

**FACTORS INFLUENCING ANTIOXIDANT PHYTOCHEMICAL STABILITY  
OF TEAS**

A Dissertation

by

YOUNGMOK KIM

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY

December 2008

Major Subject: Food Science and Technology

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**ABSTRACT**

Factors Influencing Antioxidant Phytochemical Stability  
of Teas. (December 2008)

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Tea is second only to water as the most consumed beverage in the world due to its health-promoting benefits and characteristic flavor. Even though many botanical teas such as yaupon holly (*Ilex vomitoria*) and mamaki (*Pipturus albidus*) are currently available in the tea market, only limited information is available on their polyphenolic contents containing diverse biological activities.

Identification of phenolic compounds was performed using advanced analytical technique of mass spectrometry to provide polyphenolic profile of teas. To present fundamental information of phytochemical stability during tea storage, studies to determine the impacts of tea processing, different packaging materials, and various storage conditions on the phytochemical stability were conducted. Ascorbic acid addition and lowering pH successfully decreased the reduction of phenolic compounds by reducing the rate of oxidative degradation while tea pasteurization accelerated oxidative degradation. Glass bottles were most effective to retain higher concentration of polyphenolics. Tea polyphenolics at lower temperature (3 °C) without light exposure

were higher retained in each tea. Tea cream, the complex formed by interaction between polyphenolics, caffeine, protein, and metal ions, is highly undesirable in tea industry since it causes haziness and cloudiness in tea infusion as well as deteriorates sensorial properties. Only limited information is available on tea creaming in non-fermented type teas such as green tea, yaupon holly, and mamaki even though many tea creaming compounds are present in these teas. Therefore, studies were conducted to determine the degree of contribution of the tea creaming compounds to tea creaming and to develop novel chemical methods to minimize tea creaming without detrimental effects. Methods applied in the present study defined that hydrophobic interaction was the driving force make tea cream insoluble in the solution.

Results obtained by present studies provided fundamental and practical information to the tea industry in terms of phytochemical stability and suggested storage stability of phytochemicals should be considered during processing and storage, since it could be better retained or improved by applying appropriate methods.

## **DEDICATION**

This dissertation is dedicated to tea lovers. Drink more tea!

## ACKNOWLEDGEMENTS

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## CHAPTER I

### INTRODUCTION

#### **Justification**

Next to water, tea is the most consumed beverage in the world and is enjoyed by two thirds of the world's population for its characteristic flavors and potential association with reduced risk of several major diseases such as coronary heart disease, stroke, and cancer. Ready-to-drink (RTD) tea is a growing market segment due to enhanced consumer awareness of their thirst satiety, flavor blends, and potential health benefits from its bioactive phytochemicals yet little knowledge is available on the shelf life or phytochemical stability.

RTD tea products are commonly subjected to common food processing methods such as addition of ascorbic acid to enhance flavor, acidification to improve storage stability, and terminal pasteurization steps to insure safety yet phytochemical stability is seldom a consideration in these processing regimes. As such, no published studies relate phytochemical stability during long-term RTD tea storage under various storage conditions. Since RTD tea products are rarely shipped or stored under refrigerated conditions, numerous changes may occur in these products simply due to storage time and temperature. By finding optimal processing or formulation conditions that enhance the stability of tea, their quality will be better retained prior to consumer purchase. Likewise, no studies have reported phytochemical changes or effects based on packaging

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materials. In today's markets, packaging materials such as glass, PET, and metal cans are being used for RTD teas or tea blends with little knowledge of the effects that these packages may have on preserving the antioxidant phytochemicals in the tea. Also, various storage conditions such as temperature and light exposure may be important factors that affect phytochemical stability. Lastly, tea cream which is chemical complex formed by interaction between polyphenolics and other tea creaming compounds such as caffeine, protein, and metal ions is unfavorable phenomenon for RTD tea industry since it lowers tea quality by making it hazy or cloudy. Therefore to improve phytochemical stability, a basic understanding of physical or chemical interactions among tea constituents is needed so that remediation steps can be implemented to the quality of teas throughout retail storage.

### **Objectives**

1. To identify polyphenolic compounds in three tea varieties (green tea, yaupon holly, and mamaki) using LC/ESI/MS<sup>n</sup> techniques and to evaluate contributing phytochemical compounds that related to antioxidant capacity.
2. To understand the impact of tea processing treatments on phytochemical stability and quality of three tea varieties and its stabilizing mechanism from various treatment methods during storage under a variety of retail conditions.
3. To evaluate the impact of various packaging materials on the physical quality and phytochemical stability of three teas and to investigate the chemistry behind packaging effects during storage.

4. To determine the impact of variable storage conditions (i.e. light and temperature) on the quality and phytochemical stability of three tea varieties and to investigate the physicochemical mechanisms impacting their phytochemical stability.
5. To identify the mechanism of the formation of tea cream and develop novel chemical or physical means of inhibiting tea creaming.

## CHAPTER II

### LITERATURE REVIEW

#### **Tea and Ready-to-Drink Tea Markets**

Tea (*Camilla sinensis*) is second only to water as the most consumed beverage in the world with a per capita consumption of about 120 mL/day and the consumption is exceeds that of coffee, beer, wine and carbonated soft drink (1, 2). Reasons for the popularity of tea worldwide are its use as a social drink, unique aroma and flavor characteristics and potentially from its potential health benefits against cardiovascular diseases, hypertension, cell functions, certain types of cancer, and is generally an antioxidant rich food (3–5). Tea has always had a rich history in the US with retail products ranging from bagged or loose leaf tea, powered tea, compressed tea, and more recently ready-to-drink (RTD) tea and tea-infused juices and beverages. Among those, recently RTD tea has gained popularity and dominated tea markets due to increased awareness of health benefits from antioxidant polyphenolics. In 2005, the value of tea markets, including ready to drink tea, was approximately \$ 6.8 billion in the US alone with projections close to \$10 billion by 2010 (6).

RTD tea is commonly sold in glass or plastic bottles but steel or aluminum cans are also a growing market segment based on convenience. Unlike other countries in Asia and Europe, about 80% of tea consumed in the US is served cold which is attributed to rapid market ascent in the US in relation to other countries (7, 8). Consumer shifts

towards healthy beverages have also been a driving force for RTD tea in the US. Specifically, this market has grown to \$ 1.5 billion with 10 % or more annual growth since early 2000's (8). Information is readily available on the chemical composition of green and black tea, but limited information exists neither on the phytochemical composition in RTD tea (9) nor on the stability of these compounds during processing and storage. Since RTD teas are stored in solution for extended periods of time, most often without the aid of refrigerated distribution or storage, inherent changes during storage may result that impact consumer acceptability and potential health benefits.

### **Health Benefits from Teas**

As a significant source of polyphenolic flavonoids, which exhibit potent antioxidant capacity in both *in vitro* and *in vivo*, tea has been widely consumed worldwide due to its potential health benefits mostly from polyphenolics. Numerous epidemiologic observations and experimental studies have reported that polyphenolic compounds found in tea may reduce the risk of various illnesses such as cancer and coronary heart disease (10). Cancer, which is a group of diseases caused by uncontrolled growth and spread of abnormal cells, is now North America's second leading killer after cardiovascular disease (11). Fortunately, abundant experimental investigations and epidemiological surveys have provided the evidence that polyphenolic flavonoids present in teas reduced the risk of cancer in the past decade (10). Evidence of the anticarcinogenic potential of tea polyphenols was proven by many experimental studies indicating their binding property to carcinogen and inhibiting heterocyclic amine

(carcinogenic compound in cooked meat) formation (1). An epidemiological observation conducted in Asian country where green tea is regularly consumed in large amount showed drinking green tea is directly related to prevention of cancer. For example, in a 9-year study of 8,552 Japanese adults, high green tea consumption (10 cups or more) delayed cancer onset by 8.7 years as compared to lower consumption (3 cups or lower) (12). Even though several studies were conducted to investigate cancer protective effects from black tea consumption (predominantly consumed tea in the US and European countries), black tea consumption was not as effective as green tea consumption on cancer prevention which can be attributed to green tea catechins such as epicatechin gallate, epigallocatechin gallate, and epigallocatechin that show higher anticarcinogenic property than theaflavin (13–15). The occurrence of breast cancer, which the fifth most common cancer death after lung, stomach, liver, and colon cancer and the most common cancer among women (16), was inversely related to the consumption of green tea because of estradiol (female hormone) and sex hormone-binding globulin (glycoprotein binding to testosterone and estradiol), which cause breast cancer onset, are affirmatively transformed (17). It has also been reported that tea flavonoids such as epigallocatechin gallate, epigallocatechin and theaflavin-3-3'-digallate sufficiently inhibited the growth of lung tumor (18) and the increase of 8-hydroxydeoxyguanosine formation in mouse lung DNA (19).

Many clinical studies with human subjects have reported that flavonoids present in tea lowered the incidence of coronary heart disease. In studies conducted in Europe, with 550-800 study subjects for 10 to 15 years duration, it was observed that flavonol

consumption was inversely associated with incidence of coronary heart disease (1). Additionally, according to Hertog et al. (20), a study of 12,763 men in seven countries examining the effect of tea flavonols on coronary heart disease showed flavonol intake significantly lowers the incidence of the mortality after 25 year follow-up. Another clinical trial conducted in the Scottish Heart Health Study reported that a slightly positive effect was found in tea consumption associated with coronary morbidity and all-cause mortality (21).

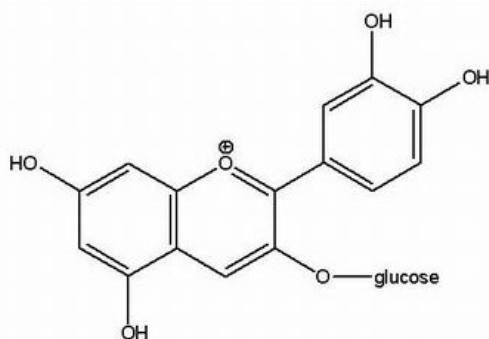
Besides two of the most serious diseases mentioned above, consumption of tea is also known to be effective for lowering incidence of numerous types of diseases and improving general human health. For example, tea consumption is inversely associated with the incidence of diseases such as atherosclerosis (1) and endothelial cell function (cell malfunction) (22). Also, tea consumption is effective for lowering obesity by stimulating hepatic lipid metabolism, inhibiting lipase, stimulating thermogenesis, modulating appetite, and synergizing with caffeine (1). It was also reported that consistent drinking of normal strength tea significantly reduced the development of hypertension (23).

### **Roles of Polyphenolics**

Polyphenolics, which are a group of chemical substances of plant secondary metabolites found in plants, play various roles. First, polyphenolics act as a colorant. The representative phenolic colorant are anthocyanin, which is a subgroup of flavonoids that contain  $C_3C_6C_3$  carbon skeleton and cover a broad range of colors including blue, purple,



violet, magenta, red, and orange (24). Even though flavonoids release colors as well, anthocyanins are the most broadly distributed pigment in the plant world. As shown in **Figure 2-1**, anthocyanins differ in the number of hydroxyl and/or methoxy groups present and sugars such as glucose, galactose, arabinose and xylose are attached to the 3 position in the C ring. When the attached sugars are hydrolyzed into aglycone and sugar, the aglycone is referred to as an anthocyanidin which is another color source along with anthocyanin. The color of anthocyanins and anthocyanidins come from excitation of a molecule by light and the strength of color is determined by the relative electron mobility in the structures. Since the two colorants have many double bonds which are readily excited, the compounds can release color readily under the presence of light (24).



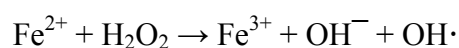
**Figure 2-1.** Structure of anthocyanin.

Secondly, polyphenols act as antimutagenic, anticarcinogenic and antimicrobial agents. Antioxidant polyphenols protect molecules against reactive intermediates such as free radicals and electrophiles formed during the metabolic activation of carcinogens and mutagens. They also play a role to remove free radicals and to facilitate additions to electron-deficient regions in electrophilic ultimate carcinogens (25), indicating why polyphenols have antimutagenic and anticarcinogenic properties. Polyphenols are

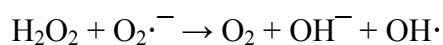
effective microbial inhibitor for various kinds of microorganisms such as *Streptococcus mutans* and *Salmonella* (26). The hydroxyl groups on the phenolic compounds are related to their toxicity to microorganisms and increased hydroxylation (turned to radical compound by oxidation) results in increased toxicity and additionally, the ability to form complex with cell wall polypeptides may give polyphenols (especially flavonoids) an antimicrobial property (27).

Lastly, polyphenolics play a role as antioxidants. Free radicals (superoxide, peroxy radical, alkoxy radical, hydroxyl radical and nitric oxide) are reactive and rapidly attack molecules in nearby cells since they contains unpaired electron (excited state), thereby resulting in the generation of another free radical (28). Among various free radicals, hydroxyl radical ( $\text{OH}\cdot$ ) is the most notorious free radical due to its tremendous reactivity. The hydroxyl radical is generated by a two step procedure (28). First, superoxide ( $\text{O}_2\cdot^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$  – non radical but has a little reactivity and can move through cell membranes) are first generated by environmental sources (cigarette smoke, pollutant, UV light, etc.) and endogeneous sources (respiration burst, enzyme reactions, autooxidation). Second, those two compounds are transformed to hydroxyl radical by two reactions called the Fenton reaction and the Haber-Weiss reaction with transition metals such as  $\text{Fe}^{2+}$  and  $\text{Ca}^+$ .

#### **Fenton reaction**



#### **Haber-Weiss reaction**



Since polyphenolics have reducing properties, they are generally considered as antioxidants. Among polyphenolics, flavonoids are known to have higher antioxidant capacity due to their ability to donate hydrogen/electrons (reduction potential), break chain reactions of lipid peroxidation (hydrogen/electron donation), and chelate transition metal ions (29). The reduction potential of flavonoids starts from *o*-dihydroxyl catechol structure in the B ring which contains several OH groups (hydrogen and/or single electron donating) and additional gallate group (e.g. epigallocatechin gallate) with OH groups (30, 31). Also, hydroxyl group in the A and C ring are known to participate in reducing activity of flavonoids. The 2, 3 double bond in the B ring with 4-oxo function in the C ring is responsible for electron delocalization (one electron in a molecule is not associated with single atom shared by more than two atoms) from the B ring (30). This structure may generate delocalized electrons and result in resonance. Thus, radicals are stabilized due to the resonance effect (30, 32). After donating a single electron for free radical quenching, the OH group also has unpaired electron (oxidized antioxidant). However, oxidized antioxidant is not reactive because phenolic compounds have one or more bulky ring substitutes that provide steric hindrance.

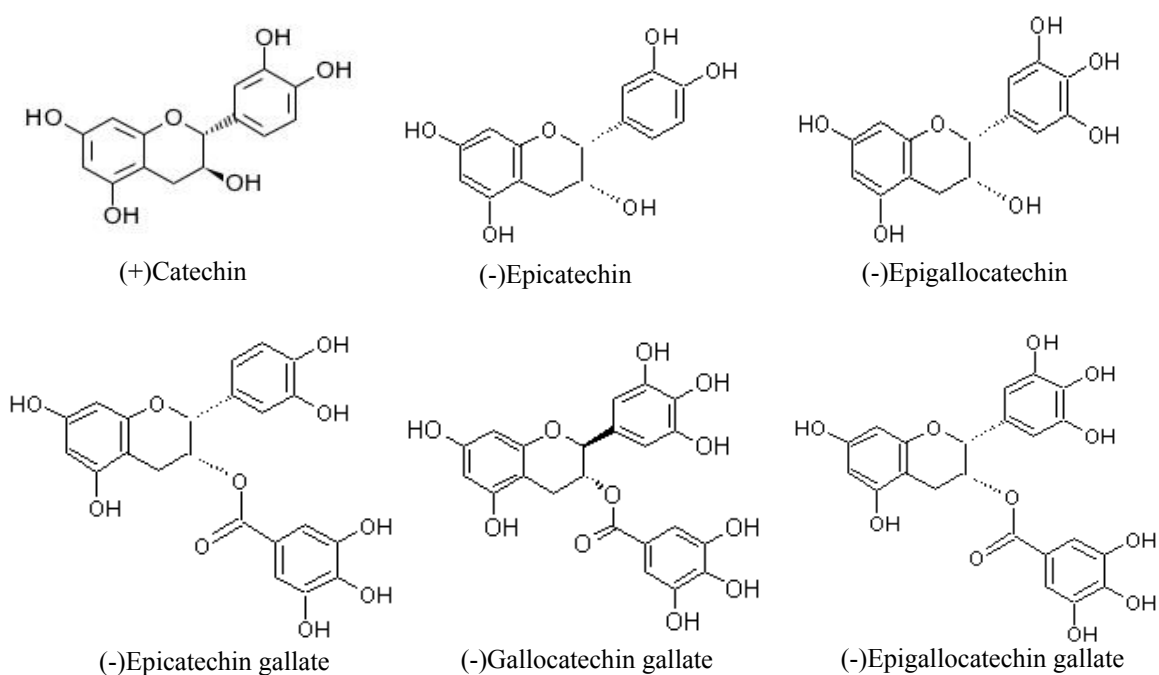
## **Phytochemicals in Teas**

### ***Green tea (Camellia sinensis)***

Green tea (*Camellia sinensis*) has long been recognized as a significant source of catechin and unique catechin derivatives found only in tea. These are called as tea

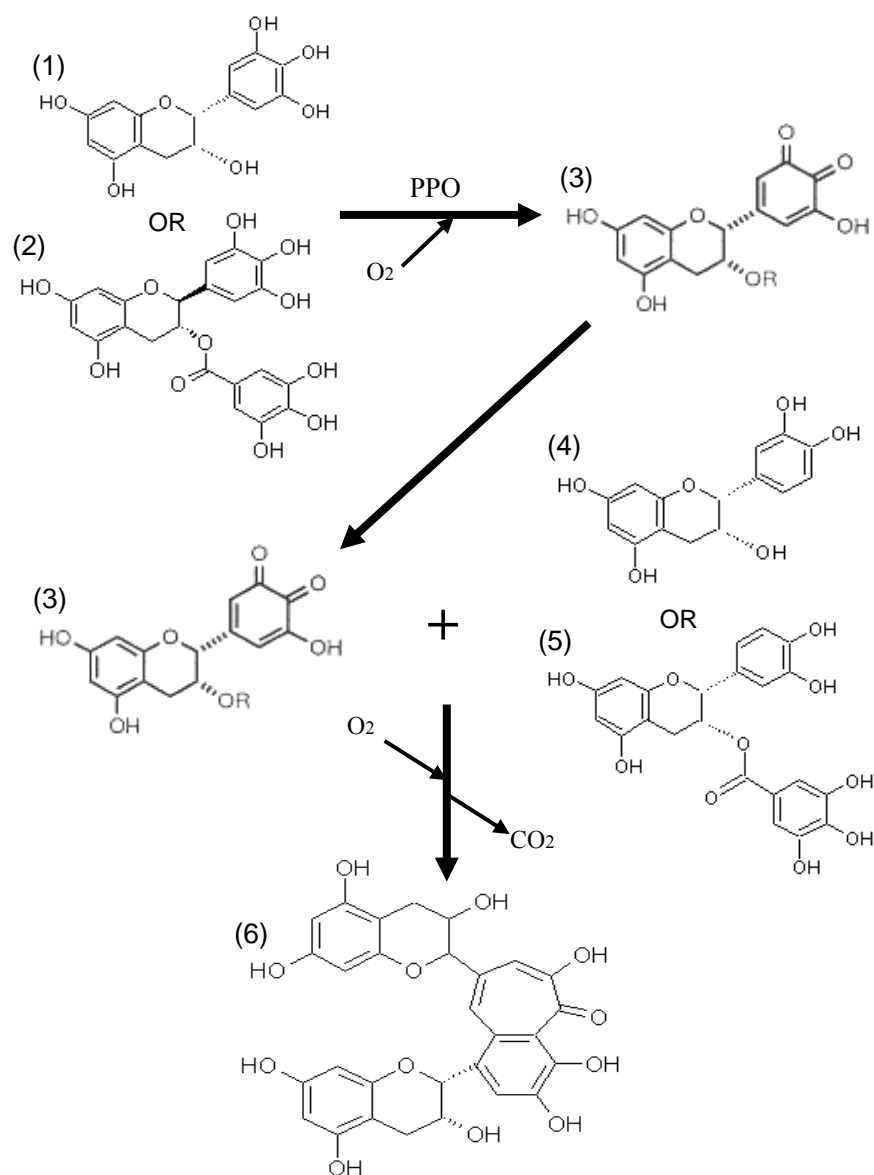
catechins, flavan 3-ols, or flavanols. These flavan-3-ol derivatives include (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), and (-)-gallocatechin gallate (GCG) that contribute to both antioxidant capacity and organoleptic properties (33, 34) (**Figure 2-2**).

