lawn, NJ) at 40°C under vacuum. The solution was transferred to a 100ml final volume using a volumetric flask and acidified deionized distilled water. After this, the samples were purified by MCX and C18 column (Waters Assoc, Milford, MA).

3.4 Catechins Extraction from Tea

According to the literature views and our preliminary experiment, the results show that green tea contained higher amount of catechins than black tea and oolong tea. Therefore, the Japanese green tea was chosen as the catechin sources in this study. Japanese green tea leaves were extracted by boiling water for 30 minutes in a beaker. The green tea was vacuum and filtered through a 70mm Whatman No.4 filter paper (Whatman Inc., Florham, NJ), then collected by a Buchner Funnel (Fisher Scientific, Fair Lawn, NJ). The solution was purified by C18 column (Waters Assoc, Milford, MA).

3.5 Total Monomeric Anthocyanins

The pH differential method was used to determine the concentration of anthocyanin according to Giusti and Wrolstad (2005). Buffer solutions were prepared using 0.1M potassium chloride at pH 1.0 and 0.4M sodium acetate at pH 4.5. Absorbance of samples at pH 1.0 and 4.5 were measured using SpectraMax 190
Microplate Reader (Molecular Devices LLC, Sunnyvale, CA) at 700nm and $\lambda_{\text{vis-max}}$.

The $\lambda_{\text{vis-max}}$, molar absorptivity ($\epsilon$) and molecular weight for each sample shows in Table 3.2.

<table>
<thead>
<tr>
<th>ACN sources</th>
<th>$\lambda_{\text{vis-max}}$ (nm)</th>
<th>Molar absorptivity ($\epsilon$)</th>
<th>Molecular weight (MW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strawberry</td>
<td>498</td>
<td>15600</td>
<td>433.2</td>
</tr>
<tr>
<td>Blackberry</td>
<td>510</td>
<td>26900</td>
<td>449.2</td>
</tr>
<tr>
<td>Eggplant</td>
<td>520</td>
<td>23700</td>
<td>465.2</td>
</tr>
<tr>
<td>Saponified-Red cabbage</td>
<td>510</td>
<td>26900</td>
<td>449.2</td>
</tr>
<tr>
<td>Red cabbage</td>
<td>526</td>
<td>26900</td>
<td>449.2</td>
</tr>
</tbody>
</table>

Table 3.2 The $\lambda_{\text{vis-max}}$, molar absorptivity ($\epsilon$) and molecular weight (MW) used in the experiment.

Calculation for absorbance of the sample as follows:

$$A = (A_{\lambda_{\text{vis-max}}} - A_{700})_{\text{pH 1.0}} - (A_{\lambda_{\text{vis-max}}} - A_{700})_{\text{pH 4.5}}$$

Monomeric anthocyanin pigment (mg/liter) = $$(A \times MW \times DF \times 1000)/ (\epsilon \times 1)$$

### 3.6 Saponification of Anthocyanins

Saponification of anthocyanins is also known as alkaline hydrolysis of anthocyanin. This method was performed according to the method described by Giusti and Wrolstad (1996). 10ml of 10% aqueous KOH was added to 1ml of sample in a screw-cap test tube. The pigment was hydrolyzed for 8 minutes at room temperature in the dark. The solution was neutralized using 2N HCl, and the hydrolysate was purified using a C18 Sep-Pak cartridge (Waters Assoc, Milford, MA). Methanol from the samples was removed on the rotary evaporator until a small drop of liquid was present. The sample was then dissolved in 0.01% HCl acidified water to obtain 100ml final volume.
3.7 Total Phenolics

The microscale Folin-Ciocalteau method was used to evaluate total phenolics (Waterhouse, 2005). First, the gallic acid standard was made by diluting 0.5ml, 1ml, 2.5ml, 5ml, and 10ml of gallic acid solution (5g/liter) into 50ml of total volume of distilled water in a glass tube. Then, 20µl of sample and 20µl of each gallic acid dilution were added to separate 3ml plastic cuvettes. All of cuvettes were filled with 1.58ml of distilled water and 100µl of FC reagent for 1 to 8 minutes incubation. Lastly, 300µl of sodium carbonate solution (Na$_2$CO$_3$) was added and the solution was gently mixed by pipeting. In order to get a better chemical reaction, the samples were incubated 2 hours at room temperature before measuring the absorbance. Absorbance was measured at 765nm using SpectraMax 190 Microplate Reader (Molecular Devices LLC, Sunnyvale, CA). The standards were measured to create the calibration curve, and to determine the corresponding gallic acid concentration of the samples.

3.8 pH Measurement

Measurement of pH was an important method to confirm that the pH values of samples were below 3.5, which is an excellent condition to maintain the anthocyanin stability. The pH was measured using the SevenCompact pH/Ion meter (Mettler-Toledo Inc., Columbus, OH). The pH meter was calibrated by pH buffer: pH 1.68, pH 4, pH 7, and pH 10 (Fisher Scientific, Fair Lawn, NJ) before measuring the samples. Insert the electrode into the samples to obtain the pH value until the value was stable. Using the distilled deionized water and wipers to clean the electrode before measuring next samples.
3.9 Color Analysis

The color of sample was measured by ColorQuest XE (HunterLab, Inc., Reston, VA) to detect transmittance and reflectance. Samples were read using illuminant D65. Measurement taken were CIE L*, a*, b*, chroma and hue angle. L* indicates the lightness or darkness (100 for complete white and 0 for complete darkness). The a* value indicates red-green colors with positive values representing red color and negative values representing green color. The b* values indicates yellow-blue color with positive values indicating yellow color and negative values indicating blue color. Chroma shows the intensity of the color, the higher value, the higher intensity. Hue angle represents the different colors of the spectrum on a 360° axis, which classifies the color of red, yellow, blue, etc.

The ColorQuest XE was calibrated before every session of use. Samples were placed in 25ml cell culture flask to measure the transmittance and reflectance. Each sample was measured 3 times and averaged.

3.10 High Performance Liquid Chromatography (HPLC)

In order to identify anthocyanins and catechins present in our samples, we used a high performance liquid chromatography (HPLC) (Shimadzu, Columbia, MD) system equipped with LC-6AD pumps and s SIL-20A autosampler coupled to a SPD-M20A Photodiode Array Detector (Shimadzu, Columbia, MD). A reverse phase Symmetry C18 (3.5µm 4.6×150mm) column (Water Corporation, Milford, MA) and a 4.6 x 22mm Symmetry 2 micro guard column (Water Corporation, Milford, MA) were used. All of samples were filtered through a 0.45µm Phenex Syringe filter
(Phenomenex, Torrance, CA) before being placed into the HPLC. Samples were analyzed using a flow rate of 0.8ml/min and a 50 µl injection volume.

The measurement of anthocyanins was performed using the Schimadzu HPLC equipped with LC-20AD pump. Separation of anthocyanins and catechins for the combination of tea with strawberries, blackberries, eggplant, saponified red cabbage, red cabbage. The linear gradient used in the analysis was from 0%-5% B 5mins, 5%-5% B 5mins, 5%-25% B 21mins, 25%-40% B 5mins, 40%-0% B 5mins. The mobile phase consisted of solvent (A) 100% acetonitrile and solvent (B) 5% (v/v) formic acid. Anthocyanins were measured at 520nm, while catechins were measured at 280nm.

3.11 Statistical Analysis

The result of monomeric anthocyanin concentration, total phenolic content and color were plotted against time and analysis of covariance (ANCOVA) was used to determined the adequacy of the degradation kinetic model at p<0.05 and a 95% confidence interval. The result of chroma, hue, and lightness were subjected to two-way Analysis of Variance (ANOVA) with an alpha< 0.05 acceptance level. All statistical analyses were performed using SPSS 21.0 software (Chicago, IL).
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Determination of Total Monomeric Anthocyanins

Five anthocyanin-rich materials were chosen to carry out stability studies including strawberries, blackberries, eggplant, saponified red cabbage, and red cabbage. These different sources were chosen due to content single but different common anthocyanidins with sugar, which are pelargonidin-3-glucoside (strawberry), cyanidin-3-glucoside (blackberry), delphinidin-3-rutinoside (eggplant), cyanidin-3-sophoroside-5-glucoside (saponified red cabbage), and acylated cyanidin-3-sophoroside-5-glucoside (red cabbage) (Figure 4.4). These anthocyanin rich materials were chosen to compare the impact on stability of the different aglycones (pelargonidin, cyanidin, delphinidin) and sugar and acyl substitutions (glucoside, rutinoside, sophoroside, and acylating groups). Moreover, these anthocyanin rich sources are readily available from different commercial sources, and they are common anthocyanin sources in people’s diet.

The total monomeric anthocyanins (TMA) content in the anthocyanin-rich materials (strawberry, blackberry, eggplant, saponified-red cabbage, and red cabbage) showed that different plant materials contain different concentrations of monomeric anthocyanin. All fresh materials, as previously reported in the literature, contained
high amount of pigment, with blackberries (118.73g/100g fresh weight) having higher anthocyanin content than the eggplant (74.9g/100g fresh weight) and strawberries (31.79mg/100g fresh weight). The red cabbage and saponified red cabbage extracts contained 289.9mg/L and 383.07mg/L monomeric anthocyanin, respectively.

Due to differences on TMA of the anthocyanin rich materials used, dilution of different materials was used to obtain the same concentration in the final research samples, which contained 30mg/L of TMA. However, the measurement of initial (Day 0) monomeric anthocyanin in each sample showed the content ranging between 20.85±0.44 to 27.29±0.10 mg/L. The measurement of monomeric anthocyanin at the initial time point (Day 0) showed a loss of total monomeric anthocyanin: 29.25% (strawberry), 21.26% (blackberry), 29.4% (eggplant), 10.53% (saponified red cabbage), and 32.63% (red cabbage). These pigment losses could be attributed to pigment degradation during the dilution step and/or during the few days storage time before the storage study began.