Polyphenolics account for up to 30 % of the dry weight of tea, of which up to 90 % of those compounds are flavanols (35), which are attributed to the high biological activity of green tea (36). Studies have demonstrated that these compounds afford protection against cardiovascular disease and cancer as well as pharmaceutical activities such as antihypertensive, antioxidative, anticarcinogenic, antiarteriosclerotic, hypocholesterolaemic and hypolipidemic properties (4, 9, 34, 37, 38).



**Figure 2-2.** Structures of six major catechins present in green tea.

Teas from the genus *Camillia* are generally divided into three main food categories based on their manufacturing processes. These include green tea (unfermented), oolong tea (partially fermented), and black tea (fully fermented), all of which can be manufactured from the same tea plant (39). In the Far East (Korea, Japan, and China), green tea is predominantly consumed as a hot infusion while black teas are more popular in most European and Western countries (40). For green tea manufacture, leaves are immediately heated or rapidly dried to inactivate polyphenol oxidase and native microflora which catalyzes the aerobic oxidation of tea catechins. This process generally protects tea catechins from oxidization (36), as long as processing steps are carried out in a timely manner. For black tea, leaves may be crushed or allowed to wither to induce oxidization and fermentation prior to drying. The characteristic color, reduced bitterness and astringency, and general flavor are derived from this process giving black tea a marked distinction from green tea (39, 41). The complex process of flavonol oxidation in black tea has been the topic of numerous studies. During fermentation, monomeric flavan-3-ols may undergo oxidative polymerization converting predominantly into bisflavanols, theaflavins, and thearubigins (36) (**Figure 2-3**). Due to these changes, green tea generally has a higher antioxidant capacity than black tea due to its higher content of flavan-3-ol monomers, specifically EGCG as the most predominant polyphenolic in green tea. Evaluation of isolated compounds also revealed that EGCG has higher antioxidant capacity than theaflavins (31, 42). However, the stability of green tea catechins in metal cans is known to be significantly lower than that of black tea (43). Furthermore, the mechanism of degradation of phytochemical stability is still unclear.



**Figure 2-3.** Oxidation scheme of flavan-3-ols in green tea to theaflavin in black tea. Number assignment: (1) (-)-epigallocatechin (EGC), (2) (-)-epigallocatechin gallate (EGCG), (3) orthoquinone derivative of EGC (R = H) and EGCG (R = galloyl group), (4) (-)-epicatechin (5) (-)-epicatechin gallate, (6) Theaflavin. Abbreviations: PPO: polyphenol oxidase.

Therefore, it is critical to understand the basic changes that may occur in RTD green teas and to improve its stability by post-processing or during storage in green tea products. Also, applying other packaging materials more widely used in RTD tea market such as glass and PET rather than canned materials needs to be considered in extending its stability in various environments.

### ***Yaupon holly (Ilex vomitoria)***

Yaupon holly (*Ilex vomitoria*) is a dioecious shrub or small tree, which is commonly grown in the moist pines and hardwood forests of the South Atlantic Gulf Coastal Plain and the Savanna Scrublands of east central Texas (44). This large shrub or small evergreen tree can grow 15 to 25 feet in height and has small, grey-green, and leathery leaves, which historically were used as tea leaves (45), most notably by Native American tribes along the Gulf and southern Atlantic coasts. Among several holly species grown in the southeastern United States, yaupon holly is important for supporting wildlife habitat for birds and animals and its fruits and foliage are a common food source for many animals (45).

Yaupon holly leaves, the only caffeine containing plant native to North America, were historically brewed into a stimulating beverage by Native Americans and later by European colonists due to the moderately high concentrations of methylxanthines such as caffeine and theobromine (46–48). As a traditional tea, yaupon holly was reported as being used as a medicinal tea by indigenous people since the early 1500's in the US (44, 49). However, the tea was also used in ceremonial activities and brewed and consumed

in high concentrations by natives as the “black drink” that purges the natives by inducing vomiting (47). When observed by Europeans, it was commonly believed that yaupon holly tea had emetic properties thus the scientific name *Ilex vomitoria* was given to yaupon holly, but no such evidence for such an effect has been recorded (47).

Few studies have reported phytochemical composition of yaupon holly but its phenolic profiles as well as its chemical and biological activities are poorly characterized in the literatures even though yaupon has a long history and has gained popularity in the North America as a tea source. Methylxanthine alkaloids (caffeine and theobromine), cinnamic acid derivative (chlorogenic acid and its isomers) and flavonoids (rutin and luteolin) were tentatively identified by HPLC-PDA analysis based on retention time and maximum wavelength of absorbance (48) and they contribute most of antioxidant capacity of yaupon holly. Furthermore, volatile compounds and organic acid such as furfural and butanedioic acid (i.e. succinic acid), respectively were found by GC-MS analysis (47). As a dioecious (the word originated from Greek “two households”) plant, yaupon holly has two unisexual reproductive organs in different genders, male and female, with the female shrubs containing higher phenolic concentrations and thus a higher antioxidant capacity (48). Herms and Mattson (49) reported that female species needs more nutrients for fruit and seed maturation and also different distribution of resource for secondary metabolism in each gender might cause different phenolic concentration in the plants.



***Mamaki (Pipturus albidus)***

Mamaki (*Pipturus albidus*), is an endemic shrub of the Hawaiian Islands with a history of use among Polynesians against stomachache and liver ailments (50, 51). Mamaki was first described by Skottsberg (52) as a shrub of small tree with white and fine hairy stems. Mamaki barks, leaves, and stems have been used for expectant mothers, blood purification, curing general debility and thrush, and as a laxative (53–54). The mamaki tea from leaves has been consumed by native Hawaiians to relieve pre-existing diseases and to control blood pressure, cholesterol, and sugar levels in human blood (55). Mamaki leaves also demonstrated anti-viral activity and were able to inhibit the growth of *Streptococcus pyogenes* and *Staphylococcus aureus* (56). Although mamaki tea has been used as a medicinal tea by native Hawaiians for curing several diseases, especially blood related diseases, and gained regional popularity in the Hawaiian Islands (51, 55), no extensive research has been done to define its chemical, physical and biological properties.

**Processing of RTD Teas**

In the manufacturing of RTD tea, several processing treatments to improve tea quality are currently being used. For example, ascorbic acid (vitamin C) is usually added as a fortificant and to improve flavor and color. Additionally, pH is lowered to expand shelf life and heat treatments are applied for pasteurization of final tea beverage product.

In European countries, adding ascorbic acid rich lemon juice to a cup of tea is very popular phenomenon for tea drinkers who love acid flavor. Additionally, ascorbic

acid is added to RTD tea to augment its preservation and nutritional quality (57). Protecting flavonoids in fruit juices from oxidative degradation during storage and processing is another advantage of adding lemon juice to tea (50 mg ascorbic acid/100 g lemon) (58, 59). During RTD tea processing, ascorbic acid is also added to lower the pH of the tea infusion, resulting in a reduction of hydrogen peroxide ( $H_2O_2$ ) production, which is toxic and causes cell death (60). Also, ascorbic acid is known to be an antioxidant, which functions by quenching free radicals including  $H_2O_2$  and inhibiting cytotoxicity caused by oxidants (61). Even though lowered pH (adding high amount of ascorbic acid) may induce better stability of tea beverage, excessive fortification might cause serious adverse effects in the human body since recommended daily allowances for ascorbic acid is 60 mg/day for most female adults and 90 mg/day for most male adults (62). Overdose of ascorbic acid may cause some potential toxicity related to intra-intestinal events and metabolites in the urinary system (63). Additionally, hyperoxaluria defined as excessive urinary oxalate and hemolysis, which is the breaking open of red blood cells causing exposure of hemoglobin into surrounding plasma, might occur when overdosed for a long duration (63).

Heat processing, which is an essential step for pasteurization of tea, has been reported to cause quality deterioration (color, flavor and taste degradation) and has become a dilemma for RTD tea manufacturing (64, 65). Since tea infusions show higher sensory quality when they are brewed with relatively low temperatures, heat pasteurization may be adversely affecting taste and flavor of RTD tea (66). Darker colors in tea infusions which are not favorable for tea consumers were obtained when

higher temperatures were applied, (65). Moreover, Kim et al. (65) reported that the concentration of major catechins in green tea such as EGCG, EGC, EC and ECG was reduced by heat treatment. The impact of heat processing on quality and polyphenolics are available on popular teas such as green and black tea, however, no study has reported the heat effects on yaupon holly and mamaki, which have different chemical composition. Long term storage effects from heat treatment should be considered for tea stability since RTD teas may be stored for several months at retail storage.

A few studies have reported that stability of green tea catechins are pH dependent (67). Organic acids such as citric acid, malic acid, succinic acid, fumaric acid, L-glutamic acid, and L-aspartic acid are generally used for adjustment of pH (33, 60). According to Chen et al. (33), green tea catechins at lower pH (less than pH 4.0) were stable during storage, while teas at neutral pH (pH 7.0) degraded faster when pH was lowered by two common organic acids such as ascorbic acid and citric acid in the manufacturing of RTD tea (33). This study showed that addition of high acid contents would confer more stability to tea beverages since a lower pH is more effective for stabilizing tea catechins. According to Aoshima and Ayabe (60), ascorbic acid fortification may reduce H<sub>2</sub>O<sub>2</sub> production in polyphenol-rich beverages such as green tea by lowering pH while no protective effect against H<sub>2</sub>O<sub>2</sub> production was found when pH of the tea beverage was neutral. Since no study has been conducted for other tea beverages such as yaupon holly and mamaki, which contain totally different chemical composition, finding the pH effect on those new teas should be the first step for commercialization.

### **Packaging Materials for RTD Teas**

RTD teas have been usually filled into glass and PET bottles and sometimes steel or aluminum cans (68). Since tea beverages readily deteriorate as a result of browning, taste and flavor changes, and loss of ascorbic acid during storage, packaging materials are always considered to protect teas from those deteriorations (69).

Polyethylene terephthalate (PET) is extensively used for various beverages such as fruit juices, sports drinks, soft drinks, and RTD teas. However, PET is also known to absorb aroma compounds present in fruit beverages and additionally, compounds from PET could influence juice flavor causing ascorbic acid loss (70). Fruits juices stored in plastic bottles contain less limonene, terpene, sesquiterpenes, and aldehydes when compared to glass bottles due to oxidation or absorption (71, 72). The oxidation may be caused by the permeable property of plastic packaging and the absorption might be caused by the fact that plastic materials could absorb aromatic compounds (70). According to Bissett and Berry, (73), glass retained more ascorbic acid to remain in the fruit juice than plastic materials during storage. Even though PET has several disadvantages as described above, it is until preferred by beverage packaging manufacturers because it is inexpensive way for beverage packaging compared to glass (70). Historically, glass has been used for premium beverages due to its perception of quality, while PET was used for routine or mass-produced beverage products. However, changes in energy costs and the price of raw materials for packages (i.e. PET resin) have greatly influenced packaging decisions by beverage manufacturers. Due to its low O<sub>2</sub> transmission rate ( $2 \text{ cm}^3 \cdot \text{mm}/\text{m}^2 \cdot \text{day} \cdot \text{bar}$ ) and mechanical resistance, PET packaging is

largely promoted in the beverage industry as replacements for glass bottles which are also heavy and fragile (74). However, unlike fruit juices, the effects of packaging material on RTD teas have not been reported even though PET is most used packaging material.

The retortable pouch (RP) which is defined as a sealed and heat resistant pouch for storing perishable food items for a long duration (75) has gained popularity due to its convenience (microwavable, lightweight, and durable) and ease of sterilization (heat resistant) in the present packaging market. Appropriately sterilized and sealed retortable pouches could prevent perishable food products from contamination with microorganisms without the aid of refrigeration. Additionally, its lightweight and durable properties provide improved protection for all kinds of food items including liquid product (75). A retortable pouch is made by laminating together a polyester film and an inelastic polypropylene film to form impermeable layer because impermeable packaging material ensures good preservation of precooked food product (76). For sterilization of cooked food in retortable pouch, the packaging containing food is heated in boiling water and additional sterilization could be added when the food in the packaging is heated in a microwave oven (76).

Commercial RTD tea contains only limited amount of antioxidant flavonoids because significant amount of antioxidants might be lost during processing treatments. According to Chen et al. (33), green tea leaves contains 8 to 15 g of total catechins in 100 mg of dried tea leaves whereas only 0.3-35 mg of catechins are present in 100 mL of commercial RTD tea . Since there are significantly lower levels of antioxidants in

commercial RTD tea compared to brewed tea, a new packaging material which directly contains brewed tea with minimal degree of processing should be considered. Glass and PET bottles could also be used to store brewed tea, however, many tea drinkers prefer to drink just hot plain tea without adding flavor (some preservatives and flavor such as ascorbic acid, citric acid, phosphoric acid and natural flavors are usually added).

Therefore, the retortable pouch could be an alternative for existing tea packaging materials because the retortable pouch is microwavable (easily heated), easy to carry, durable, resistant to extreme temperature (boiling and freezing), inexpensive, and lightweight. Also, commercial sterilization (no need of extra preservation) would be available instead of pasteurization (need additional preservation method such as refrigeration) if retortable pouches are used for RTD tea because the brewed tea has pH of 4.5 through 7.

### **Conditions for RTD Tea Storage**

Most RTD teas are stored for durations from a few weeks to years until they are consumed. During transportation, RTD teas are generally stored at room temperature in a truck due to the previous heat treatment while teas are stored either at room or refrigeration temperature at retail storage. Since controlled temperature storage adds considerable cost to the tea product, most of RTD teas are stored at room temperature. Several studies have shown the effects of storage temperature on other beverages the effects of temperature on physical and phytochemical stability but the influence on RTD teas have not been extensively studied. Hawthorn fruit drinks showed higher

phytochemical stability for 24 week when kept at 4 °C compared to other temperature (23 and 40 °C) while the stabilized phenolic compounds in hawthorn were (-)-epicatechin, isoquercetin and chlorogenic acid, which also present in tea drinks (77). Also, total active vitamin C (L-ascorbic acid and dehydroascorbic acid) in grape fruit juice was also higher at the lowest temperature (10 °C) studied during 12-week storage (78). However, according to the study of Rodriguez et al. (79), ascorbic acid degradation of an alcoholic orange juice beverage was not influenced by temperature while other quality parameters such as degree of browning, accumulation of furfural, and limonene content were highly correlated with temperature difference (4, 25 and 40 °C).

Besides temperature, light exposure is also an important factor in influencing the quality of RTD tea during storage because light is one of the important environmental factors that influence the stability of food products (80). Some RTD teas are covered by all of the packaging material/label to improve consumer appeal while some are barely covered to show the own color of the product. This means that the products can be exposed to the light depending on the type of packaging materials or packaging method. There is considerable information available on the effects of light on foods and other beverages, and these changes vary with the type of food and the conditions under which they were stored. For example, ascorbic acid was not affected by light exposure in juice samples during 52 days of storage at 8 °C if the storage bottle was closed with airtight lids (81). However, the study conducted by Andrews and Driscoll, (82), showed ascorbic acid of commercial juice was better retained in foil-covered bottles than clear bottles during 18 days of storage at 3 °C. Ascorbic acid content in green tea was also more

retained when it was stored in packaging covered with aluminum foil (83). Even though considerable information is available as described above, no study has reported the light effect on phytochemical stability of RTD teas yet.

Quality deteriorations in teas during storage such as loss of ascorbic acid, flavor loss, altered color from olive green to brown, and taste conversion from well-balanced and complex to flat with a loss of briskness have been well documented and have all been shown to be influenced by exposure to light and temperature during storage (83). However, there is limited information available on phytochemical changes due to exposure to light and temperature during storage.

### **Tea Cream Formation**

Caffeine, one of several methylxanthines naturally present in a wide range of food and beverage products such as tea and coffee, is known to stimulate the central nervous system and has been reported to improve cognitive performance, alertness, and reaction time (84–86). Caffeine also acts as an antioxidant. Furthermore, when caffeine was removed from a caffeine-rich espresso coffee, the antioxidant value was reduced by 25-30 % (42). Tea contains about 2.5-3.5 % caffeine by dry weight while fermented tea leaves contain more caffeine than unfermented teas (86). In healthy does, Caffeine has shown beneficial effects on the human body as a stimulating compound, however, from a chemical interaction point of view, it was reported that caffeine may interact with polyphenolic compounds to form an undesirable precipitate in brewed teas known as tea cream (87). Since tea cream depreciates not only sensory properties such as color,



astringency, appearance, and taste but also bioactive properties of tea by loss of compounds that contribute those properties (64, 87), it is undesirable reaction for RTD tea production. Particularly, the muddy, hazy appearance and increased turbidity of tea infusion is considered as a significant problem in the tea manufacturing industry (88).

Tea cream in black tea is generated by the reaction of caffeine or protein with oxidized polyphenolics containing galloyl esters followed by other compounds in tea like carbohydrates and metal ions, such as calcium, that bind to the already formed tea cream (88–92). The presumptive binding reaction of galloyl esters was supported in studies where galloyl esters of theaflavins and thearubigins decreased by ester hydrolysis and the concentration of black tea cream was reduced (93). Studies on tea cream have reported that production of black tea cream with caffeine or protein interactions with oxidized polyphenolics, however, limited information is available on green tea creaming.

Green tea cream may be formed by polyphenolic-caffeine or polyphenolic-protein complexation. The formation of complexes between polyphenol and caffeine may occur as hydrophobic interactions and hydrogen bonding between hydroxyl groups of polyphenol (i.e. Flavan-3-ols in green tea) and the keto-amide group of caffeine (94). The complexes between polyphenols and protein may also be affected by hydrophobic interactions and/or hydrogen bonding between hydroxyl groups of flavan-3-ols in green tea and proline-containing peptides (95–99). Additionally, protein-caffeine complexes may occur with the binding of proline-containing peptides and the keto-amino group of caffeine (100). The tea creaming compounds such as polyphenolics, caffeine, and protein form stacked complexes, which are held together with hydrogen bonds and

further bound to other purine alkaloids such as paraxanthine, theophylline, theobromine and theacrine (100, 101). Caffeine-polyphenol complexation is enhanced with the aid of metal ions (102), which bind to already formed complexes and attract polyphenols due to their high binding affinity to metals. Furthermore, chlorogenic acid, which is predominant phenolic compound found in yaupon holly and mamaki teas, may also form tea cream by a similar hydrogen bonding mechanism (101, 103).