Figure 4.1 shows that solutions containing a combination of anthocyanins and catechins consistently measured a lower concentration of TMA after time 0, although the amount of anthocyanin added to all samples was exactly the same. This is probably attributed to the method used for TMA quantitation, which is based on the changes in absorbance at different pH. Anthocyanin pigments undergo reversible structural transformation with a change in pH manifested by strikingly different spectra. The colored oxonium form predominates at pH 1.0 and the colorless hemiketal form at pH 4.5 (Giusti and Wrolstad, 2001). The pH-differential method is based on this reaction, and permits rapid measurement of the total anthocyanins. However, this method is affected by the presence of polymerized degraded pigments and other interfering compounds. In this research, due to copigmentation, the
combination of anthocyanins and catechin produced large polymerized compounds, which impacted the anthocyanin structural transformation. Therefore, the monomeric anthocyanin content decreased with increasing concentrations of catechin, which is a polyphenolic.

Over the 70 days of storage in the dark at 4°C, the TMA stability was monitored and the results are presented in Figure 4.1 and Table 4.1. The TMA seems to degrade during the 70 days storage. The TMA content in the control group of all anthocyanin rich materials shows no significant differences over time (p>0.05), indicating that the anthocyanin from the different sources’ were relatively stable in control solutions. However, there was little but significant degradation over time for the treatment samples. Figure 4.1 shows that the higher concentration of catechins consistently showed lower concentration of TMA over time. This degradation was statistically significant different (p<0.05, ANCOVA and regression analysis) for anthocyanin combined with catechin. The results showed that not only the method used for TMA quantitation impacted the content of TMA, but also there some certain degradation of TMA over 70 days storage, especially when the anthocyanin was combined with higher amounts of catechins.
Figure 4.1 Changes in total anthocyanins in the combination of strawberry of anthocyanins and catechins over 70 days of storage.

Figure 4.2 Changes in total anthocyanins in the combination of blackberry of anthocyanins and catechins over 70 days of storage.
Figure 4.3 Changes in total anthocyanins in the combination of eggplant of anthocyanins and catechins over 70 days of storage.

Figure 4.4 Changes in total anthocyanins in the combination of red cabbage of anthocyanins and catechins over 70 days of storage.
Figure 4.5 Changes in total anthocyanins in the combination of saponified-red cabbage of anthocyanins and catechins over 70 days of storage. The graphs show that there was no significant degradation of monomeric anthocyanin from the control group in different material (p>0.05); however, the catechin treatment samples show significant degradation of monomeric anthocyanins (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1:1</th>
<th>1:5</th>
<th>1:10</th>
<th>1:20</th>
<th>1:50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strawberry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>24.57±0.39</td>
<td>23.58±0.40</td>
<td>23.92±0.20</td>
<td>18.92±6.50</td>
<td>21.49±0.20</td>
<td>22.27±0.80</td>
</tr>
<tr>
<td>Day 70</td>
<td>25.07±0.85</td>
<td>22.76±1.40</td>
<td>18.00±1.75</td>
<td>15.331.38</td>
<td>12.45±1.84</td>
<td>14.84±4.14</td>
</tr>
<tr>
<td>Blackberry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>25.01±1.48</td>
<td>24.83±0.16</td>
<td>24.31±0.41</td>
<td>23.29±0.21</td>
<td>22.66±0.08</td>
<td>21.64±1.75</td>
</tr>
<tr>
<td>Day 70</td>
<td>24.92±1.26</td>
<td>23.58±0.53</td>
<td>19.97±0.47</td>
<td>17.50±2.09</td>
<td>14.85±1.48</td>
<td>12.89±2.84</td>
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<td>Eggplant</td>
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<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>23.32±0.54</td>
<td>23.09±0.23</td>
<td>23.83±1.24</td>
<td>20.86±0.95</td>
<td>20.43±0.18</td>
<td>15.54±0.36</td>
</tr>
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<td>Day 70</td>
<td>22.31±0.85</td>
<td>19.75±0.21</td>
<td>14.54±0.82</td>
<td>12.07±0.17</td>
<td>9.13±0.89</td>
<td>11.78±0.91</td>
</tr>
<tr>
<td>Saponified-RC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>27.29±0.10</td>
<td>27.48±0.24</td>
<td>26.76±0.45</td>
<td>26.67±0.38</td>
<td>26.31±0.26</td>
<td>26.53±0.43</td>
</tr>
<tr>
<td>Day 70</td>
<td>32.73±0.51</td>
<td>28.32±1.79</td>
<td>22.65±0.09</td>
<td>17.37±0.22</td>
<td>20.09±0.51</td>
<td>16.93±0.39</td>
</tr>
<tr>
<td>Red Cabbage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>20.85±0.44</td>
<td>20.18±0.46</td>
<td>19.42±2.21</td>
<td>18.73±1.26</td>
<td>19.71±0.79</td>
<td>22.36±1.71</td>
</tr>
<tr>
<td>Day 70</td>
<td>23.98±0.61</td>
<td>20.83±0.79</td>
<td>17.41±1.44</td>
<td>17.56±1.38</td>
<td>15.54±0.42</td>
<td>20.09±2.46</td>
</tr>
</tbody>
</table>