Since concentrated levels of polyphenolics and caffeine in tea infusion cause tea cream more frequently (104), it is preferred to reduce amount of polyphenolics and caffeine to prevent forming of tea cream in RTD tea. This is why RTD tea contains less polyphenolics than freshly brewed tea (87). By reducing levels of polyphenolics and caffeine, the potential health benefits from drinking RTD tea have diminished. To improve those benefits and stability of RTD tea, other efforts that do not reduce beneficial compounds from the tea beverage need to be considered.

Oxygen is essential for humans and other living organisms to function and the oxidative mechanism is important for the cells to survive in the body. However, when oxygen is transformed to free radicals which are highly reactive oxygen molecule, the oxygen can destroy cell membranes of all the living organisms due to the presence of unpaired electron. Oxidation process induced by free radicals causes deterioration of food quality by changing color and flavor as well as reducing nutritional value (105). However, oxidation has been adapted to improve wine's overall quality by enhanced the taste and flavor from forced oxidation during wine aging process. Currently, two oxidation methods are being used in wine industry, microoxygenation and macroaeration,

to improve overall quality of wines. Microoxygenation is the technique used in winemaking process to add small amount of oxygen in a controlled manner. Added oxygen plays a role in oxidation, condensation, and polymerization reactions mainly on phenolic compounds in red wine processing leading to the formation of new pigments and polymeric compounds in stabilizing red wine's characteristic color (106).

Additionally, the treatment induces the oxidation of phenolic and volatile compounds and improves the sensorial quality of wines (106). Macroaeration is being used in wine industry for producing softer and less astringent wines and stabilizes color by either simply splashing wine against the wall or directly injecting air into the wine.

Macroaeration is applied only for red wine but not white wines because relatively higher phenolic concentration and lower pH of red wines protects quality degradation from oxidation process while lower phenolic concentration and higher pH of white wines induces more quality deterioration from oxidation. Since these oxidation techniques have shown to have beneficial effects on overall quality of wines, modified oxidation for tea infusions could be introduced as a method to prevent or reduce tea cream formation without detrimental effects on tea quality.

## CHAPTER III

### IDENTIFICATION OF ANTIOXIDANT POLYPHENOLIC COMPOUNDS IN THE THREE TEA VARIETIES USING LC/ESI/MS<sup>N</sup> ANALYSIS

#### **Introduction**

With the current and growing popularity of tea in all its current forms, many studies have been conducted that demonstrate its health benefits derived from its rich source of antioxidant polyphenols, namely flavan 3-ols and flavonols in green tea, theaflavins and thearubigins in black tea, and chlorogenic acid and its isomers in botanical teas including yerba maté. Since different teas contain their own characteristic compounds, health benefits from each tea are also different based on the biological properties of the compounds. For example, black tea, which is the most consumed tea beverage in the world, contains oxidized catechins (about 3-10 % of theaflavins and >20 % of thearubigins of the total solid) while green tea contains about 30 % of tea catechins (flavan 3-ols). Especially for green tea, most of the studies have focused only on the major compounds, flavan 3-ols, even though significant amount of other health promoting compounds such as flavonols (quercetin, kaempferol, myricetin, and apigenin) are present mostly as glycosidic form. Moreover, the polyphenolic identification of novel botanical teas such as yaupon holly (*Ilex vomitoria*), a dioecious shrub of the southern US, and mamaki (*Pipturus albidus*), an endemic shrub of the Hawaiian Islands, are not fully elucidated. Therefore, the antioxidant capacity and polyphenolics of traditional green tea (*Camellia sinensis*) were compared to those of

botanical teas using advanced HPLC/ESI/MS<sup>n</sup> techniques followed by proper fractionation method for more accurate identification and quantification and for determination of the degree of contribution to antioxidant capacity. This study is an important preliminary study to determine what portion of phenolic compounds in teas induce health promoting properties and how different compounds work for which aspects of biological activity.

## **Materials and Methods**

### ***Tea preparation***

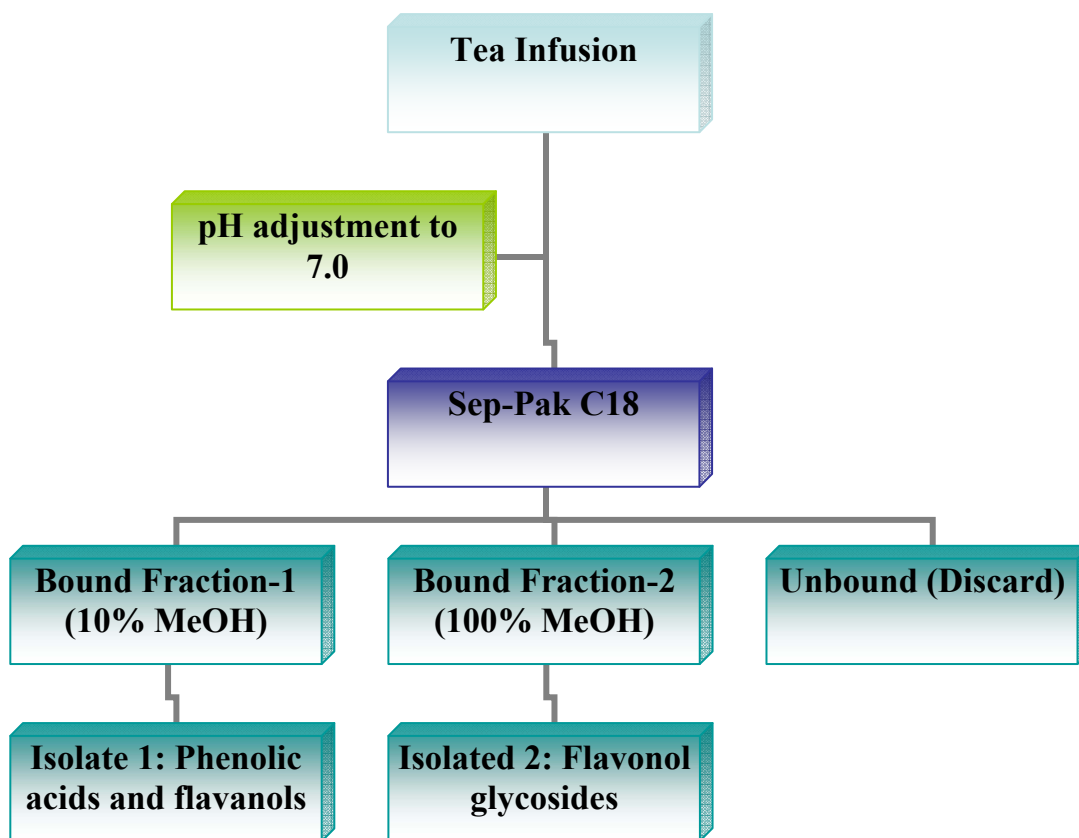
Green tea leaves were harvested and hot air dried in Hwagae, Korea during June 2006. Yaupon holly leaves were wild harvested in Texas in July 2006. Mamaki leaves were harvested in Hawaii in August 2004 and kindly donated from University of Hawaii at Manoa. Yaupon holly and mamaki leaves were dried in hot air (90 °C) for 4 hrs prior to shipping to Texas A&M University. Each tea was finely powdered with a mortar and a pestle for efficient polyphenolic extraction prior to hot water infusion, whereby tea infusions were prepared by pouring hot water (90 °C) directly onto the leaves (1:100 ratio, w:v) with constant stirring. The water for tea brewing was previously purified using a Milli-Q water system (Billerica, MA) and autoclaved for 25 min to achieve sterilization. After 10 minutes of infusion, the leaf was filtered through cheesecloth and then allowed to cool to 25 °C for final filtrations first through Whatman #4 filter paper

followed by a 1 cm bed of diatomaceous earth under a slight vacuum to remove suspended particles.

### *Fractionations of teas*

Due to the diversity of polyphenolics present in each tea, the polyphenolics was fractionated based on their affinity to C<sub>18</sub> cartridge and solubility in organic solvents. A preliminary study was conducted with the two fraction methods to determine more efficient fractionation method on each tea. These preliminary trials identified the first isolation method as the most efficient for green tea and yaupon holly while the second method was used for mamaki infusion.

The polyphenol separation of green tea and yaupon holly was conducted using the fractionation method previously described by Schütz et al. (107) with modification. The pH of each prepared tea infusion was adjusted to neutral (pH 7.0) with sodium hydroxide solution (0.3 M) and 5 mL of each tea sample was applied to a C<sub>18</sub> Sep-Pak reversed phase cartridge, which was previously activated by of 5 mL of 100 % methanol and then rinsed with 5mL of deionized water. After discarding unbound fraction, phenolic acids (fraction 1) was eluted with 10 mL of 10 % methanol and flavonoids (fraction 2) was eluted by rising with 10 mL of 100 % methanol. The eluates were evaporated under slight vacuum until the organic solvent was removed. Isolate 1 was appropriately diluted with water before the injection to HPLC and LC/ESI/MS<sup>n</sup> and isolate 2 was dissolved in 5 mL of water and then diluted with appropriate amount of water prior to injection. This methodology was briefly illustrated in **Figure 3-1**.

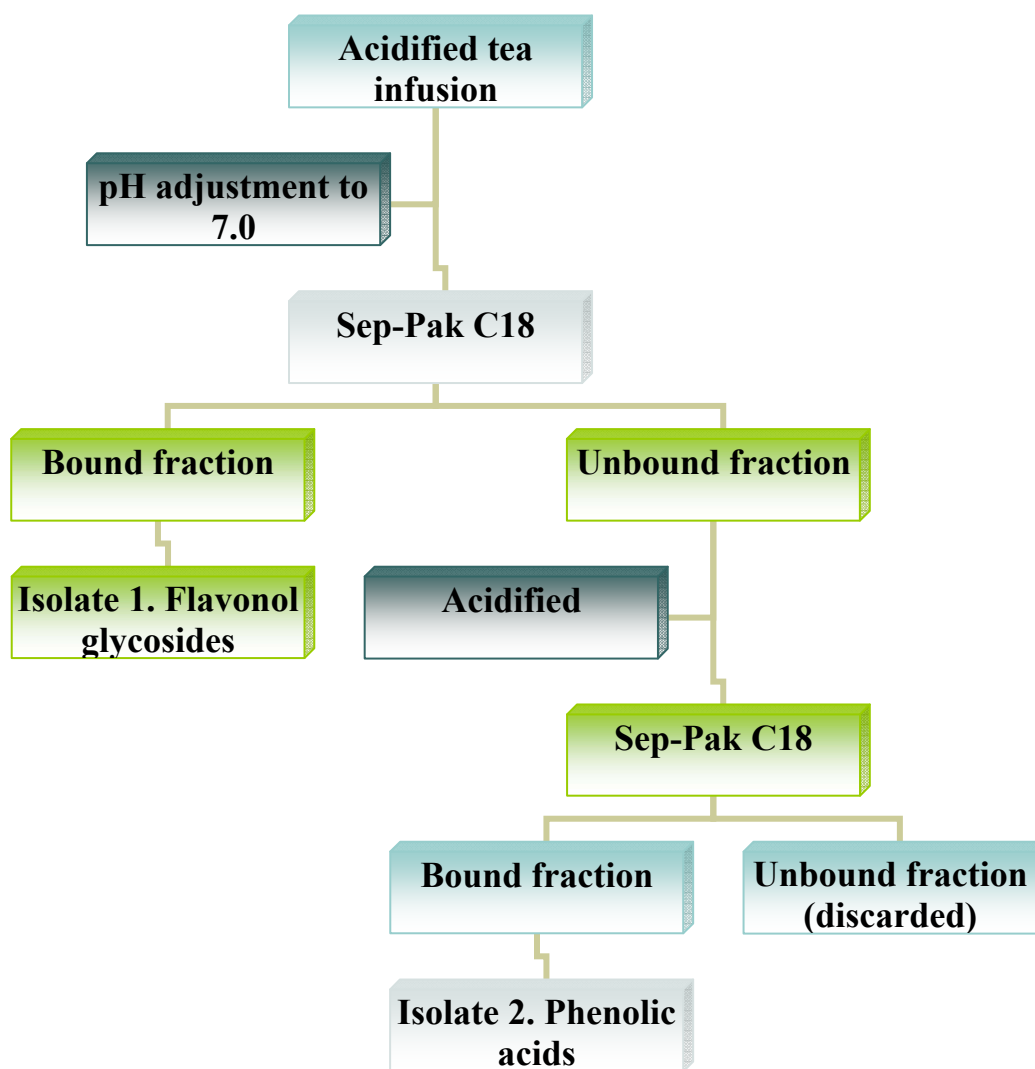


**Figure 3-1.** The design for green tea and yaupon holly fractionations and classification of polyphenolics present in methanolic extracts of tea varieties.

For the mamaki fractionation, tea was brewed using distilled water at pH 3.0. After cooling, a known amount of the tea infusion was taken to a beaker and pH of each infusion was adjusted to neutral (pH 7.0) with sodium hydroxide solution (0.3 M). Neutralized tea infusion (4 mL) was loaded onto an activated C<sub>18</sub> Sep-Pak reversed phase cartridge. Bound fraction was eluted with 10 mL of 100 % methanol (fraction 1, flavonoids) and unbound fraction was collected into a beaker which contained a few drops of acetic acid in order to acidify the unbound fraction immediately. After washing the same C<sub>18</sub> cartridge used for elution of fraction 1 with water, the acidified unbound

fraction was eluted with 10 mL of 100 % methanol (fraction 2, phenolic acids). Both fraction 1 and 2 were evaporate under reduced pressure and dissolved in 4 mL of water.

This methodology was briefly illustrated in **Figure 3-2**.



**Figure 3-2.** The design for mamaki fractionation and classification of polyphenolics present in methanolic extracts of tea varieties.



### ***Phytochemical analysis using HPLC and LC/ESI/MS<sup>n</sup>***

Individual polyphenolics was first analyzed by HPLC as described by Lee and Talcott, (108) with a slight modification. Each isolate was first diluted 3-fold with deionized water and filtered through a 0.45  $\mu\text{m}$  PTFE filter (Whatman, Clifton, NJ) prior to injection. Polyphenolic separations was conducted on a Waters 2695 alliance HPLC system using a Water 996 photodiode array (PDA) detector with a Dionex 250 x 4.6 mm Acclaim 120-C<sub>18</sub> column run at 0.8 mL/min. A gradient mobile phase consisted of Phase A (100 % H<sub>2</sub>O) and Phase B (60 % Methanol and 40% H<sub>2</sub>O) each adjusted to pH 2.4 using *o*-phosphoric acid. The gradient started by running 0 % Phase B for 1 min, 0-30 % Phase B over 30 min, 30-80 % Phase B in 15 min, 80-100 % Phase B in 15 min for a total run time of 70 min. The column was equilibrated with 100 % phase A for 2 min prior to the next sample injection. Phenolic compounds were detected and quantified at 280 nm against external standards of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-gallocatechin, chlorogenic acid, caffeic acid, caffeine, *p*-coumaric acid, ferulic acid, kaempferol, rutin, and quercetin all procured from Sigma Adrich (Sigma Chemical Co., St. Louis, MO).

Mass spectrometric analysis was carried out to define structural information of individual polyphenolics present in tea varieties based on molecular masses and fragment ions. After filtering through a 0.45  $\mu\text{m}$  PTFE filter (Whatman, Clifton, NJ), polyphenolics in the isolations was first detected by Finnigan Surveyor PDA plus detector (ThermoFisher, San Jose, CA) and evaluated on a Thermo Finnigan LCQ Deca XP Max MS<sup>n</sup> ion trap mass spectrometer system (ThermoFisher, San Jose, CA)

equipped with Finnigan Surveyor MS pump and Finnigan Surveyor Autosampler plus. Polyphenolics in each tea infusion were separated on the Dionex 250 x 4.6 mm Acclaim 120-C<sub>18</sub> column. The mobile phase consisted of Phase A (100 % H<sub>2</sub>O w/ 0.5 % formic acid (5 mM ammonium formate), v/v) and Phase B (100 % methanol w/ 0.5 % formic acid, v/v) run at 0.6 mL/min. Polyphenolics were separated using a gradient elution system that held mobile phase A for 1 min and then changed phase B 0 % to 50 % in 10 min; 50 % to 70 % in 5 min; 70 % to 100 % in 60 min and then returned to the original condition in 10 min for the next injection. The MS was operated in negative ion mode and fitted with an atmospheric pressure electrospray ionization (ESI) source.

Electrospray voltage was set to 3300 V with sheath gas flow rate of 60 units/min and a capillary gas temperature of 250 °C. Other ESI-MS parameters were as follows; auxiliary gas (N<sub>2</sub>), 5 units/min; capillary voltage, 1.5 V. Mass spectrometry data was obtained in the full scan mode ( $m/z$  200-2000) and fragment ions were additionally acquired in MS<sup>2</sup> and MS<sup>3</sup> mode.

### ***Antioxidant capacity***

Antioxidant capacity of each tea infusion was measured using the ORAC (Oxygen Radical Absorbance Capacity) run according to Talcott and Lee, (109) and adapted to work with a 96-well BMG Labtech FLUOSTAR Optima microplate reader (Offenburg, Germany). This method is based on the principle inhibiting the decay of fluorescence in the presence of the peroxy radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride. The rate of fluorescence decay was monitored every 2

min for 70 min by calculating the area under the decay curve. Antioxidant capacity was quantified by using a standard curve of Trolox (6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) that is a cell-permeable, water soluble analog of vitamin E with potent antioxidant properties. Each sample was suitably diluted (400X) in pH 7.0 phosphate buffer before pipetting into a 96-well microplate. Data was represented in micromoles of Trolox equivalents per gram of tea infusion.

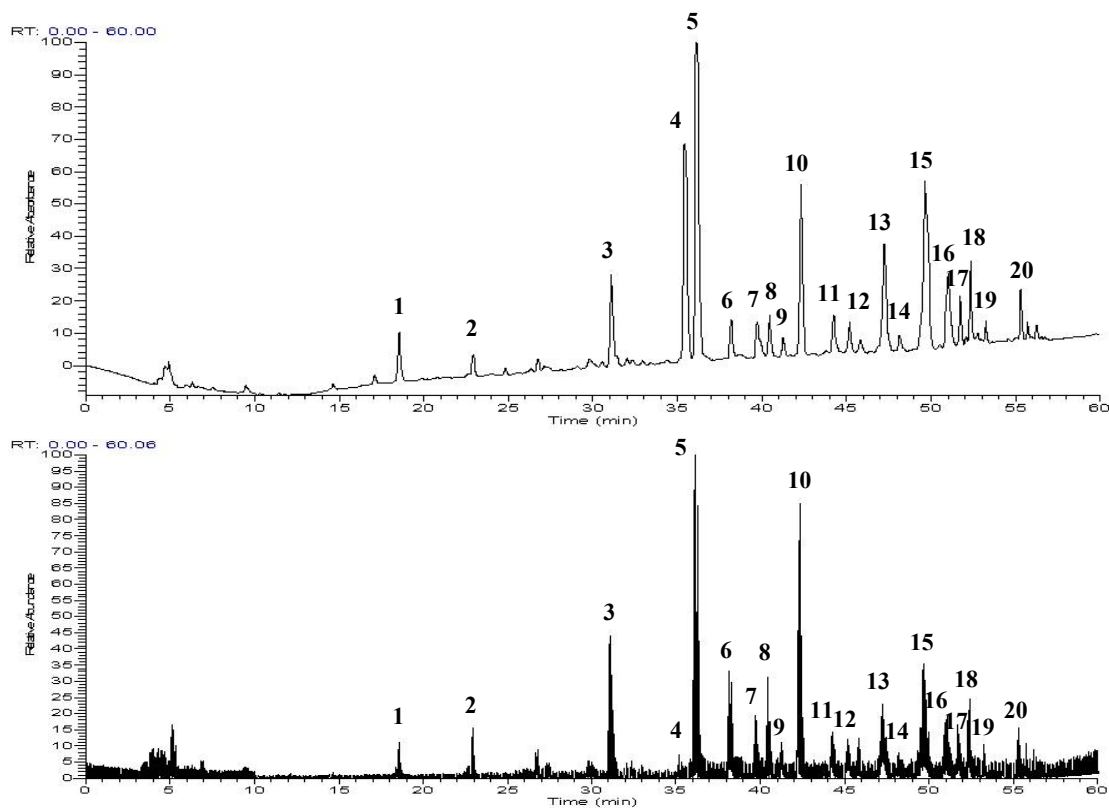
### *Statistical analysis*

Data represent the mean triplicate analysis using ANOVA (analysis of variance) with JMP 5 statistical software (110). Mean separation was conducted using the LSD test ( $P < 0.05$ ).

## **Results and Discussion**

### *Polyphenolic identification of green tea infusion by LC/ESI/MS<sup>n</sup> analysis*

Before separation was conducted using fractionation, green tea was analyzed using LC/ESI/MS<sup>n</sup> to identify phenolic compounds in the original infusion. Utilizing LC/ESI/MS<sup>n</sup> analysis, 20 phenolic compounds were identified based on retention time, absorbance spectrum, external standard, and mass fragmentation pattern (**Figure 3-3**).



**Figure 3-3.** Chromatogram determined by PDA analysis at 280 nm (top) and total ion current (TIC) analyzed by LC/ESI/MS<sup>n</sup> (bottom) of phenolic compounds present in green tea infusion. For peak assignment see **Table 3-1**.

In most instances, mass fragmentation data was coupled with characterizations in previous studies of tea infusions (111–113) and MS data shown in **Table 3-1**.

**Table 3-1.** LC/ESI/MS<sup>n</sup> characteristics of phenolic compounds present in green tea infusion.

Peak	RT	$\lambda_{\text{max}}$	Compound	[M-H] <sup>-</sup>	MS <sup>2</sup> (m/z)	MS <sup>3</sup> (m/z)
1	18.50	274	5-galloylquinic acid	343	169, <b>191</b> <sup>a</sup>	85, 127
2	22.96	232, 270, 324	(-) Gallocatechin	305	<b>179</b> , 221, 261	
3	31.14	243, 269, 322	(-) Epigallocatechin	305	<b>179</b> , 221, 261	
4	35.44	257	4-caffeoylquinic acid	353	191, <b>179</b>	135, 151, 163
5	36.13	258	(-) Epigallocatechin gallate	457	<b>169</b> , 305, 331	125
6	38.19	241, 278	(-) Epicatechin	289	179, 205, <b>245</b>	125
7	39.75	241, 275	(-) Gallocatechin gallate	457	<b>169</b> , 305, 331	
8	40.47	241, 276	(-) Epigallocatechin 3-methyl gallate	471	<b>183</b> , 287, 305	125
9	41.16	241, 271	(-) Epicatechin 3,5-digallate	593	<b>289</b>	169, 125
10	42.32	240, 275	(-) Epicatechin gallate	441	<b>289</b>	169, 125
11	44.25	228, 241, 271	Apigenin glycoside	563	353, 443, <b>473</b> , 503	353, 383
12	45.17	227, 241, 271	Apigenin glycoside	563	353, <b>443</b> , 473, 545	353, 383
13	47.28	241, 262, 354	Myricetin 3-glycoside	479	<b>317</b>	
14	48.13	222, 242, 271	Dehydro-isovitexin $\beta$ -glucoside <sup>b</sup>	593	341, <b>413</b> , 473	293
15	49.67	242, 255, 354	Quercetin 3-glucosyl-rhamnosyl-galactoside	771	<b>301</b>	162, 293
16	50.98	230, 242, 354	Quercetin 3-rutinoside (rutin)	609	<b>301</b>	151, 179
17	51.73	228, 242, 266	kaempferol glycoside	755	<b>285</b>	
18	52.39	229, 242, 265	Kaempferol 3-rutinoside	593	<b>285</b>	162
19	53.23	225, 243, 315	Unknown quercetin conjugate	901	741, 301	
20	56.24	223, 244, 315	Unknown quercetin conjugate	901	741, 301	

<sup>a</sup>. Ions in boldface indicate the most intense product ion for MS<sup>3</sup> fragmentation.

<sup>b</sup>. Tentatively identified.

Peak 1 was identified as 5-galloylquinic acid, which has been previously reported in significant concentration in green tea (114). No authentic standard is available for this

compound, but the deprotonated molecule ( $[M-H]^-$ ) was observed at  $m/z$  343 and it was fragmented to yield  $MS^2$  spectrum with ions at  $m/z$  191 (the amu of a quinic acid) and  $m/z$  169 (gallic acid) confirming its identification.

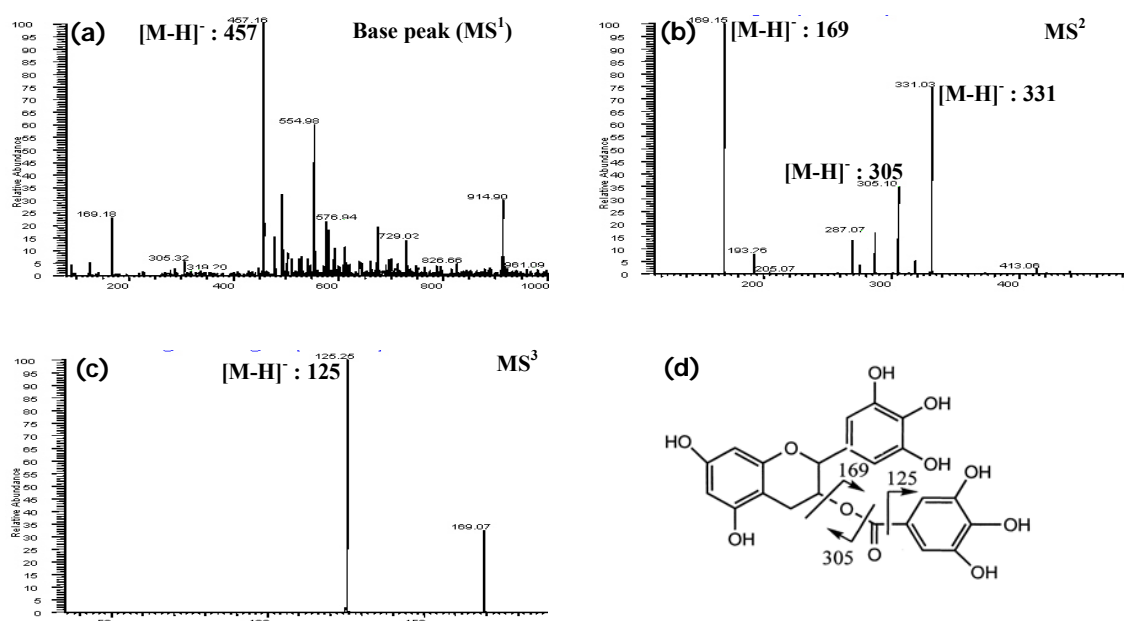
Peak 2 was determined as (-)-gallocatechin, which was previously observed in green tea (115). The deprotonated ion at  $m/z$  305 was obtained and  $MS^2$  fragments at  $m/z$  179, 221, and 261 were obtained. This result was in agreement with Del Rio et al. (112).

Peak 3 was identified as (-)-epigallocatechin (EGC), which is one of the most prevalent tea catechins in green tea infusion (115). Even though EGC has the same  $[M-H]^-$  at  $m/z$  305 and fragmentations at  $m/z$  179, 221, and 261 as observed in peak 2, its identification was confirmed by comparing to retention time and  $\lambda_{max}$  of authentic standard.

Peak 4 was regarded as 4-caffeoylquinic acid (cryptochlorogenic acid), which was previously observed in all *Camellia sinensis* teas including green tea (116). Three chlorogenic isomers, 3, 4, or 5-caffeoylquinic acid, showed exactly the same  $[M-H]^-$  at  $m/z$  353 and fragmented to yield  $MS^2$  and  $MS^3$  spectra with ions at  $m/z$  191 and 179 and 135, 151, and 163, respectively. The identification of these three isomers was based on the order of retention time, which was previously reported by several studies (107, 117, 118). The fragmented ion at  $m/z$  179 represents subtraction of the caffeoyl moiety from quinic acid.

Peak 5 was identified as (-)-epigallocatechin gallate (EGCG) with the parent ion of  $[M-H]^-$  at  $m/z$  457 and  $MS^2$  yielded fragments at  $m/z$  169, 305, and 331 with further ion at  $m/z$  125 by  $MS^3$  fragmentation. The fragmentation process of EGCG by  $MS/MS$

was described in **Figure 3-4**. The parent ion  $[M-H]^-$  at  $m/z$  457 was first obtained by MS, which is the strongest signal among green tea mass spectra, and it was additionally deprotonated by MS/MS obtaining ions at  $m/z$  169 (free gallic acid), 305 (epigallocatechin moiety), 331. By the final MS<sup>3</sup> fragmentation, fragment ion at  $m/z$  125 which is dihydroxy phenol moiety was obtained as a result of gallic acid fragmentation as shown in **Figure 3-4** (d).



**Figure 3-4.** Negative ion mass spectra of parent ion ( $m/z$  457, (a)) and deprotonated ions by additional MS<sup>2</sup> ( $m/z$  169, 305, and 331, (b)) and MS<sup>3</sup> (125, (c)) on EGCG. and (d) represents fragmentation of EGCG by MS/MS analysis.

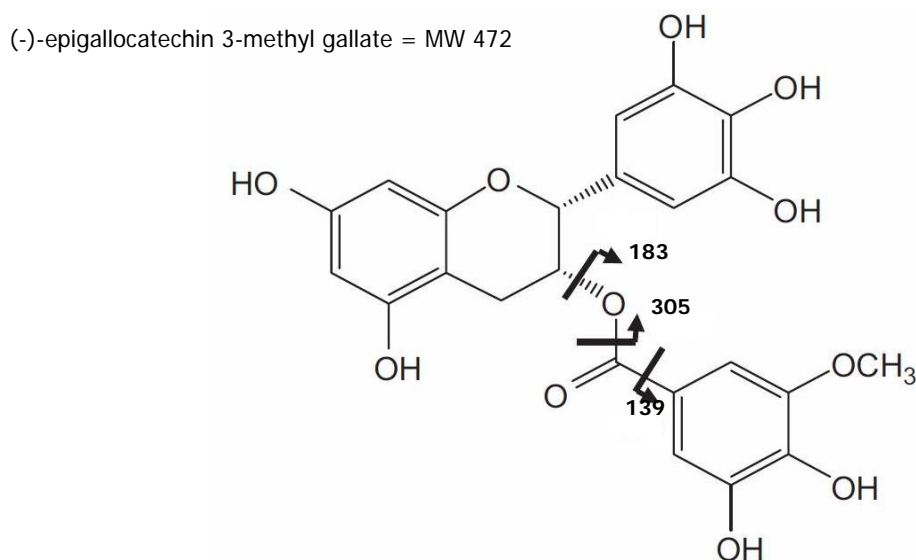
Peak 6 was determined as (-)-epicatechin based on its retention time, similar absorbance spectrum and fragmentation pattern of  $[M-H]^-$  at  $m/z$  289 with MS<sup>2</sup> fragment ion at  $m/z$  179, 205, and 245 and it was additionally fragmented to yield MS<sup>3</sup> spectrum

with ion at  $m/z$  125. Four epimeric isomers of (+)-catechin have been found in green tea such as (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin gallate (EGCG) but (+)-catechin, which has two asymmetric carbon atoms in the C ring, is present in green tea infusion at relatively small concentrations due to its ease of epimerization during the hot brewing process (9).

Peak 7 was defined as (-)-gallocatechin gallate (GCG) at the same MS/MS fragmentation pattern with EGCG which showed a  $[M-H]^-$  at  $m/z$  457 and  $MS^2$  fragmentation at  $m/z$  169, 301, and 331. Studies have reported that the retention time of EGCG is less than GCG (119–122) and likewise EGCG was eluted at 3.63 min earlier than GCG.

Peak 8 was identified as (-)-epicatechin 3-methyl gallate, which its presence in green tea was previously reported by Wang et al. (122) and Nishitani and Sagesaka, (123). The parent ion  $[M-H]^-$  at  $m/z$  471 and ions at  $m/z$  183, 287, and 305 by  $MS^2$  with a further fragmented ion at  $m/z$  125 by  $MS^3$  indicates (-)-epicatechin 3-methyl gallate was formed by addition of a methyl group to galloyl group of EGCG, which in accordance with Wang et al. (122) (**Figure 3-5**).





**Figure 3-5.** Proposed structure and fragmentation of (-)-epigallocatechin 3-methyl gallate by MS/MS analysis [Wang et al. (122)].

Peak 9 was tentatively identified as (-)-epicatechin 3,5-digallate with a  $[M-H]^-$  ion at  $m/z$  593 and predominant  $MS^2$  ion at  $m/z$  289 and this identification is in agreement with Wang et al. (122) that reported the presence of this compound in green tea infusion using LC/ESI/ $MS^n$  analysis.

Peak 10 was defined as (-)-epicatechin gallate (ECG) based on the comparison with absorbance spectrum and retention of authentic external standard, and MS/MS fragmentation. This compound showed a  $[M-H]^-$  ion of  $m/z$  441 and additional fragmentation yielded  $m/z$  289 by  $MS^2$  and  $m/z$  169 and 125 by  $MS^3$  analysis. The presence of ECG in green tea has been detected and identified with LC/ESI/ $MS^n$  analysis by many studies that showed the same fragmentation pattern confirming the identification of ECG in this study (112, 113, 121, 124).

Peak 11 and 12 were tentatively identified as apigenin glycosides (glycosylflavonoids), which have been detected in various plants including herbal teas (125, 126) yet no studies reported its presence in green tea. Most of the aglycone of the glycosylflavonoids is either apigenin or luteolin and likewise, the compounds detected in the present study may be apigenin glycosylflavonoids based on its fragment pattern. The  $[M-H]^-$  in both cases was  $m/z$  563, which yielded a  $MS^2$  fragmentation at  $m/z$  353, 443, 473, and 503. Apigenin glycosylflavonoids at  $m/z$  563 first yielded the ions of  $[M-H-60]^-$  and  $[M-H-120]^-$  by fragmentation indicating the presence of pentose substitution. Since  $[M-H-90]^-$  and  $[M-H-120]^-$  were the most prevalent ions in  $MS^2$  ( $m/z$  473 [100],  $m/z$  443 [82], and  $m/z$  503 [74] in peak 11 and  $m/z$  443 [100],  $m/z$  473 [59], and  $m/z$  503 [not detected] in peak 12), numbers in parenthesis represent relative intensity of fragmented ions, both peaks may contain arabinose instead of pentose as a glycoside due to lack of  $[M-H-60]^-$ , which is evidence of presence of pentose (127).

Peak 13 has a  $[M-H]^-$  at  $m/z$  479, which yielded a major  $MS^2$  fragment at  $m/z$  317 indicating this compound is myricetin 3-glycoside on the basis of fragment pattern reviewed by Atoui et al. (111) who detected the presence of myricetin 3-glycoside in green tea. Myricetin, an aglycone of myricetin 3-glycoside, was detected at  $m/z$  317 (myricetin MW = 318) by  $MS^2$  fragmentation with the loss of its sugar molecule.

Peak 14 was tentatively identified as dehydro-isovitexin  $\beta$ -glucoside due to its fragmentation pattern.  $MS/MS$  fragmentation yielded a  $[M-H]^-$  at  $m/z$  593 and additional fragmentation produced  $MS^2$  and  $MS^3$  ions at  $m/z$  413 ( $[M-H-180]^-$ ) by cleavage of the glycosidic bond at C-2' and 293 ( $[M-H-180-120]^-$ ) by additional fragmentation of ion at

$m/z$  120 from glucose, respectively (128). Isovitexin (apigenin 6-*C*-glycoside, [M-H-180+H<sub>2</sub>O]<sup>-</sup>) was identified as aglycone and its presence in green tea was previously reported by (129, 130).

Peak 15 was tentatively identified as quercetin 3-glucosyl-rhamnosyl-galactoside on the basis of its fragment pattern. This was confirmed by the MS/MS fragmentation, which yielded a [M-H]<sup>-</sup> at  $m/z$  771 and a prominent MS<sup>2</sup> ion at  $m/z$  301 indicating loss of aglycone quercetin. The MS<sup>3</sup> fragmentation yielded ions at  $m/z$  162 and 293 and the loss of ion at  $m/z$  162 indicates a hexosyl residue was present (131).

Peak 16 was defined as quercetin 3-rutinoside (rutin) due to its a [M-H]<sup>-</sup> at  $m/z$  609 and on the basis of the mass spectrum, absorbance spectrum, and retention time in comparison to authentic standard. A prominent MS<sup>2</sup> ion at  $m/z$  301 indicated loss of sugar molecule (glucose-rhamnose) from aglycone quercetin as observed with peak 15. The presence of rutin in green tea has been extensively reviewed by many literatures and the concentration of rutin is highest among all flavonol glycosides in green tea (124, 132, 133).

Peak 17 was tentatively identified as kaempferol glycoside because it produced a [M-H]<sup>-</sup> at  $m/z$  285 indicating loss of glycoside from the aglycone kaempferol. The lost glycosides could be galactose, glucose-rhamnose, or glucose but no further identification was obtained from MS<sup>3</sup> fragmentations.