Table 4.1 Total monomeric anthocyanin content (mg/L) in the control and treatment samples ± standard deviation at the start (Day 0) and after 70 days of refrigerated storage in the dark. RC: Red cabbage.
Obviously, the Day 30 TMA content in the red cabbage showed significant differences between the control, 1:1, and 1:5 and in the ratio 1:10, 1:20, and 1:50; however, the TMA of the Day 45 came back to the range of 15-23 mg/L. This phenomena at Day 30 might be due to an experimental error. There were several reasons to cause the experimental error, including improper handling, machine error, lost samples, and temperature change. Overall, the TMA content still followed the trend line during the 70 days storage.

4.2 Determination of Total Phenolic

The total phenolic content of control and treatment groups from different anthocyanin sources was measured and compared (Table 4.2 and Figure 4.2). All anthocyanin rich materials (control group) used for the different treatments contained low levels of total phenolic, ranging from 5.43 (saponified-red cabbage) to 81.5 (red cabbage) mg/L of phenolic, calculated as gallic acid equivalents (GAE). As expected, the addition of the catechin as copigment increased the total phenolic content (p<0.05). Overall, the total phenolic content consistently increased from treatment ratio 1:1 to 1:50 in all anthocyanin rich materials: strawberry (S), blackberry (B), eggplant (E), saponified-red cabbage (SR), and red cabbage (RC). The highest amount of total phenolics of ratio 1:50 from different material at the initial of testing (Day 0) were: 1369.79±155.86 (S), 1292.98±61.26 (B), 1334.24±92.76 (E), 1305.31±6.48 (SR), and 1250.88±46.64 (RC) mg/L.

Over the 70 days of storage in the aluminum box at 4°C, the degradation of total phenolic content in blackberry and eggplant was statistically not significantly
different (p>0.05, ANCOVA and regression analysis) for control and treatment samples, which means that total phenolic content remained stable at the blackberry and eggplant. Total phenolic content show a linear correlation $R^2$>0.9 from control to highly catechin treatment. However, the statistical results show there was slight degradation of total phenolic content in the strawberry, saponified-red cabbage, and red cabbage (p<0.05). After the 70 days storage, control group of different materials still contained the lowest level of total phenolic due to no catechin treatment. The total phenolic content of the control groups at Day 70 were: 13.62±6.06 (S), 23.52±9.51 (B), 63.38±8.61 (E), 6.98±0.61 (SR), and 81.50±13.99 (RC) mg/L. The ratio of 1:50 still had the highest level of total phenolic content: 1523.79±104.76 (S), 1416.74±27.23 (B), 1382.14±37.96 (E), 1463.36±79.02 (SR), and 1573.52±73.25 (RC) mg/L.

Figure 4.6 Changes in total phenolic content in the combination of strawberry of anthocyanins and different catechins treatment over 70 days of storage.
Figure 4.7 Changes in total phenolic content in the combination of blackberry of anthocyanins and different catechins treatment over 70 days of storage.

Figure 4.8 Changes in total phenolic content in the combination of eggplant of anthocyanins and different catechins treatment over 70 days of storage.
Figure 4.9 Changes in total phenolic content in the combination of saponified-red cabbage of anthocyanins and different catechins treatment over 70 days of storage.