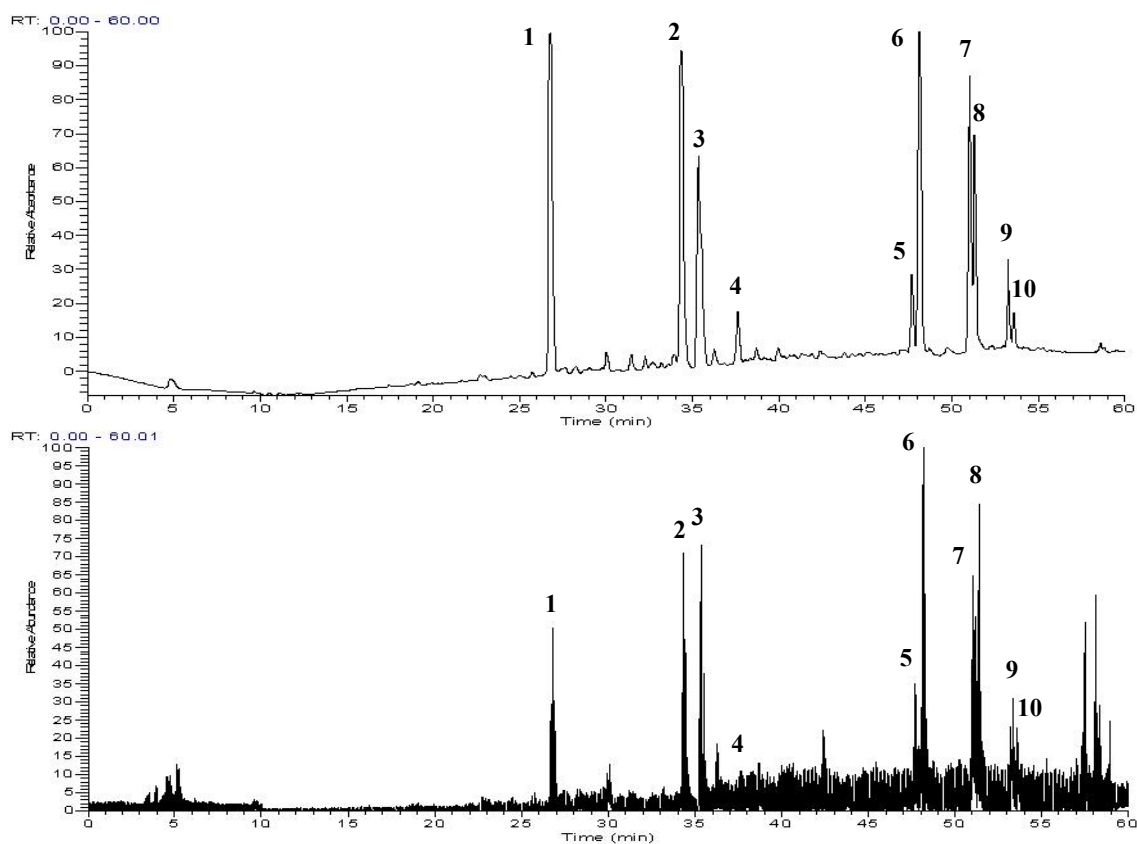
Peak 18 was identified as kaempferol 3-rutinoside due to its MS/MS fragmentation at  $m/z$  593 and the main MS<sup>2</sup> fragmentation was found at  $m/z$  285 (kaempferol MW = 286) indicating loss of hexose-rhamnose with no intermediate ion at

$m/z$  447 (monoglycoside). The same fragmentation pattern and presence in green tea is in agreement with Atoui et al. (111) and Kiehne and Engelhardt, (130).

Peak 19 and 20 had a  $[M-H]^-$  at  $m/z$  901, which yielded a major  $MS^2$  fragmentation at  $m/z$  741 and 301. The fragment ion at  $m/z$  301 corresponds to the aglycone quercetin as observed in identification of peak 15 and 16. This was previously identified by Del Rio et al. (112) who confirmed the identification of unknown quercetin conjugate.

#### ***Polyphenolic identification of yaupon holly infusion by LC/ESI/MS<sup>n</sup> analysis***

Polyphenolics present in yaupon holly infusion were identified by LC/ESI/MS<sup>n</sup> analysis. The identification was in agreement with Bravo et al. (117) and Markowicz et al. (132) who previously reported polyphenolic constituents in yerba mate, which is one of *Ilex* genus including yaupon holly, using the same analysis. Polyphenolic characterization of yaupon holly by HPLC analysis has been reported by studies by Fuller et al. (47) and Palumbo et al. (48). However, there is no information available on the polyphenolic identification of yaupon holly performed by LC/ESI/MS<sup>n</sup> analysis. In total, 10 polyphenolic compounds were detected by LC/MS and identified based on retention time, absorbance spectrum, external standard, and MS fragmentation pattern **(Figure 3-6)**.



**Figure 3-6.** Chromatogram determined by PDA analysis at 280 nm (top) and total ion current (TIC) analyzed by LC/ESI/MS<sup>n</sup> (bottom) of phenolic compounds present in yaupon infusion. For peak assignment see **Table 3-2**.

All the identifications evaluated in the present study were described in **Table 3-2**.

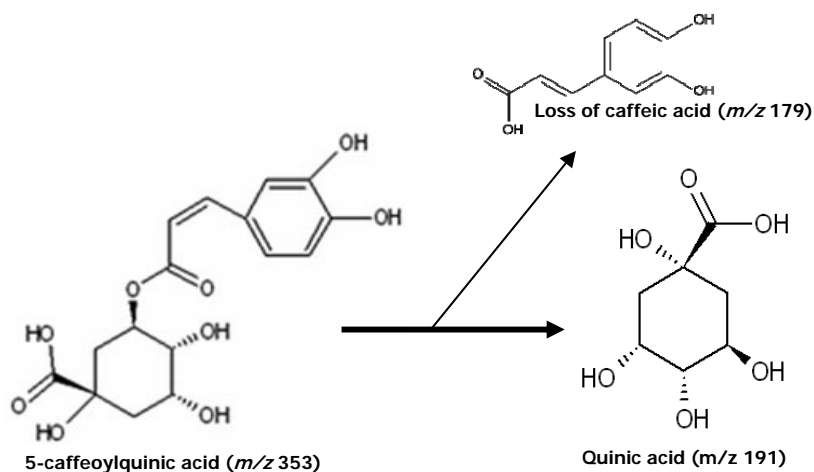
**Table 3-2.** LC/ESI/MS<sup>n</sup> characteristics of phenolic compounds present in yaupon holly infusion.

Peak	RT	$\lambda_{\max}$	Compound	[M-H] <sup>-</sup>	MS <sup>2</sup> ( <i>m/z</i> )	MS <sup>3</sup> ( <i>m/z</i> )
1	26.76	248, 307	3- <i>O</i> -caffeoylquinic acid	353	<b>179</b> <sup>a</sup> , 191	85, 127, 135
2	34.35	249, 304	5- <i>O</i> -caffeoylquinic acid	353	<b>179</b> , 191	85, 127, 135
3	35.34	247, 325	4- <i>O</i> -caffeoylquinic acid	353	<b>179</b> , 191	85, 127, 135
4	37.62	231, 249, 324	Kaempferol 3-rhamnoside	447	<b>301</b>	
5	47.70	243, 326	3,4-dicaffeoylquinic acid	515	<b>353</b>	135, 179, 191
6	48.14	244, 328	3,5-dicaffeoylquinic acid	515	<b>353</b>	135, 179, 191
7	51.04	255, 346	Quecetin 3-rutinoside (rutin)	609	<b>301</b>	179
8	51.33	245, 328	4,5-dicaffeoylquinic acid	515	<b>353</b>	135, 179, 191
9	53.28	233, 243, 265	Kaempferol 3-rutinoside	593	<b>285</b>	
10	53.60	230, 243, 347	Isorhamnetin 3-rutinoside	623	<b>315</b>	287, 300, 315

<sup>a</sup>. Ions in boldface indicate the most intense product ion for MS<sup>3</sup> fragmentation.

Peak 1, 2, and 3 were identified as 3, 5, and 4-caffeoylquinic acid, respectively. All have similar spectra and the same fragment patterns, which produce a [M-H]<sup>-</sup> at *m/z* 353 and prominent MS<sup>2</sup> ions at *m/z* 179, 191. This is in accordance with Bravo et al. (117) and matches the mass spectra of 3, 5, and 4-caffeoylquinic acid, a known compound in yerba maté infusion. Chlorogenic acid (5-caffeoylquinic acid), which corresponds to caffeic acid esterified to quinic acid was fragmented by losing caffeoyl moiety at *m/z* 179 (caffeic acid) to quinic acid at *m/z* 191 (**Figure 3-7**). The elution profile of three detected caffeoylquinic acids was reported by many literatures and they all agreed on the elution order of 3-caffeoylquinic acid (neochlorogenic acid), 5-caffeoylquinic acid (chlorogenic acid), and 4-caffeoylquinic acid (cyrptochlorogenic acid)

(112, 117, 118, 133). So, likewise, peak 1, 2, and 3 were identified followed by the order and retention time matched with chlorogenic acid standard (5-caffeoylquinic acid).

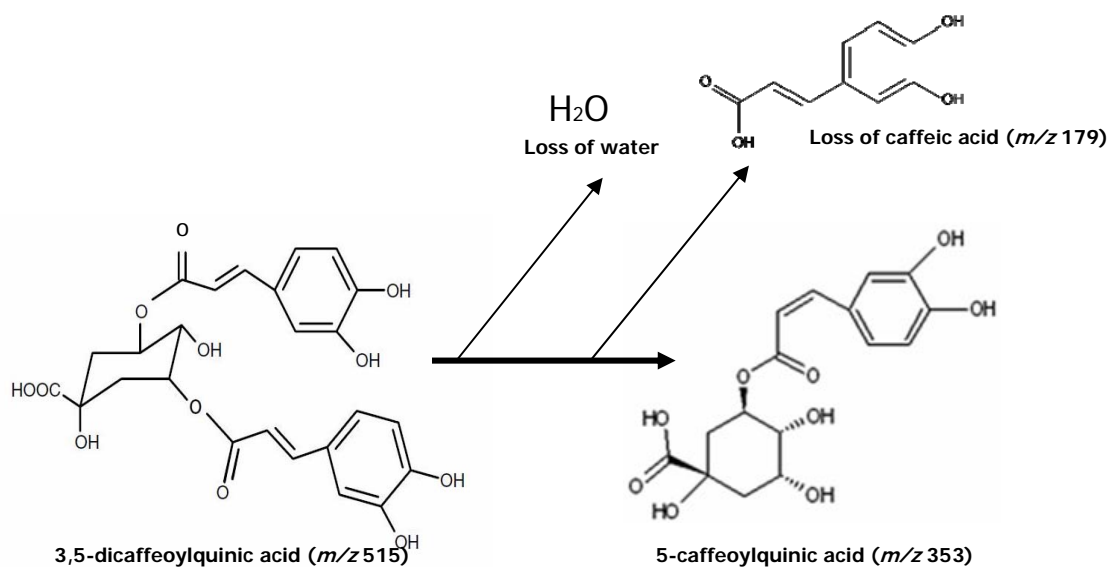


**Figure 3-7.** Scheme of fragmentation of 5-caffeoylquinic acid (chlorogenic acid) in yaupon holly infusion.

Peak 4 was identified as kaempferol 3-glycoside and had a molecular ion at  $m/z$  447, which is common to kaempferol and luteolin glycosides (117). However, fragmented ion at  $m/z$  301 (quercetin aglycone) suggests that this compound is quercetin rhamnoside (quercetin MW = 302) due to the fragmentation by loss of rhamnose.

Peak 5, 6, and 8 were identified as 3,4, 3,5, and 4,5-dicaffeoylquinic acid, which all produced a  $[M-H]^-$  at  $m/z$  515 and additional fragmentation yielded  $MS^2$  ion at  $m/z$  353 and  $MS^3$  ions at  $m/z$  135, 179, and 191. Dicaffeoylquinic acid, which was reported as a major constituent present in yerba mate, fragmented in  $MS^2$  yielding an ion at  $m/z$  353, which was obtained by the loss of dehydrated caffeic acid molecule (117, 134)

(**Figure 3-8**). Judging from their retention times of dicaffeoylquinic acid in  $C_{18}$  column previously reported by Bravo et al. (117), Clifford and Ramirez-Martinez, (135), and Yoshimoto et al. (136), the peaks of 5, 6, and 8 were determined as 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, respectively.



**Figure 3-8.** Scheme of fragmentation of 3,5-dicaffeoylquinic acid in yaupon holly infusion.

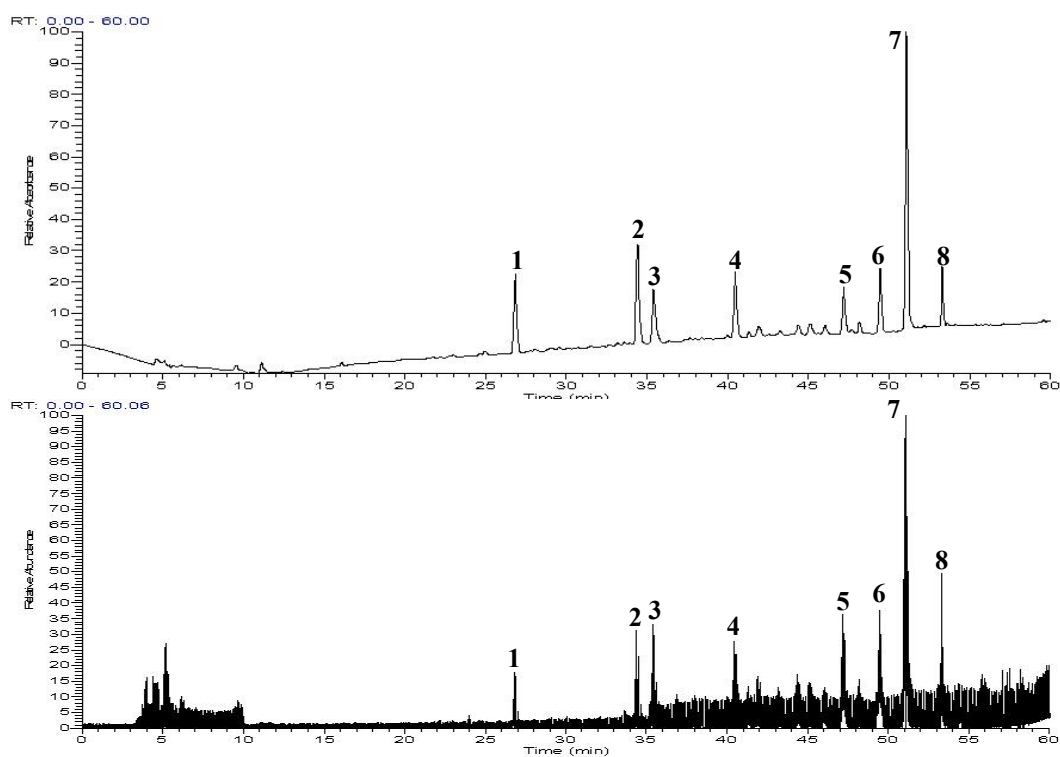
Peak 7 and 9 were determined as quercetin 3-rutinoside and kaempferol 3-rutinoside, respectively as observed in green tea infusions on the basis of the retention time compared to the same compound in green tea and their same fragmentation patterns of peak 7 (a  $[M-H]^-$  at  $m/z$  593, and the main  $MS^2$  fragmentation at  $m/z$  285) and peak 9 (a  $[M-H]^-$  at  $m/z$  609, and the main  $MS^2$  fragmentation at  $m/z$  301).



Peak 10 was identified as isorhamnetin 3-rutinoside, which is widely found in plant kingdom including various herbal teas (137, 138). This peak produced a  $[M-H]^-$  at  $m/z$  623, which yielded a  $MS^2$  fragment ion at  $m/z$  271, 300, and 315. According to Parejo et al. (139), the glucosidic bond of *O*-rhamnoglucoside is readily cleaved in the negative mode with producing fragment ion at  $m/z$  315 by the neutral loss of a rhamnoglucosyl moiety from the deprotonated molecule ( $[M-H-308]^-$ ). Moreover, rutinosides usually show a high relative intensity of the aglycone fragment (usually 100%) as observed in the peak 10 compared to other glycosides (139).

#### ***Polyphenolic identification of mamaki infusion by LC/ESI/MS<sup>n</sup> analysis***

In total, eight polyphenolic compounds were detected in mamaki infusion using LC/ESI/MS<sup>n</sup> analysis based on their retention time, absorbance spectrum, external standard, and MS fragmentation pattern (**Figure 3-9**). As observed in polyphenolic identification of yaupon holly infusion, three caffeoylquinic acids and two flavonol glycosides were the major compounds in mamaki infusion and others such as apigenin glycosides and were also observed (**Table 3-3**).



**Figure 3-9.** Chromatogram determined by PDA analysis at 280 nm (top) and total ion current (TIC) analyzed by LC/ESI/MS<sup>n</sup> (bottom) of phenolic compounds present in yaupon infusion. For peak assignment see **Table 3-3**.

**Table 3-3.** LC/ESI/MS<sup>n</sup> characteristics of phenolic compounds in mamaki infusion.

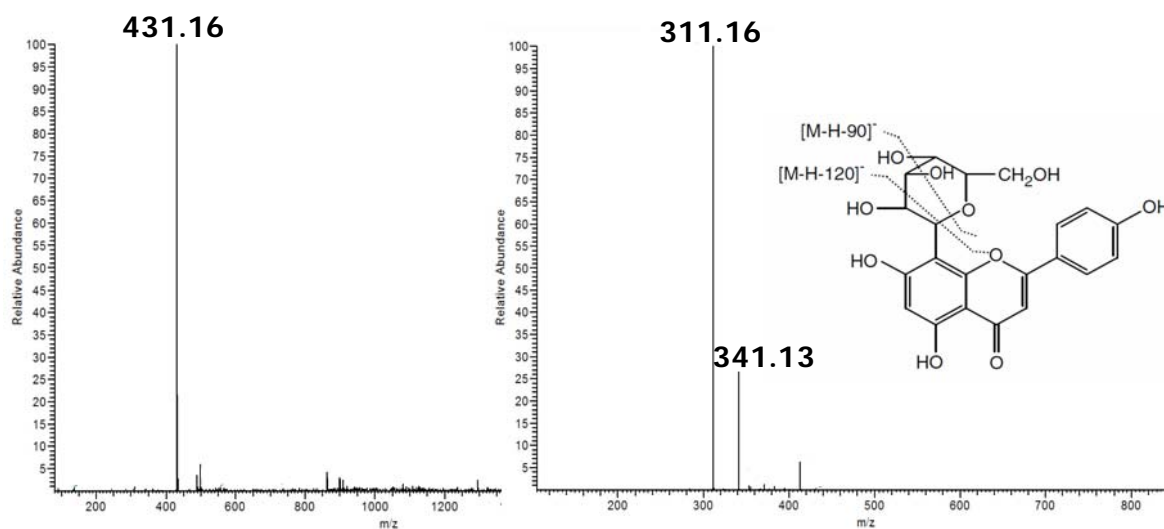
Peak	RT	$\lambda_{\max}$	Compound	[M-H] <sup>-</sup>	MS <sup>2</sup> (m/z)	MS <sup>3</sup> (m/z)
1	26.86	251, 326	3- <i>O</i> -caffeoylquinic acid	353	135, <b>179</b> <sup>a</sup> , 191	127
2	34.41	232, 249, 326	5- <i>O</i> -caffeoylquinic acid	353	135, <b>179</b> , 191	127
3	35.40	233, 250, 326	4- <i>O</i> -caffeoylquinic acid	353	135, <b>179</b> , 191	127
4	40.48	231, 248, 328	6- <i>C</i> -hexosyl apigenin	593	<b>353</b> , 473, 503	353
5	47.20	230, 242, 336	Apigenin 7-glucoside	431	<b>311</b> , 341, 283	
6	49.46	242, 270, 337	Apigenin 7-glucoside	431	<b>311</b> , 341, 283	
7	51.08	233, 255, 354	Quercetion 3-rutinoside	609	<b>301</b>	179
8	53.30	231, 243, 346	Kaempferol 3-rutinoside	593	<b>285</b>	

<sup>a</sup>. Ions in boldface indicate the most intense product ion for MS<sup>3</sup> fragmentation.

Peak 1, 2, and 3, were readily identified as 3,5, and 4-caffeoylquinic acid, respectively as observed in yaupon holly infusion by comparing to fragmentation pattern, retention time, absorbance spectra of the three compounds in yaupon holly and authentic standard of 5-caffeoylquinic acid.

Peak 4 was tentatively identified as *C*-hexosyl apigenin, which showed a  $[M-H]^-$  at  $m/z$  593 and fragment  $MS^2$  ions at  $m/z$  473 ( $[M-H-120]^-$ ) and 503 ( $[M-H-90]^-$ ), indicating the presence of *C*-hexosyl unit on its flavonoids structure and the  $MS^3$  ion at  $m/z$  353 (aglycone + 83) represents that apigenin (MW 270) is present as an aglycone (140).

Peak 5 and 6 were identified as apigenin 8-glucoside on the basis on the same fragmentation pattern reviewed by Sánchez-Rabaneda et al. (141) who found a  $[M-H]^-$  at  $m/z$  431 and the most prominent  $MS^2$  ions at  $m/z$  311 and 341 as observed in peaks 5 and 6. The typical fragmentation of *C*-glycoside by  $[M-H-120]^-$  and  $[M-H-90]^-$  indicates that a deprotonated molecule ( $m/z$  431) lost its glycoside from the aglycone apigenin (**Figure 3-10**).



**Figure 3-10.** Chromatograms of deprotonated and MS<sup>2</sup> ions of apigenin 8-glucoside from peak 5 and 6 detected from LC/ESI/MS<sup>n</sup> analysis of mamaki infusion.

Peak 7 and 8 were readily identified as quercetin 3-rutinoside (rutin) and kaempferol 3-rutinoside as observed in polyphenolic identification in yaupon holly infusion based on the same fragmentation pattern and similar retention time.

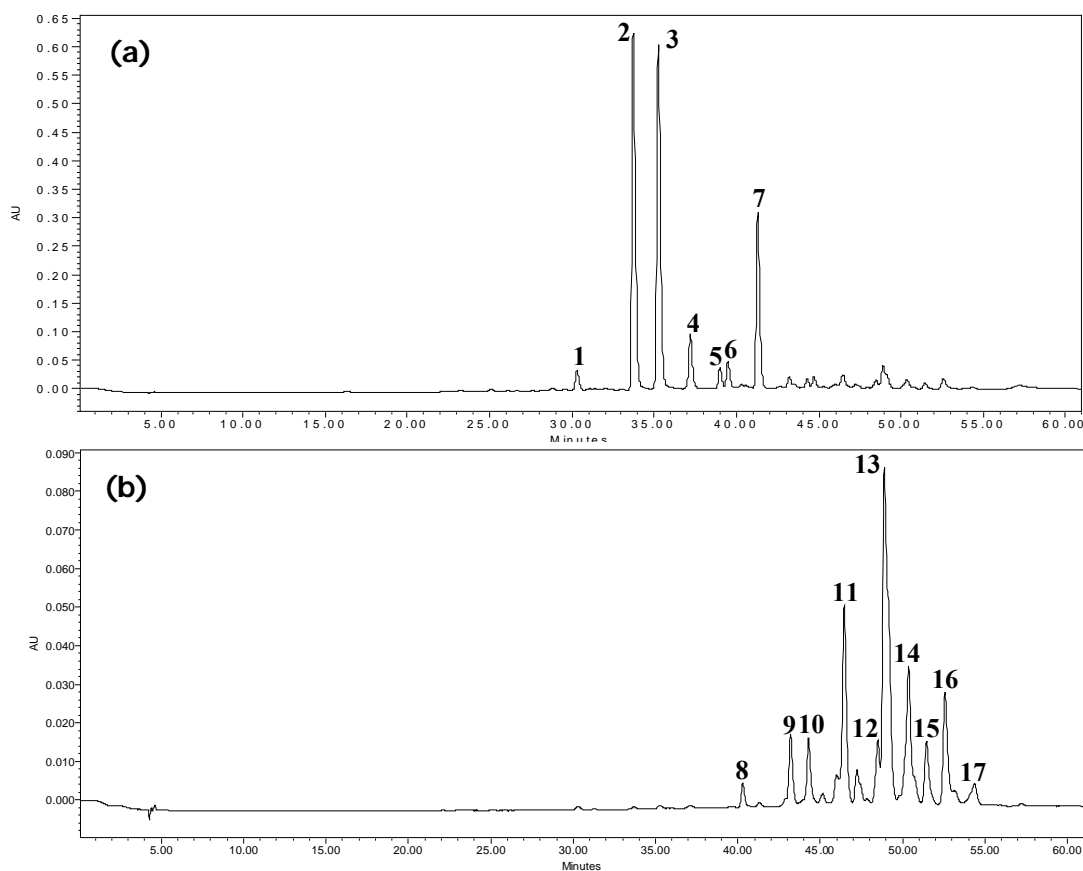
***Identification and quantification by HPLC-PDA analysis of phenolic fractions separated from green tea infusion***

Green tea contains various classes of polyphenols such as flavan-3-ols (tea catechins) and flavonols (usually present as glycosidic form) demonstrating different health-promoting potentials mainly from their antioxidant activities. Most of studies have focused on health benefit in vivo or biological activities in vitro by flavan-3-ols but not on flavonols even though they are present at significant concentration (up to 2-3 % of the water soluble solids in both green and black tea) (142). The main flavonoids

present in green tea are flavanols, which take account up to 30 % of dry weight, and flavonols, which have gained attention due to their excellent pharmaceutical properties such as antioxidative, anticarcinogenic and antiarteriosclerotic functionalities. According to Bors et al. (143), flavonols act as an antioxidant due to the structure of conjugated keto group on double bonded C ring with adjacent hydroxyl group(s) (1 to 3) on the B ring. Moreover, flavonol glycoside is structurally more stable compared to flavonols (144), changes in flavonols may play a more important role to impact the stability of tea beverage during storage. Therefore, an investigation of flavonol glycoside needs to be conducted by comparing flavanols following an appropriate separation method.

Polyphenolics were fractionated into neutral fractions as described in **Figure 3-1**. The pH of green tea infusion was previously adjusted to neutral (pH 7) to extract neutral polyphenolics such as catechins and flavonols. The polyphenolic containing infusion was passed through C<sub>18</sub> Sep-Pack cartridge which was preconditioned with methanol and distilled water for neutral fraction. The unbound fraction which contained interfering compounds such as sugars and organic acids was not included in this procedure. Neutral fraction 1 (Fraction 1) absorbed on the solid phase was eluted with 10 % methanol followed by elution with 100 % methanol for neutral fraction 2 (Fraction 2). Fraction 1 contained flavan 3-ols while flavonol glycosides were obtained in fraction 2. This difference was due to different affinity of polyphenolics to C<sub>18</sub> cartridge and partition based on different solubility in methanol solutions.

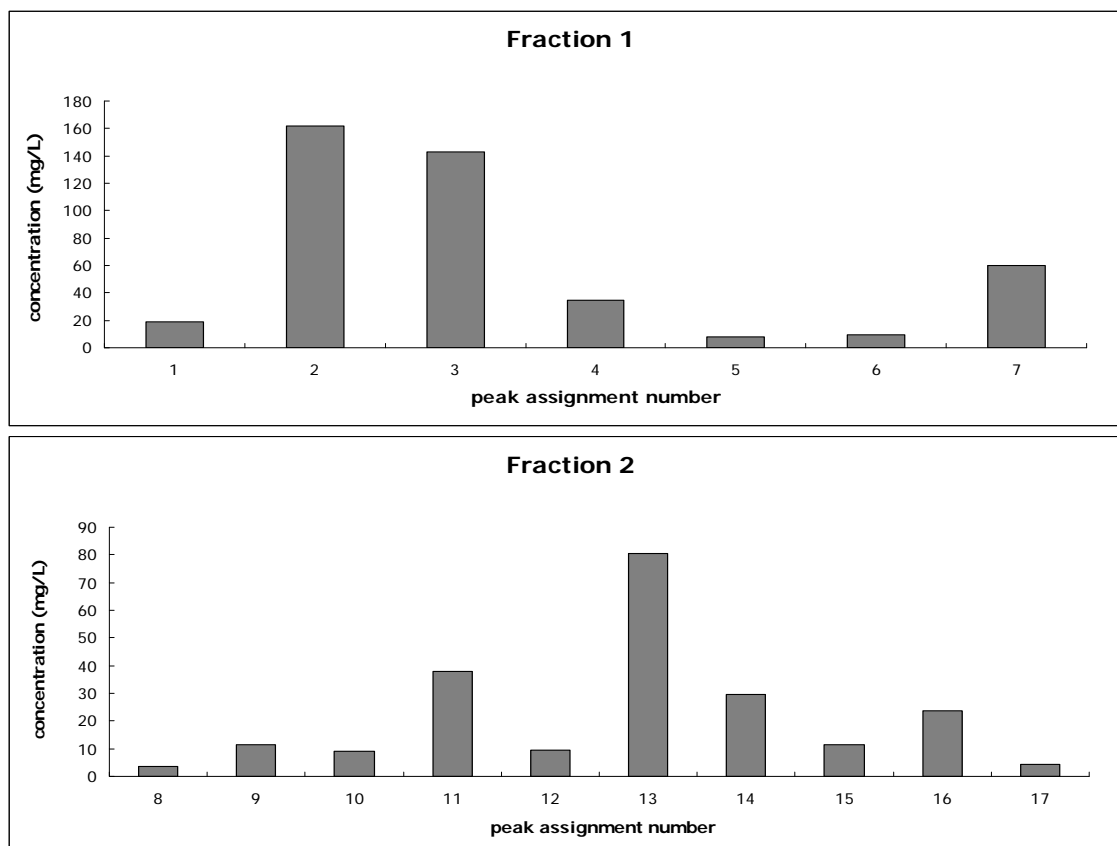
Polyphenolics present in green tea infusion were separated into two groups: flavan-3-ols including caffeine and flavonols as shown in **Figure 3-11**. As a result of



**Figure 3-11.** Chromatograms of polyphenolics and caffeine present in green tea infusion separated by the first fraction method. (a) flavan-3-ols and caffeine in fraction 1 determined at 280 nm and (b) other flavonoids (mostly flavonols) in fraction 2 at 360 nm by HPLC-PDA analysis. Peak assignment: **Fraction 1 (a)**. 1. (-)-epigallocatechin, 2. caffeine, 3. (-)-epigallocatechin gallate, 4. (-)-epicatechin, 5. (-)-gallocatechin gallate, 6. (-)-epigallocatechin 3-methyl gallate, 7. (-)-epicatechin gallate. **Fraction 2 (b)**. 8. 6-*C*-hexosyl apigenin, 9. Apigenin 6-*C*-arabinosyl-8-*C*-glucosyl, 10. Apigenin 6-*C*-glucosyl-8-*C*-arabinosyl, 11. Myricetin 3-glycoside, 12. Quercetin 3-glucosyl-rhamnosyl-galactoside, 13. Quercetin 3-rutinoside (rutin), 14. kaempferol glycoside, 15. Kaempferol 3-rutinoside, 16. Unknown quercetin conjugate, 17. Unknown quercetin conjugate.

fractionation, a total of 6 flavan-3-ols were separated from green tea infusion while 10 flavonol glycosides were found in fraction 2. The presence of all the flavan-3-ols detected in fraction 1 such as EGC, EGCG, EC, GCG, EGC 3-methyl gallate, and ECG were previously reported and they have been shown to be the predominant phenolic compounds in green tea (34, 121, 111, 145).

Caffeine (1,3,7-trimethylpurine-2,6-dione hydrate) was present at the highest concentration (162.08 mg/L) of all the compounds found in green tea while the most prevalent phenolic compound was EGCG that was present at the concentration of 142.89 mg/L (**Figure 3-12**) in the first fraction. As previously reported by several studies, green tea was one of the important sources providing naturally occurring caffeine. In the second fraction, rutin (quercetin 3-rutinoside) was present at the highest concentration (80.44 mg/L), which was previously reported its presence in green tea (142). In green tea, 44.6 % of phenolic compounds was determined as flavonol glycosides while the flavan 3-ols concentration took account 55.4 % of total polyphenolics.



**Figure 3-12.** Concentration (mg/L) of flavan-3-ols and caffeine in fraction 1 and flavonol glycosides in fraction 2. For the number assignment see **Figure 3-11**. The concentration of flavonol glycosides from 8 to 17 in fraction 2 was expressed as rutin equivalent (RE).

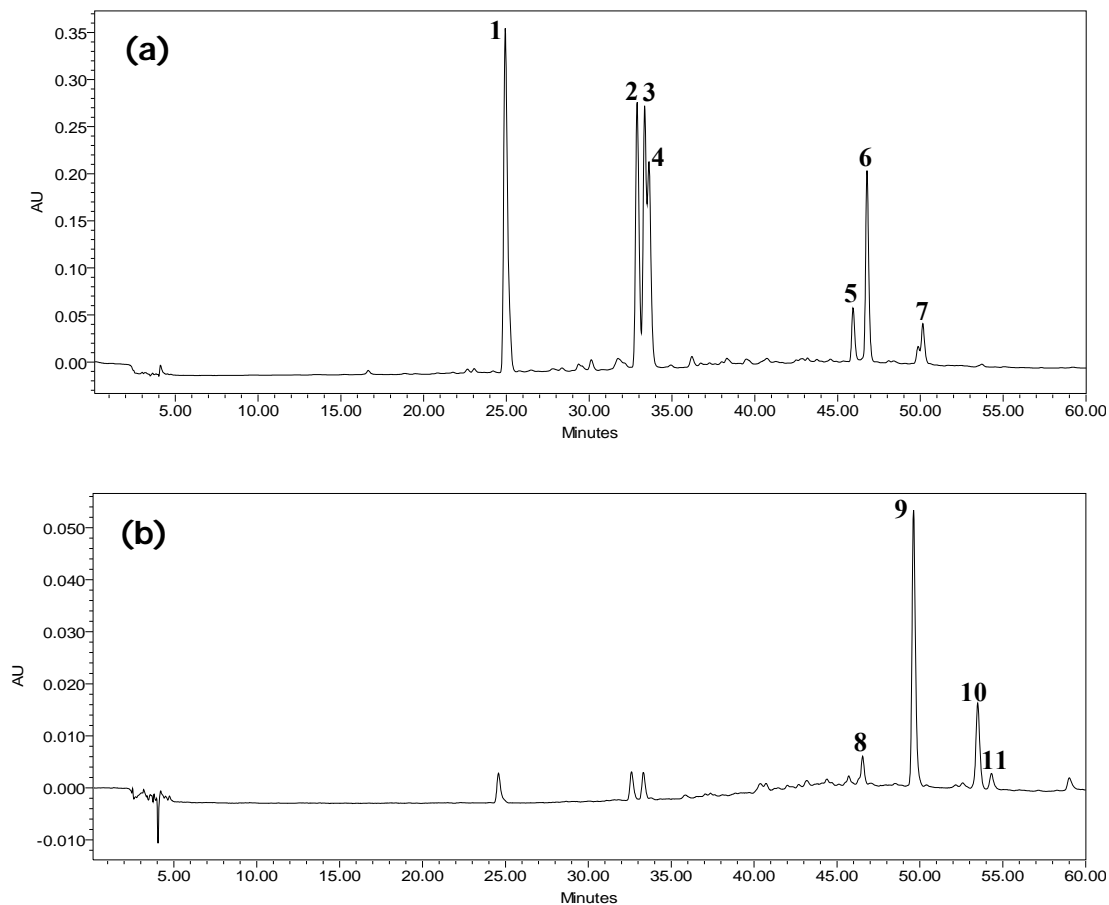
***Identification and quantification by HPLC-PDA analysis of phenolic fractions separated from yaupon holly infusion***

As described in **Figure 3-1**, the pH of yaupon holly infusion was neutralized before passing through C<sub>18</sub> cartridge to ionize phenolic acids in the infusion (chlorogenic acid and its isomers). The ionized phenolic acids and other neutral compounds in yaupon holly infusion (flavonols) were entrapped in the neutral cartridge and eluted with two

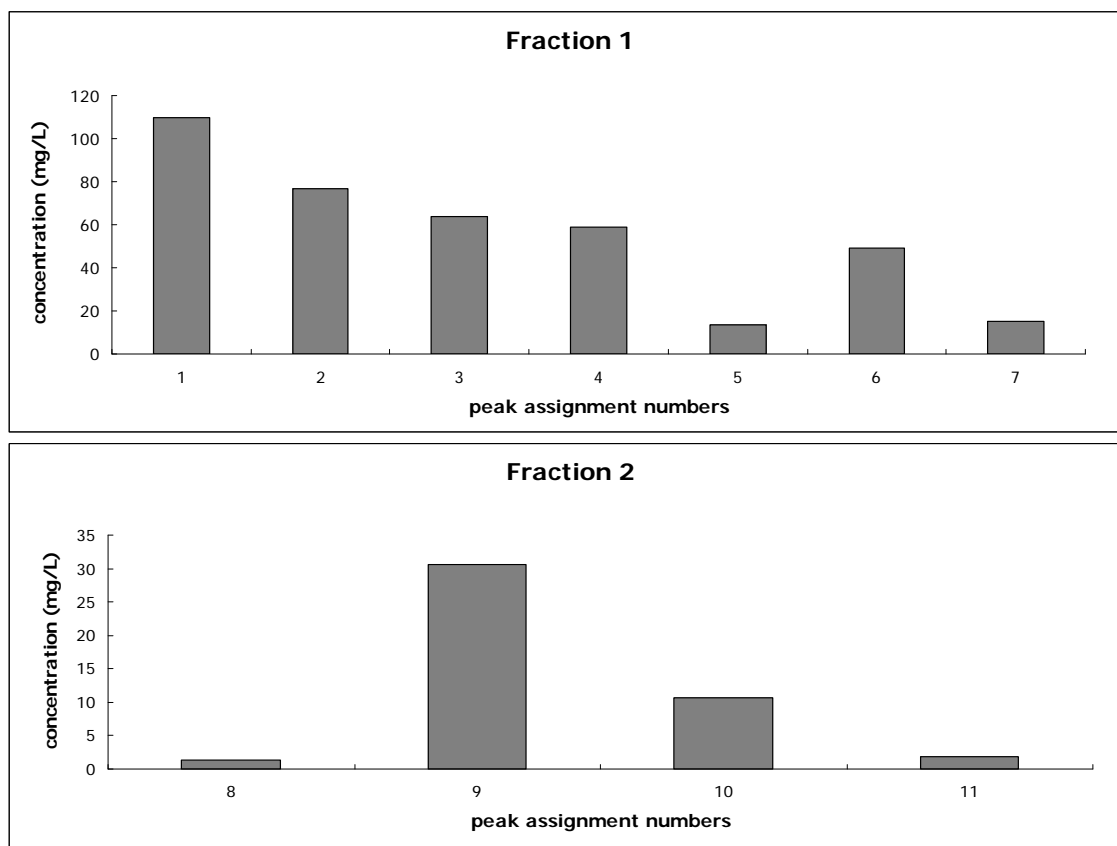


different methanolic solutions (10 and 100 % methanol) based on different solubility and affinity to C<sub>18</sub> cartridge.

The presence of two different groups of polyphenolic compounds in yaupon holly was reported by Palumbo et al. (48) who described that hydrocinnamic acids and flavonoids were the only two groups found in yaupon holly. Likewise, by fractionation, caffeoylquinic acids and dicaffeoylquinic acids were separated from the infusion and their identifications were determined based on the LC/ESI/MS<sup>n</sup> analysis of yaupon holly infusion and compared to authentic standards (**Figure 3-13**). In fraction 2, four flavonols glycosides were found as observed in LC/ESI/MS<sup>n</sup> analysis. In fraction 1, 3-caffeoylquinic acid (neochlorogenic acid) was present at the highest concentration (109.73 mg/L) followed by 5-caffeoylquinic acid (chlorogenic acid, 76.90 mg/L) and 4-caffeoylquinic acid (64.02 mg/L) and total concentration of four dicaffeoylquinic acids was 136.95 mg/L (**Figure 3-14**). Caffeine was also found in fraction 1 but the concentration (58.94 mg/L) was significantly lower than that of green tea. In fraction 2, rutin was present at the highest concentration (30.64 mg/L) while other flavonols were detected at relatively low concentrations, especially kaempferol 3-rhamnoside and isorhamnetin 3-rutinoside (1.40 and 1.92 mg/L, respectively). In yaupon holly infusion, hydrocinnamic acids such as caffeoylquinic acids and dicaffeoylquinic acids were predominant compounds which take 88.1 % of total polyphenolics while flavonols glycosides hold only 11.9 %.