Figure 4.10 Changes in total phenolic content in the combination of red cabbage of anthocyanins and different catechins treatment over 70 days of storage.
### Table 4.2: Total phenolic content (mg/L) in the control and treatment samples ± standard deviation at the start (Day 0) and after 70 days of refrigerated storage in the dark. RC: Red cabbage.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1:1</th>
<th>1:5</th>
<th>1:10</th>
<th>1:20</th>
<th>1:50</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strawberry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>27.57±15.62</td>
<td>51.69±6.27</td>
<td>195.48±15.32</td>
<td>321.57±21.08</td>
<td>598.95±27.85</td>
<td>1369.79±155.86</td>
</tr>
<tr>
<td>Day 70</td>
<td>13.62±6.06</td>
<td>58.57±20.71</td>
<td>192.71±23.64</td>
<td>323.00±16.65</td>
<td>633.21±40.87</td>
<td>1523.33±104.76</td>
</tr>
<tr>
<td><strong>Blackberry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>29.12±17.38</td>
<td>32.21±12.56</td>
<td>165.71±7.90</td>
<td>320.10±13.99</td>
<td>540.05±38.44</td>
<td>1292.98±61.26</td>
</tr>
<tr>
<td>Day 70</td>
<td>23.52±9.51</td>
<td>46.0±8.332</td>
<td>167.02±10.84</td>
<td>349.17±43.03</td>
<td>643.57±55.57</td>
<td>1416.74±27.23</td>
</tr>
<tr>
<td><strong>Eggplant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>49.21±6.49</td>
<td>57.52±4.52</td>
<td>178.7±29.07</td>
<td>366.02±22.15</td>
<td>586.14±29.71</td>
<td>1334.24±92.76</td>
</tr>
<tr>
<td>Day 70</td>
<td>63.38±8.61</td>
<td>13.83±31.66</td>
<td>243.17±22.88</td>
<td>415.95±24.48</td>
<td>664.81±36.33</td>
<td>1382.14±37.96</td>
</tr>
<tr>
<td><strong>Saponified-RC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>5.43±10.17</td>
<td>37.88±8.40</td>
<td>175.21±3.43</td>
<td>303.95±11.43</td>
<td>568.57±13.64</td>
<td>1305.31±66.48</td>
</tr>
<tr>
<td>Day 70</td>
<td>6.98±0.61</td>
<td>57.48±9.80</td>
<td>180.57±11.21</td>
<td>339.10±53.76</td>
<td>697.62±32.80</td>
<td>1463.36±79.02</td>
</tr>
<tr>
<td><strong>Red Cabbage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>55.69±6.15</td>
<td>91.69±4.56</td>
<td>197.17±14.50</td>
<td>324.14±7.75</td>
<td>562.00±75.92</td>
<td>1250.88±46.64</td>
</tr>
<tr>
<td>Day 70</td>
<td>81.50±13.99</td>
<td>13.64±0.81</td>
<td>198.43±23.40</td>
<td>349.90±50.08</td>
<td>589.79±82.93</td>
<td>1573.52±73.25</td>
</tr>
</tbody>
</table>

4.3 Color Analysis

#### 4.3.1 Initial Color Characteristics of the Anthocyanin/Catechin Solutions

To evaluate how the catechin acts as copigment in the anthocyanin rich materials, color characteristics were compared between control and different treatment ratio samples. The initial (Day0 and Day1) color characteristics of the effects of catechin on color were described on Table 4.3. L* indicates the lightness or darkness with 100 for complete white and 0 for complete darkness. Chroma shows the intensity of the color, the higher value, the higher intensity. Hue angle represents the different colors of the spectrum on a 360° axis, which classifies the color of red, yellow, blue, etc.

We immediately measured the color characteristic after adding the catechin at room temperature, and storing the samples at 4°C for one day. The solution
equilibrated during this first day. Thus, the lightness (L*) and hue angle of all control and treatments samples decreased slightly, and the chroma increased slightly in the first day.

Comparing the control of different anthocyanin-rich materials, acylated-anthocyanins from red cabbage provided a darkest intense red color (L*=34.62, chroma=67.60, hue angle=16.80). Non-acylated anthocyanins from blackberries showed a darker orange/red color at higher intensity (L*=47.79, chroma=70.21, hue angle=38.61) as compared to other non-acylated anthocyanins from strawberries, eggplant, and saponified-red cabbage. One interesting observation was that the anthocyanin after the alkaline hydrolysis, when comparing the saponified-red cabbage and red cabbage, showed brighter and much lower intensity color characteristics (L* increased 98% and chroma decreased 43%) in saponified materials.

Addition of catechin acts as a copigment to the anthocyanin solutions resulted in a darker, higher intensity, and red color at lower lightness, higher chroma and hue angle value (p<0.05). This phenomena acted significant different (p<0.05) when adding higher concentration of catechin (1:50). Copigmentation of anthocyanin generally results in a hyperchromic effect, which is the enhancement of color intensity, and decreases in lightness, which becomes darker (Mazza and Miniati, 1993; Gonzalez-Manzano et al., 2009). The lightness of all materials consistently decreased when more catechin was added from 1:1 to 1:20; however, the chroma and the hue angle obviously increased up to 40% and 160% (saponified-red cabbage), respectively, when the anthocyanin:catechin ratio was 1:50. The original light red color turned into darker, higher intensity, orange/red color. The results showed that it is possible to produce high intensity red and orange/red color by using catechin as copigment.
<table>
<thead>
<tr>
<th>Natural Text</th>
<th>Numeric Text</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strawberry</strong></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td><em><em>Lightness (L</em>)</em>*</td>
<td>66.35±0.06</td>
</tr>
<tr>
<td><strong>Chroma</strong></td>
<td>35.44±0.05</td>
</tr>
<tr>
<td><strong>Hue angle</strong></td>
<td>45.59±0.02</td>
</tr>
<tr>
<td><strong>Blackberry</strong></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td><em><em>Lightness (L</em>)</em>*</td>
<td>47.79±0.16</td>
</tr>
<tr>
<td><strong>Chroma</strong></td>
<td>45.67±0.08</td>
</tr>
<tr>
<td><strong>Hue angle</strong></td>
<td>70.21±0.04</td>
</tr>
<tr>
<td><strong>Eggplant</strong></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td><em><em>Lightness (L</em>)</em>*</td>
<td>52.02±0.06</td>
</tr>
<tr>
<td><strong>Chroma</strong></td>
<td>50.84±0.10</td>
</tr>
<tr>
<td><strong>Hue angle</strong></td>
<td>60.40±0.19</td>
</tr>
<tr>
<td><strong>Saponified-RC</strong></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td><em><em>Lightness (L</em>)</em>*</td>
<td>68.38±0.02</td>
</tr>
<tr>
<td><strong>Chroma</strong></td>
<td>68.95±0.04</td>
</tr>
<tr>
<td><strong>Hue angle</strong></td>
<td>38.68±0.08</td>
</tr>
<tr>
<td><strong>Red Cabbage</strong></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td><em><em>Lightness (L</em>)</em>*</td>
<td>34.62±0.04</td>
</tr>
<tr>
<td><strong>Chroma</strong></td>
<td>34.55±0.12</td>
</tr>
<tr>
<td><strong>Hue angle</strong></td>
<td>67.60±0.04</td>
</tr>
</tbody>
</table>

Table 4.3 The initial (Day0 and Day1) color characteristic of five different anthocyanin rich materials in the control and treatment samples ± standard. RC: Red cabbage.
4.3.2 Color Stability in the Anthocyanin/Catechin Solutions

Color naturally degrades over time, especially in natural food products. All of the anthocyanins used in this study were obtained from natural fruits and plants. In many storage studies, natural colorants have been found to fade away or lose tinctorial strength (Kammerer et al, 2006). There are certain environment conditions that speed up degradation. For example, heat and pH play the important roles in the degradation of anthocyanins. Exposure to high temperature and high pH have been reported to accelerate the degradation (Romero and Bakker, 2000; Wrolstad et al., 2002). Thus, we used sodium citrate buffer to keep the pH between 3.3 and 3.7; also, we stored the samples at 4°C in the dark condition to observed the color stability by using catechin act as copigment in the anthocyanin rich materials. Due to the solution equilibrated from Day0 to Day1, we observed the color characteristic and analyzed the degradation from Day1 to Day70 (Figure 4.3). Overall, the color characteristics of Day 30 in all materials show slightly out of the trend line, compared to other time points. This phenomena suggests that there might had some experimental error at Day 30. There were several possible reasons cause the experimental error, including improper handling, machine error, lost of sample, and temperature change. Overall, the color characteristic still follows the trend line during the 70 days storage.

Lightness (L*):

Over 70 days of storage, the acylated anthocyanin (red cabbage) still had lower lightness compared to other non-acylated anthocyanin. Acylation of anthocyanin is believed to increase the color and pigment stability (Giusti and Wrolstad, 2003). The lightness of red cabbage with and without copigment was significantly (p<0.05) lower than the lightness of other materials (Figure 4.7).
For all the materials (strawberry, blackberry, eggplant, saponified-red cabbage, and red cabbage) lightness significantly (p<0.05) decreased when more catechin was added. The copigmentation with catechin resulted in darker color compared to those without copigment (control group). The regression analysis of control and different anthocyanin:catechin ratios was significant different (p<0.05), which means that there were only slight effect of catechin on the lightness stability. However, the lightness of ratio 1:50 from saponified-red cabbage decreased 8% over 70 days storage. Therefore, the cyanidin 3- sophoroside-5 glucoside was less stable in high concentration of catechin than other anthocyanins.

**Chroma:**

The acylated anthocyanin (red cabbage) had an interesting effect on the chroma (Figure 4.7). The effect of copigment on the chroma was slightly changed at lower catechin concentrations (1:1 to 1:20). However, the chroma of anthocyanin from was significant (p<0.05) enhanced by adding the highest amounts of catechin (1:50). The possible reason might be that the acid group on the cyanidin 3-sophoroside-5 glucoside (red cabbage) already had intramolecular copigmentation before the catechin binding on the anthocyanin molecule. Acylation of anthocyanin in believed to increase the color and pigment stability (Giusti and Wrolstad, 2003).

For all the materials (strawberry, blackberry, eggplant, saponified-red cabbage, and red cabbage), chroma value increased significantly (p<0.05) when more catechin was added. The copigmentation with catechin resulted in higher intensity of color compared to those without copigment (control group). The regression analysis of control and different anthocyanin:catechin ratio was significantly different (p<0.05), which means that there were certain effects of catechin on the chroma stability.
The change in chroma over time was significant (p<0.05), for all acylated anthocyanin (red cabbage) and non-acylated anthocyanin (strawberry, blackberry, eggplant, and saponified-red cabbage), both with and without copigment. Interestingly, the degradation of chroma from strawberries, blackberries and eggplant was significant (p<0.05) with and without copigment (Figure 4.3-4.5). Especially in the strawberries, the degradation of chroma in ratio of 1:20 was higher than the control (Equation: y=-0.0109x+61.59, R^2=0.78 (control); y=-0.2232x+71.11, R^2=0.99). The results showed that the chroma from pelargonidin-3-glucoside has significant (p<0.05) degradation on ratio 1:20.