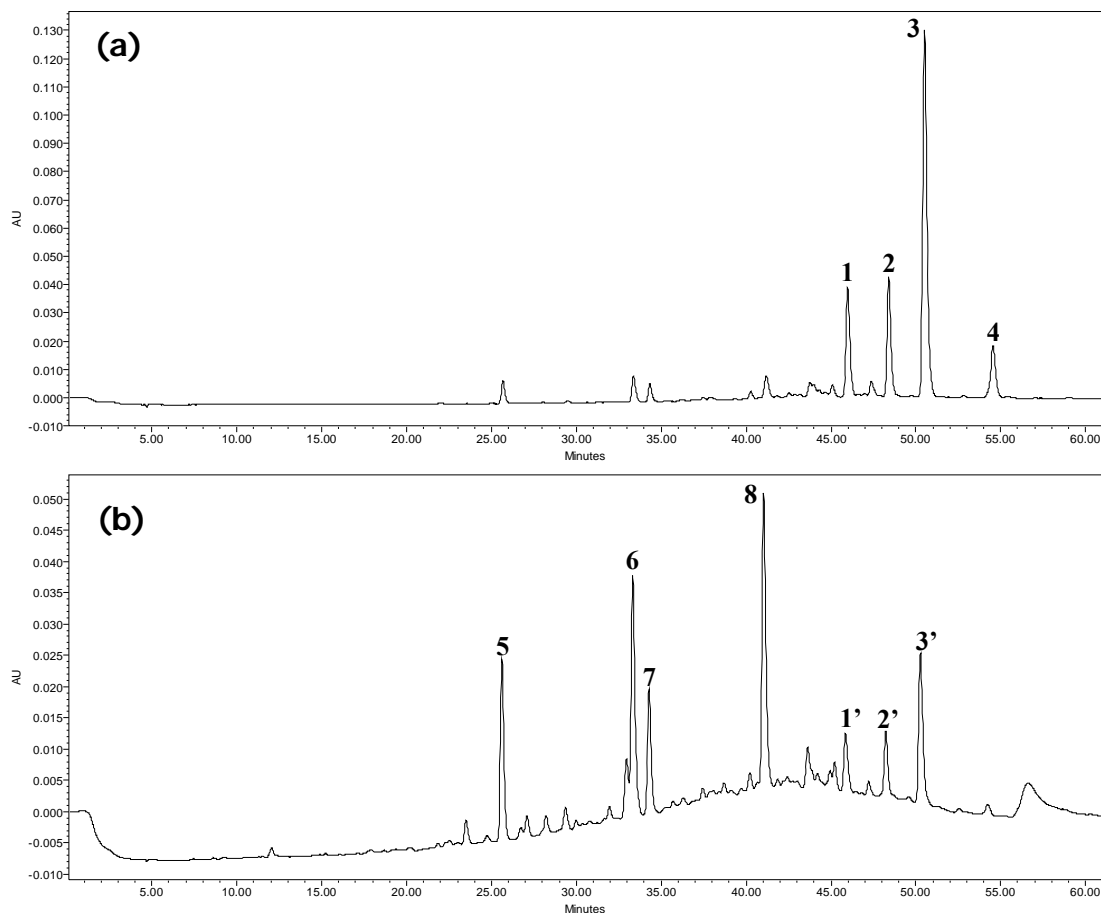
**Figure 3-13.** Chromatograms of polyphenolics and caffeine present in yaupon holly infusion separated by the first fraction method. (a) hydrocinnamic acids and caffeine in fraction 1 determined at 280 nm and (b) other flavonols in fraction 2 at 360 nm by HPLC-PDA analysis. Peak assignment: **Fraction 1 (a)**. 1. 3-caffeoylquinic acid, 2. 5-caffeoylquinic acid, 3. caffeine, 4. 4-caffeoylquinic acid, 5. 3, 5-dicaffeoylquinic acid, 6. 3, 5-caffeoylquinic acid, 7. 4, 5-caffeoylquinic acid. **Fraction 2 (b)**. 8. Kaempferol 3-rhamnoside, 9. Quercetin 3-rutinoside (rutin), 10. Kaempferol 3-rutinoside, 11. Isorhamnetin 3-rutinoside.



**Figure 3-14.** Concentration (mg/L) of hydrocinnamic acids and caffeine in fraction 1 and flavonol glycosides in fraction 2. For the number assignment see Figure 3-13. The concentration of flavonols from 8 to 11 in fraction 2 was expressed as rutin equivalent (RE).

***Identification and quantification by HPLC-PDA analysis of phenolic fractions separated from mamaki infusion***

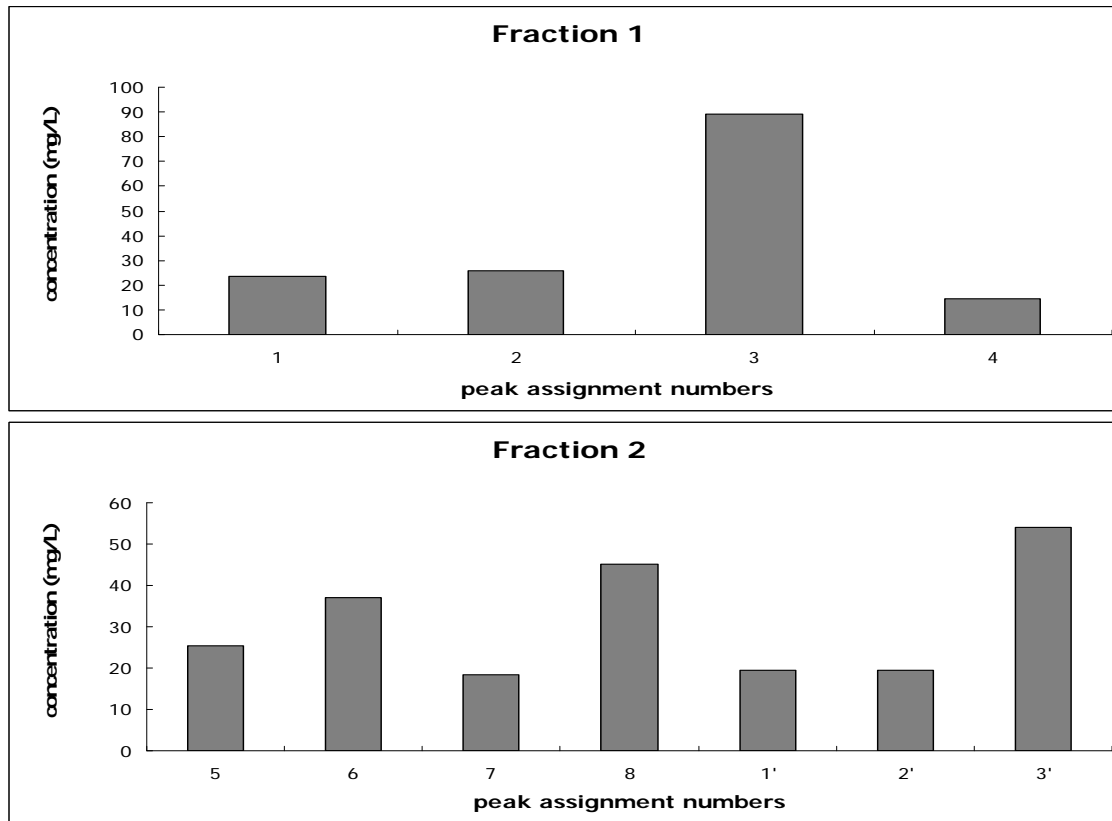
Recently polyphenolics present in mamaki were identified using LC/MS analysis by Kartika et al. (146) who reported the presence of chlorogenic acid, rutin and (+)-catechin, but (+)-catechin was not found in the present study (**Figure 3-15**). As observed in polyphenolic identification of yaupon holly infusion, chlorogenic acid and its isomers



**Figure 3-15.** Chromatograms of polyphenolics present in mamaki infusion separated by the second fraction method. (a) flavonols in fraction 1 determined at 280 nm and (b) hydrocinnamic acids and unfractionated flavonols (1', 2', and 3') in fraction 2 at 360 nm by HPLC-PDA analysis. Peak assignment: **Fraction 1 (a)**. 1. Apigenin 7-glucoside, 2. Apigenin 7-glucoside, 3. Quercetion 3-rutinoside (rutin), 4. Kaempferol 3-rutinoside, **Fraction 2 (b)**. 5. 3-caffeoylquinic acid, 6. 5-*O*-caffeoylquinic acid, 7. 4-*O*-caffeoylquinic acid, 8. cinnamic acid derivative. **Unfractionated flavonols**. 1'. Apigenin 7-glucoside, 2'. Apigenin 7-glucoside, 3'. Quercetion 3-rutinoside (rutin).

were detected in fraction 2 but the concentration of 3, 5, and 4-caffeoylquinic acids was lower by 23, 48, and 29 %, respectively in mamaki infusion. The fractionation of mamaki infusion was experimentally determined after applying two different fraction methods as described in **Figure 3-1, 2**. The method described in **Figure 3-2** showed higher efficiency in separating two phenolic groups of mamaki infusion and the extraction of chlorogenic acid and its isomers was higher by 71 % when the proposed fraction method was used.

In fraction 1, four flavonol glycosides (two apigenin 7-glucosides, quercetin 3-rutinoside (rutin), and kaempferol 3-rutinoside) as observed in LC/ESI/MS<sup>n</sup> phenolic identification of mamaki infusion. Rutin from both fraction 1 and 2 and kaempferol 3-rutinoside, also detected in yaupon holly infusion, were present at higher concentrations by 78.6 and 65.5 %, respectively (**Figure 3-16**). In mamaki infusion, hydrocinnamic acids such as caffeoylquinic acids took account of 33.9 % of total polyphenolics while flavonols glycosides hold 66.1 % as predominant phenolic compounds.

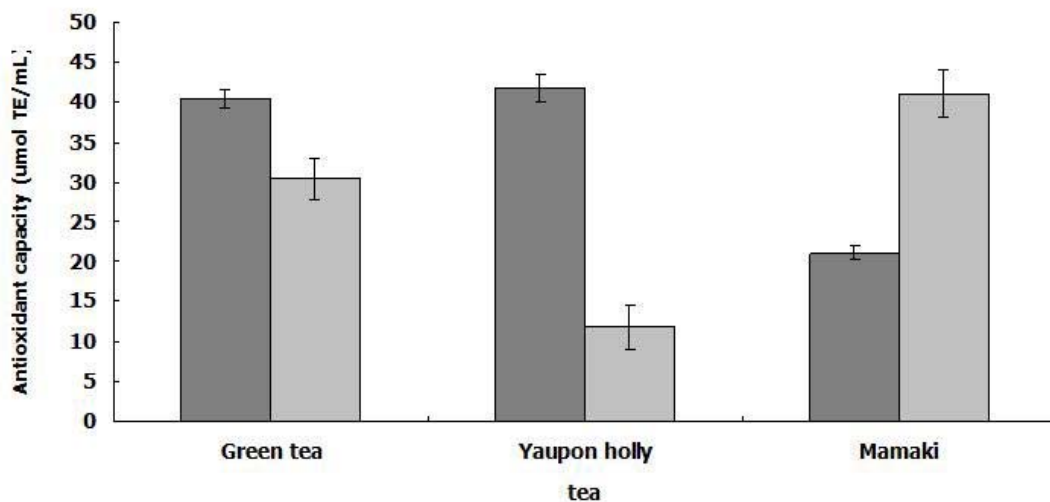


**Figure 3-16.** Concentration (mg/L) of flavonols in fraction 1 and hydrocinnamic acids and unfractionated flavonols in fraction 2 from mamaki infusion. Peak number assignment is shown Figure 3-15. The concentration of flavonols from 1 to 4 in fraction 1 and 1' to 3' in fraction 2 was expressed as rutin equivalent (RE).

***Antioxidant capacity in two different fractions from green tea, yaupon holly, and mamaki infusions***

Antioxidant capacity mostly affected by each tea's polyphenolic concentration was determined by ORAC assay. In green tea, flavan 3-ols containing fraction 1 showed higher antioxidant capacity by 25 % than flavonols containing fraction 2 (**Figure 3-17**). Caffeine, a white crystalline xanthine alkaloid, is known as an antioxidant (42, 147) and

may contribute to the total antioxidant capacity of fraction 1. The antioxidant capacity of caffeine to scavenge hydroxyl radicals was confirmed by ESR (electron spin resonance) spin trapping assay (147) and was shown to come from caffeine reacting with a hydroxyl radical forming an oxygen centered radical with a reaction rate constant close to formate, which is a known hydroxyl radical scavenger (148). However, the antioxidant capacity of caffeine was lower than all the flavan-3-ols in green tea in the order of EGCG > EGC > ECG > gallic acid > EC > catechin > caffeine and the antioxidant capacity of EGCG was 28-fold higher than that of caffeine when their peroxy radical scavenging capacity were determined by ORAC assay (149). Therefore, the antioxidant capacity of fraction 1 was mostly from flavan-3-ols and it was higher than that of fraction 2. The average antioxidant capacity of the 4 most prevalent flavan-3-ols (EGCG, EGC, ECG, and EC) was 16 % higher than that of the 3 most prevalent flavonols such as myricetin, quercetin, and kaempferol when determined using VCEAC (vitamin C equivalent antioxidant capacity) assay by Kim and Lee, (150). However, the antioxidant capacity of their glycosides, which are the most prevalent, were significantly lower than their aglycones due to the absence of a free 3-OH group in the C ring (151) and/or steric hindrance from sugar substituents (152). For example, rutin showed a significantly lower antioxidant capacity compared to its aglycone quercetin (28.7 % of quercetin) and this is because the bulky sugar group attached to quercetin causes the coplanarity loss of the B ring, which inhibits rutin from using its full delocalization potential and induces lower radical scavenging capacity compared to its aglycone (151, 153).



**Figure 3-17.** Antioxidant capacity ( $\mu\text{mol TE/mL}$ ) from fraction 1 and 2 of green tea, yaupon holly, and mamaki infusions determined by ORAC assay. Abbreviation used: TE. Trolox equivalent.

In yaupon holly, hydrocinnamic acids containing fraction 1 showed higher antioxidant capacity by 71.9 % than that of fraction 2 which contains four flavonols. Among cinnamic acid derivatives, chlorogenic acid showed relatively low antioxidant capacity and showed hierarchy of antioxidant potential as: *p*-coumaric acid > sinapic acid > ferulic acid > rosmarinic acid > hydrocaffeic acid > caffeic acid > *o*-coumaric acid > *m*-coumaric acid > chlorogenic acid > cinnamic acid (151). However, even the highest antioxidant among cinnamic acid derivatives, *p*-coumaric acid, showed lower antioxidant capacity by 23.5 % compared to that of flavan-3-ols. Furthermore, chlorogenic acid has lowest antioxidant capacity among all hydrocinnamic acid derivatives because esterification of quinic acid and the carboxyl group of caffeic acid in



forming chlorogenic acid reduces antioxidant capacity and the antioxidant capacity of chlorogenic acid was 14 % higher than that of rutin (151).

The antioxidant capacity of each tea's major fraction (fraction containing its predominant compounds that is fraction 1) was not significantly different from each other. In mamaki, rutin was present at the highest concentration among all the phenolic compounds found in the infusion. The antioxidant capacity was higher in fraction 2 by 48.7 % than in fraction 1. Since the polyphenolic compounds in mamaki infusion were partially separated, the antioxidant capacity in fraction 2 was influenced by flavonol glycosides. From the result of this fractionation, it was found that most of antioxidant capacity comes from chlorogenic acid and its mono- and di-isomers in yaupon infusions while flavonol glycosides were the major influence to total antioxidant capacity of mamaki infusion.

## CHAPTER IV

### IMPACT OF TEA PROCESSING METHODS ON PHYTOCHEMICAL STABILITY AND QUALITY OF THREE TEA VARIETIES DURING STORAGE

#### **Introduction**

Tea is second only to water as the most consumed beverage in the world due to the fact that it has not only health-promoting properties but also an appealing flavor and thirst quenching properties popular with consumers. Polyphenolics such as tea catechins and amino acid such as theanine (the major component of amino acid in green tea) (154), play a major role in taste, overall flavor, and color of green tea. Depending on the manufacturing process, the tea variety, and the growing location these organoleptic properties of tea may vary and potentially impact the processing and shelf-life of ready-to-drink (RTD) teas.

In the commercial tea market several popular teas such as green tea and black tea are readily available and have been extensively studied in effort to identify those compounds that contribute to tea quality and potential health-promoting properties. However, botanical teas are a growing market segment in the areas of bagged, loose leaf, and water-infused solutions and only a few have been extensively studied for their chemical composition and potential for augmenting human health. Among the many botanical teas currently available, yaupon holly (*Ilex vomitoria*), and mamaki (*Pipturus albidus*) are regarded as marketable species native to the US with only limited information available on their composition and stability during storage.

Currently, several processing methods are being used to improve overall quality of RTD teas. Heat pasteurization is applied to prevent microbial growth during storage (65) and ascorbic acid (vitamin C) is added to RTD teas to enhance color and stability (59, 60), and pH of the tea infusion is lowered to improve storage stability (9). However, the effects on phytochemical stability by these processing methods have focused only on some popular teas such as green tea and black tea, whereas no information regarding to ascorbic acid addition, pH adjustment, and heat treatment on phytochemical stability is available on other botanical teas. Therefore these studies investigated the phytochemical stability influenced by various processing methods of two non-fermented botanical teas (yaupon holly and mamaki) as compared to the more familiar non-fermented green tea. This study provides fundamental information regarding to storage stability in relation to processing methods in terms of phytochemical stability.

## **Materials and Methods**

### ***Tea preparation and processing***

The tea leaves of green tea, yaupon holly, and mamaki were prepared as described in chapter III. The prepared tea infusion (1:100, w/v) was stayed at room temperature until the temperature of each infusion reached to 25 °C prior to processing. To examine the effect of ascorbic acid addition to botanical teas, three different concentrations of ascorbic acid (30, 60, and 90 mg) were fortified to 240 mL of each tea infusion with continuous stirring for 10 min while untreated group of tea infusion was

kept as control (0 mg). The amount of ascorbic acid used in the present study was based on recommended dietary allowance of vitamin C from the institute of medicine of the US national academy of sciences. To explore the phytochemical changes under different pH environment during storage, pH of each tea infusion was adjusted to 3, 4 and 5 using either 0.3 M hydrochloric acid to lower pH or 0.3 M sodium hydroxide solution to elevate pH. The amount of solutions to change pH of tea infusions was recorded and the dilution factor was taken account into calculations to keep up the same concentration with the tea infusions used in other treatments. Variable heat treatments were applied to investigate the effects of different pasteurization times on the phytochemical stability of tea infusions. Each tea infusion (100 mL each) was transferred to lidded aluminum container and watertightened using metal foil tape. The infusion held in a water bath for 0, 30, 60, and 120 sec at 95 °C. This heat treatment was repeated three times. During heating, the aluminum containers containing tea infusions were submerged at least about 10 cm below the water surface. After cooling down to 25 °C, the heated infusions were transferred to three individual PET bottles (100 mL each) which was previously sterilized as described in chapter III.