**Hue angle (h):**

The change in hue angle over time was significant (p<0.05), for all acylated anthocyanin (red cabbage) and non-acylated anthocyanin (strawberry, blackberry, eggplant, and saponified-red cabbage), both with and without copigment (catechin). The effect of adding catechin on the hue angle was also significant (p<0.05) change when adding the different concentration of catechin (Figure 4.3-4.7). This result showed that the color turned red/orange using a high concentration of catechin and it was stable in all material.

Interestingly, the hue angle from the strawberries and blackberries consistently decreased when the ratio was 1:1 to 1:20; however, the hue angle suddenly enhanced to the value of 43 to 58. In the strawberries, comparing the hue of the control to the ratio 1:50, the hue increased 5% and 19% in Day1 and Day 70, respectively (Figure 4.3). This result showed that the copigmentation of anthocyanin and catechin significantly (p<0.05) changed the color from red to red/orange.
Figure 4.11 Comparison of color stability in strawberries with different catechin treatment. Bars represent the standard error of the mean (n=3).
Figure 4.12 Comparison of color stability in blackberries with different catechin treatment. Bars represent the standard error of the mean (n=3).
Figure 4.13 Comparison of color stability in eggplant with different catechin treatment. Bars represent the standard error of the mean (n=3).

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Figure 4.14 Comparison of color stability in saponified red cabbage with different catechin treatment. Bars represent the standard error of the mean (n=3).
Figure 4.15 Comparison of color stability in red cabbage with different catechin treatment. Bars represent the standard error of the mean (n=3).
4.4 High Performance Liquid Chromatography Analysis

HPLC coupled to PDA was used to determine the major anthocyanins and major catechins profiles present in the anthocyanin rich materials with and without the catechin. Catechin chromatograms profiles of catechin standard and Japanese green tea are presented in Figure 4.4. Comparing the Japanese green tea used in this study to the catechin standard, the major catechins from the Japanese green tea, were: epigallocatechin (EGC), epicatechingallate (EGCG), caffeine, epicathchin (EC), gallocatechingallate (GCG), epicatechingallate (ECG), and catechingallate (CG).

Figure 4.16 HPLC chromatograms (280nm) of catechin standards and Japanese green tea. Peak identities are as follow: 1) Gallic acid (GA); 2) Epigallocatechin (EGC); 3) Epicatechingallate (EGCG); 4) Caffeine; 5) Epicathchin (EC); 6) Gallocatechingallate (GCG); 7) Epicatechingallate (ECG); 8) Catechingallate (CG). The linear gradient used in the analysis was from 0%-5% B 5mins, 5%-5% B 5mins, 5%-25% B 21mins, 25%-40% B 5mins, 40%-0% B 5mins. The mobile phase consisted of solvent (A) 100% acetonitrile and solvent (B) 5% (v/v) formic acid.
Anthocyanins chromatograms profiles of the control and anthocyanin:catechin ratio 1:50 from five different materials of initial (Day 0) and Day 70 were presented in Figure 4.5-4.9. The materials used in this study have very distinctive profiles, and the identity of the different peaks was based on previously published literature. As expected, the anthocyanins from strawberry, blackberry, eggplant, saponified red cabbage, and red cabbage were: pelargonidin-3-glucoside, cyanidin 3-glucoside, delphinidin-3-rutinoside, cyanidin 3-sophoroside-5 glucoside, and acylated cyanidin 3-sophoroside-5 glucoside.

The addition of catechin did not seem to affect the non-acylated anthocyanin from strawberry, blackberry, eggplant, and saponified-red cabbage (Figure 4.5 to 4.8). The HPLC chromatograms (520nm) showed that the anthocyanin at the beginning and the anthocyanin remained after 70 days storage were almost the same. Therefore, the addition of catechin did not dramatically impact the non-acylated anthocyanin at the condition of 4°C in the dark.
Figure 4.17 Strawberry HPLC chromatograms (520nm and 280nm) of control and anthocyanin:catechin ratio 1:50 at Day 0 and Day 70.

Figure 4.18 Blackberry HPLC chromatograms (520nm and 280nm) of control and anthocyanin:catechin ratio 1:50 at Day 0 and Day 70.
**Figure 4.19** Eggplant HPLC chromatograms (520nm and 280nm) of control and anthocyanin:catechin ratio 1:50 at Day 0 and Day 70.

<table>
<thead>
<tr>
<th>Control</th>
<th>Day 0, 520nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>280nm</td>
</tr>
<tr>
<td></td>
<td>Day 70, 520nm</td>
</tr>
<tr>
<td></td>
<td>280nm</td>
</tr>
<tr>
<td>1:50</td>
<td>Day 0, 520nm</td>
</tr>
<tr>
<td></td>
<td>280nm</td>
</tr>
<tr>
<td></td>
<td>Day 70, 520nm</td>
</tr>
<tr>
<td></td>
<td>280nm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control</th>
<th>Day 0, 520nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>280nm</td>
</tr>
<tr>
<td></td>
<td>Day 70, 520nm</td>
</tr>
<tr>
<td></td>
<td>280nm</td>
</tr>
</tbody>
</table>

**Figure 4.20** Saponified-Red cabbage HPLC chromatograms (520nm and 280nm) of control and anthocyanin:catechin ratio 1:50 at Day 0 and Day 70.

<table>
<thead>
<tr>
<th>Control</th>
<th>Day 0, 520nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>280nm</td>
</tr>
<tr>
<td></td>
<td>Day 70, 520nm</td>
</tr>
<tr>
<td></td>
<td>280nm</td>
</tr>
<tr>
<td>1:50</td>
<td>Day 0, 520nm</td>
</tr>
<tr>
<td></td>
<td>280nm</td>
</tr>
<tr>
<td></td>
<td>Day 70, 520nm</td>
</tr>
<tr>
<td></td>
<td>280nm</td>
</tr>
</tbody>
</table>
Figure 4.9 and Table 4.4 showed the change of acylated anthocyanin chromatograms with and without the catechin over 70 days storage. At the beginning (Day 0), adding catechins might cause the peak 1-3 decreased and peak 5 and 6 increased. This chromatograms showed there might be copigment interaction with anthocyanins when adding higher concentration of catechin. Over 70 days storage, the peak area of No.1 in both control and 1:50 treatment greatly decreased compared to other peaks. The peak 1 in control and 1:50 treatment decreased 25% and 15%, respectively. Moreover, the peak area of peak 6 increased 15% after the 70 days period storage in the 1:50 treatment. McDougall et al. (2007) identified the first peak in the red cabbage chromatograms was cyanidin-3-\textit{O}-diglucoside-5-\textit{O}-glucoside, which is non-acylated anthocyanin. Our result showed that this non-acylated anthocyanin probably interacted with catechin to produce larger molecule during storage. Overall, this result showed that catechin can possibly act as copigment with non-acylated or/and acylated anthocyanin during the storage.
Figure 4.21 Red cabbage HPLC chromatograms (520nm and 280nm) of control and anthocyanin:catechin ratio 1:50 at Day 0 and Day 70.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Ret. time (mins)</th>
<th>Day 0 Control</th>
<th>Day 0 1:50</th>
<th>Day 70 Control</th>
<th>Day 70 1:50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32.4</td>
<td>9.6%</td>
<td>6.9%</td>
<td>7.2%</td>
<td>5.9%</td>
</tr>
<tr>
<td>2</td>
<td>37.8</td>
<td>4.7%</td>
<td>4.2%</td>
<td>4.2%</td>
<td>3.7%</td>
</tr>
<tr>
<td>3</td>
<td>38.1</td>
<td>8.8%</td>
<td>6.8%</td>
<td>7.3%</td>
<td>6.5%</td>
</tr>
<tr>
<td>4</td>
<td>40.6</td>
<td>19.3%</td>
<td>19.4%</td>
<td>20.1%</td>
<td>13.8%</td>
</tr>
<tr>
<td>5</td>
<td>41.8</td>
<td>9.0%</td>
<td>13.5%</td>
<td>13.4%</td>
<td>16.4%</td>
</tr>
<tr>
<td>6</td>
<td>42.1</td>
<td>40.0%</td>
<td>46.9%</td>
<td>49.9%</td>
<td>53.5%</td>
</tr>
</tbody>
</table>

Table 4.4 The retention (Ret.) time and the area percentage of 6 peaks in the red cabbage chromatograms of Day 0 and Day 70 from control and 1:50 treatment.
CHAPTER 5

CONCLUSION

In summary, our results showed that it is possible to enhance the color of anthocyanins by addition of catechins, to obtain a brilliant red/orange color and a functional food with increased phenolic and antioxidant content. Our results also show that the acylated anthocyanin from red cabbage was overall more stable than other non-acylated anthocyanins. Catechin acting as copigment could reduce the degradation of certain type of anthocyanin, and increase the color stability during the storage period. There was little degradation of monomeric anthocyanins, phenolic and chroma from non-acylated anthocyanin over 70 days storage when the anthocyanin:catechin was above 1:10. However, even after the color degraded slightly over time, the color of the anthocyanin-catechin combinations remained darker and more intense than the solutions with anthocyanins alone. In addition, our results show that anthocyanins would be a great alternative to the use of artificial colors in beverage products, which indicated that the potential health benefits remain in the product giving the consumer maximum benefits and value.
REFERENCE


anthocyanin-rich extracts on the inhibition of colon cancer cell growth. Journal of agricultural and food chemistry, 56(20), 9391-9398.