Each triplicated tea infusion was stored at room temperature (25 °C) in individual PET bottles covered with aluminum foil to exclude the effect from light. A small amount of tea infusions were pulled out biweekly for the first 12 weeks and monthly for the last 12 weeks. Samples were taken from the PET bottles for chemical analysis at each time point through a rubber septum using a sterilized needle to prevent microbial contamination or air intake to the containers.

### ***Chemical analyses***

Identification and quantification of individual polyphenolics in green tea, yaupon holly, and mamaki infusions were determined using reversed phase HPLC analysis as described in chapter III. Antioxidant capacity was measured by ORAC assay by the methods illustrated in chapter III. Total soluble phenolics (TSP) concentration (a measure of total metal ion reducing capacity) including contributions from ascorbic acid was measured using Folin-Ciocalteu assay (155). For each tea infusion, 50  $\mu$ L of tea infusion was mixed with 1 ml of 0.25 N Folin-Ciocalteu reagent in a test tube and thoroughly mixed by vortex for 30 sec. After 3 minutes reaction time, 1 ml of 1N sodium carbonate was added to form a water-soluble chromophore for a distinguishable blue color. After standing for 7 minutes, all the extractions were transferred to a Spectramax 96-well and absorbance (Softmax PRO, Sunnyvale, CA) was read at 726 nm after 2 hours. TSP was quantified in mg/L Gallic acid equivalents (GAE).

L-ascorbic acid was determined using reverse phase HPLC as previously described by Gökmen et al. (156). Briefly, samples for analysis were prepared by diluting tea infusion 3-fold with a 3 % citric acid solution, filtered through a 0.45  $\mu$ m Whatman syringe filter and injected into a Waters Alliance 2695 HPLC system equipped with a Waters 996 PDA detector (Waters Corp, Milford, MA). Separation was made on a Dionex 250 x 4.6 mm Acclaim 120-C<sub>18</sub> column with detection at 254nm with an isocratic flow at a rate of 1 mL/min. 0.2 M potassium phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>) at pH 2.4 adjusted with *o*-phosphoric acid was used as a mobile phase. L-ascorbic acid was identified based on the

similar spectra and retention time by comparing with an external standard of L-ascorbic acid purchased from Sigma Chemical Co. (St. Louis, MO).

### ***Statistical analysis***

Data was analyzed as a 3 x 4 x 10 full factorial analysis including three treatments (ascorbic acid fortification, heat treatment, and pH adjustment) with four different processing methods at 10 sampling times for each tea. Data represent the mean triplicate analysis using ANOVA (analysis of variance) with JMP 5 statistical software (110). Mean separation was conducted using the LSD test ( $P < 0.05$ ).

## **Results and Discussion**

### ***Influence of ascorbic acid addition on the stability of polyphenolics and caffeine in green tea infusion during storage***

Ascorbic acid is fortified to RTD teas by adding ascorbic acid containing citrus extracts to mimic citrus flavor or directly adding food grade ascorbic acid and to improve overall stability during storage and to enhance color. In the 1980s, the recommended dietary allowance for ascorbic acid was 60 mg/day for adult males as proposed by the food and nutrition board and national research council in 1980 and reconfirmed in 1989 (157). However, several studies claimed in the mid 1990s through present that the amount (60 mg) was not high enough to reduce the risk of chronic diseases such as cancer and cardiovascular disease through antioxidant mechanism

(157–159). The authors recommended that an intake of 90-100 mg/day is required to decrease the risk of chronic disease of adult males and females (158). Thus, in the present study, two concentrations of ascorbic acid based on old and new recommendations (60 and 90 mg/240 mL) were used as comparing with smaller amount (30 mg/240 mL) to compare their *in vitro* effect on phytochemical stability of two botanical teas comparing to green tea. The original ascorbic acid content in each tea was displayed in **Table 4-1**.

**Table 4-1.** Initial ascorbic acid concentration in green tea, yaupon holly, and mamaki infusion.

Tea	Green tea	Yaupon holly	Mamaki
<b>Concentration</b>	211.95 ± 30.80 <sup>a</sup>	200.15 ± 15.97	186.62 ± 26.76

<sup>a</sup>. Data was expressed as mg/L ± standard deviation of n=9.

Eleven phenolic compounds along with caffeine were identified by comparison of retention time and similarity of spectra to authentic standards of gallic acid, EC, ECG, EGCG, EGC, GCG, quercetin, rutin, kaempferol, and caffeine. The initial content of these identified compounds were illustrated in **Table 4-2**.

**Table 4-2.** Initial concentrations of polyphenolics and caffeine present in green tea infusion determined by HPLC analysis.

No.	Compounds	Initial content (mg/L)
1	Gallic acid	6.06 ± 0.56 <sup>a</sup>
2	(-)-epigallocatechin	204.64 ± 6.83
3	Caffeine	460.79 ± 13.58
4	(-)-epigallocatechin gallate	882.78 ± 30.38
5	(-)-epicatechin	192.23 ± 5.42
6	(-)-gallocatechin gallate	71.18 ± 2.49
7	(-)-epicatechin gallate	207.62 ± 11.51
8	Myricetin 3-glycoside	19.82 ± 2.36 <sup>b</sup>
9	Quercetin 3-rutinoside (rutin)	72.28 ± 2.28
10	Quercetin 3-glycoside	26.16 ± 2.37
11	Kaempferol 3-glycoside	13.69 ± 1.02
12	Kaempferol 3-rutinoside	19.26 ± 0.41

<sup>a</sup>. Data are expressed as mean concentration (mg/L) ± standard deviation of n=9.

<sup>b</sup>. The values between no.8 and 12 were expressed as quercetin equivalent.

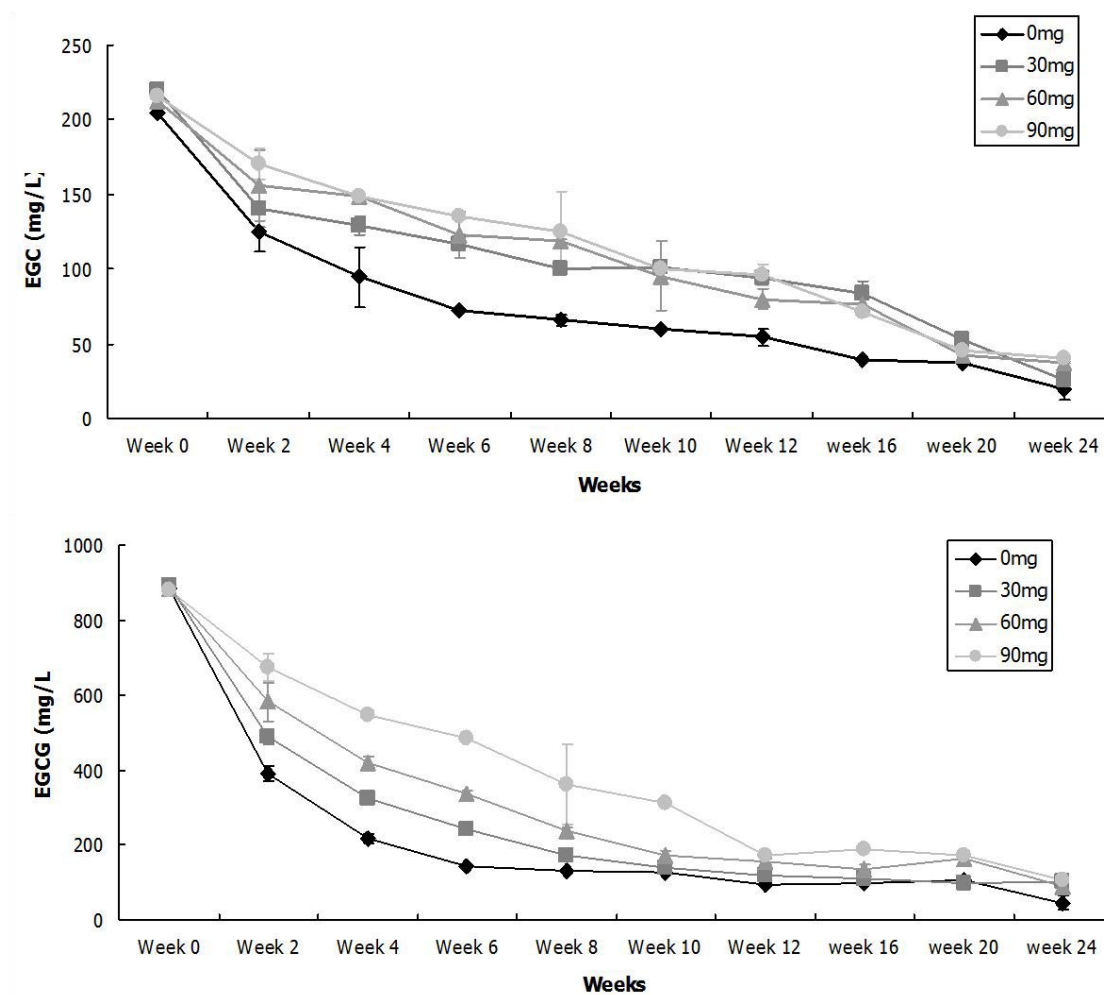
Caffeine was initially found at the concentration of 460.79 mg/L and small reduction (15 %) was observed in control during 24 week storage showing no significant difference with ascorbic acid fortified tea infusions (data not shown). Caffeine is stable in under ordinary conditions (no extreme pH and temperature) during storage due to its stable crystalline structure. According to Tanaka and Kouno, (160), caffeine in black tea was not significantly influenced by oxidation treatment while tea catechins were



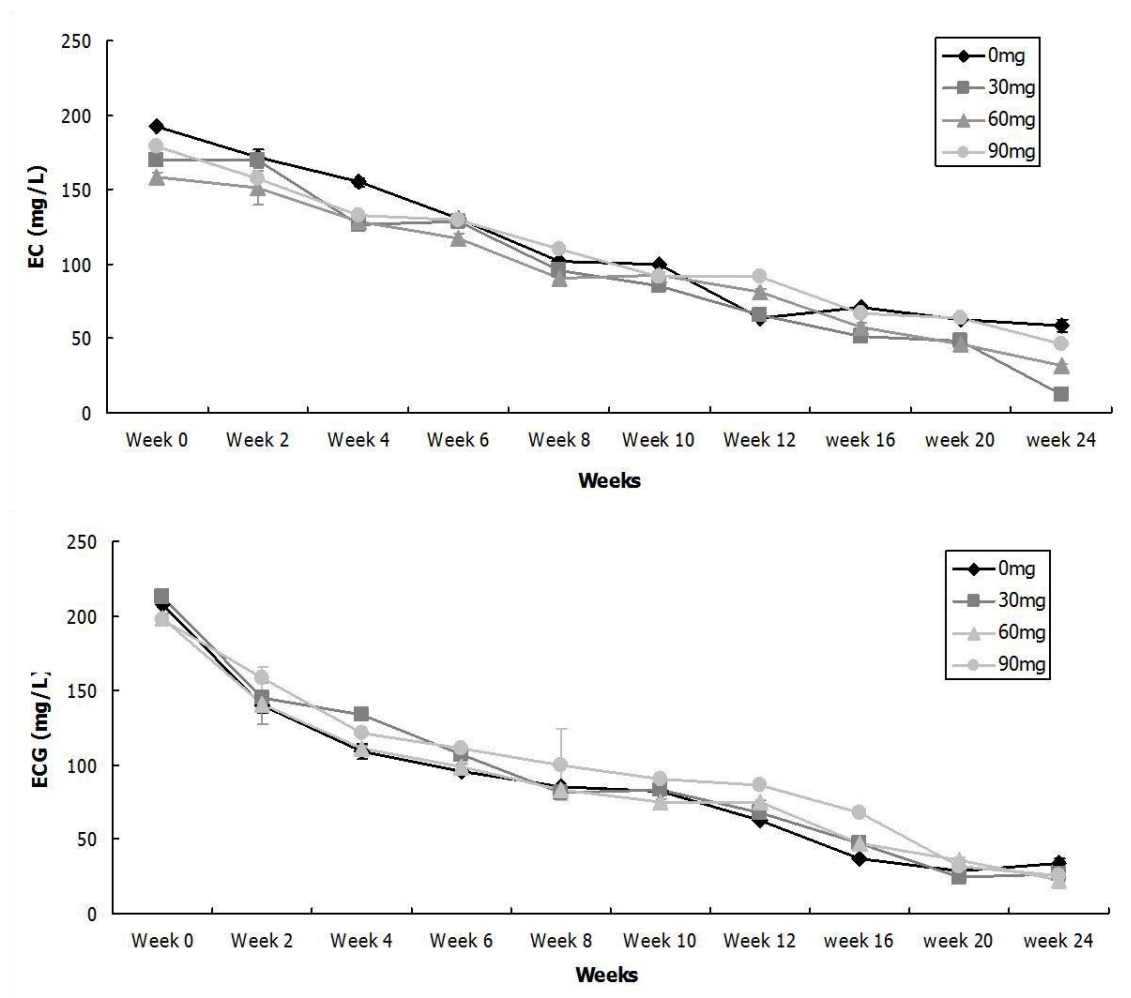
degraded. Likewise, in the present study caffeine showed significantly lower reduction compared to tea catechins.

Ascorbic acid addition was effective to reduce the rate of degradation of gallo-flavanols such as EGC and EGCG which contain galloyl group on their B rings (161). EGC concentration was higher at all the ascorbic acid fortified infusions than control during 20 week storage (**Figure 4-1**). Until Week 10, EGC and EGCG were more retained at the sample with 60 and 90 mg of ascorbic acid addition. During 12 week storage, 90 mg of ascorbic acid addition was most effective in retaining EGCG. No significant difference between control and samples with 30 and 60 mg of ascorbic acid between Week 10 and 20.

Catechol-flavanols (ECG and EC) in green tea infusion were less influenced by ascorbic acid addition compared to gallo-flavanols during 24 week storage. EC concentration of the control was higher until 4 weeks of storage by 19, 17, and 14 % than 30, 60, and 90 mg, respectively but after Week 4, no difference was observed until Week 20 (**Figure 4-2**). Likewise, no difference in ECG was observed between control and lower ascorbic acid addition (30 and 60 mg) throughout the storage while higher stabilizing effect was found when 90 mg of ascorbic acid was added to the infusion between Week 6 and 20.



**Figure 4-1.** Changes in EGC (top) and EGCG (bottom) (mg/L) affected by 30, 60, and 90 mg of ascorbic acid addition to 240 mL of green tea infusion during 24 week storage. Error bars represent standard error of the mean (n=3). Abbreviations used: EGC. (-)-epigallocatechin, EGCG. (-)-epigallocatechin gallate.



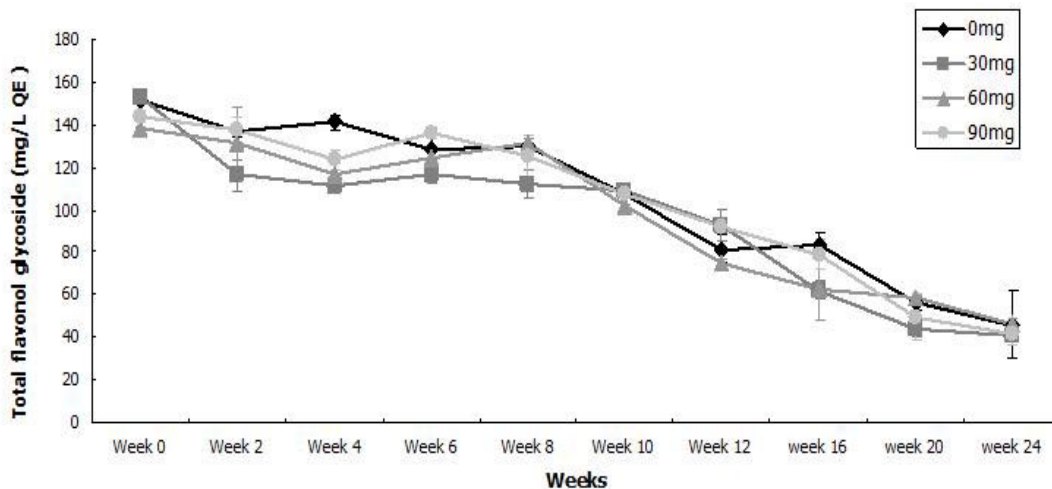
**Figure 4-2.** Changes in EC (top) and ECG (bottom) (mg/L) affected by 30, 60, and 90 mg of ascorbic acid addition to 240 mL of green tea infusion during 24 week storage. Error bars represent standard error of the mean (n=3). Abbreviations used: EC. (-)-epicatechin, ECG. (-)-epicatechin gallate.

Gallo-flavanol, EGCG and EGC, which accounted for about 70 % of the total flavan 3-ols in green tea, are known to be more unstable to oxidation than catechol-flavanol. Since those are decomposed at higher rate than catechol-flavanols during storage, the protective effect by adding ascorbic acid was higher in gallo-flavanols.

During the process of oxidation, polyphenolic compounds can lose a hydrogen atom from one of their many hydroxyl groups to form a semiquinone radical with an unpaired electron on the oxygen atom (161). The radicals form more freely on the B ring contains three hydroxyl groups such as EGC and EGCG than others have only one or two hydroxyl groups on their B rings (161, 162). In green tea products in PET bottles,  $H_2O_2$  (hydrogen peroxide) was generated from polyphenolics including tea catechins with air contact at 25 °C and it was produced during storage due to the autooxidation process of polyphenolics although there was no more air contact (60, 163, 164). The quick production of  $H_2O_2$  was because polyphenolics were readily oxidized with dioxygen ( $O_2$ ) under the presence of transition metals resulting in the formation of the corresponding quinones (163). This indicates that tea handling from brewing to bottling must be done under oxygen free environment to prevent  $H_2O_2$  production in green tea product since only a short contact of tea infusion with air induces  $H_2O_2$  production. In the present study, it was concluded that flavan 3-ols in green tea infusions were reduced by oxidative degradation due to the presence dissolved oxygen and reaction of  $H_2O_2$  in green tea infusion by air contact during handing and using air transferable PET bottles. Even though hydrogen peroxide is not a free radical itself, it is classified as reactive oxygen species (ROS) due to its oxidizing ability from its pro-oxidant action (165). Also, the presence of  $H_2O_2$  is very harmful in biological system since  $H_2O_2$  catalyze the production of superoxide ( $O_2^{\cdot-}$ ) and hydroxyl radical ( $OH\cdot$ ) under the presence of transition metals, which is the most reactive free radical (165). The polyphenol degradation by the attack of produced  $H_2O_2$  during storage is a problem for the

polyphenol-rich product including green tea since considerable amount of  $H_2O_2$  is generated with high concentration of polyphenolics (166). As a result of the present study, it was proved that antioxidant ascorbic acid was effective to reduce the rate of oxidative degradation during storage by the stabilizing reaction of ascorbic acid due to the quenching produced radicals in green tea infusion.

Total flavonol glycoside from 6 flavonol glycosides (myricetin-3-glycoside, quercetin-3-rutinoside, quercetin-3-glycoside, kaempferol-3-glycoside, and kaempferol-3-rutinoside) decreased during 24 week storage and no effect from ascorbic acid addition was observed as observed in catechol-flavanol changes. Total flavonol glycoside was reduced by 70 % at control and no difference was observed at the end of 24 week storage regardless of the amount of ascorbic acid added to the infusion (**Figure 4-3**). The lack of an effect of ascorbic acid addition on flavonol glycosides stability may be due to the lower reactivity of glycosides compared to the aglycones (151, 167) and/or only 1 or 2 hydroxyl group(s) on the B ring of identified flavonol glycoside in green tea as observed in catechol-flavanols changes.

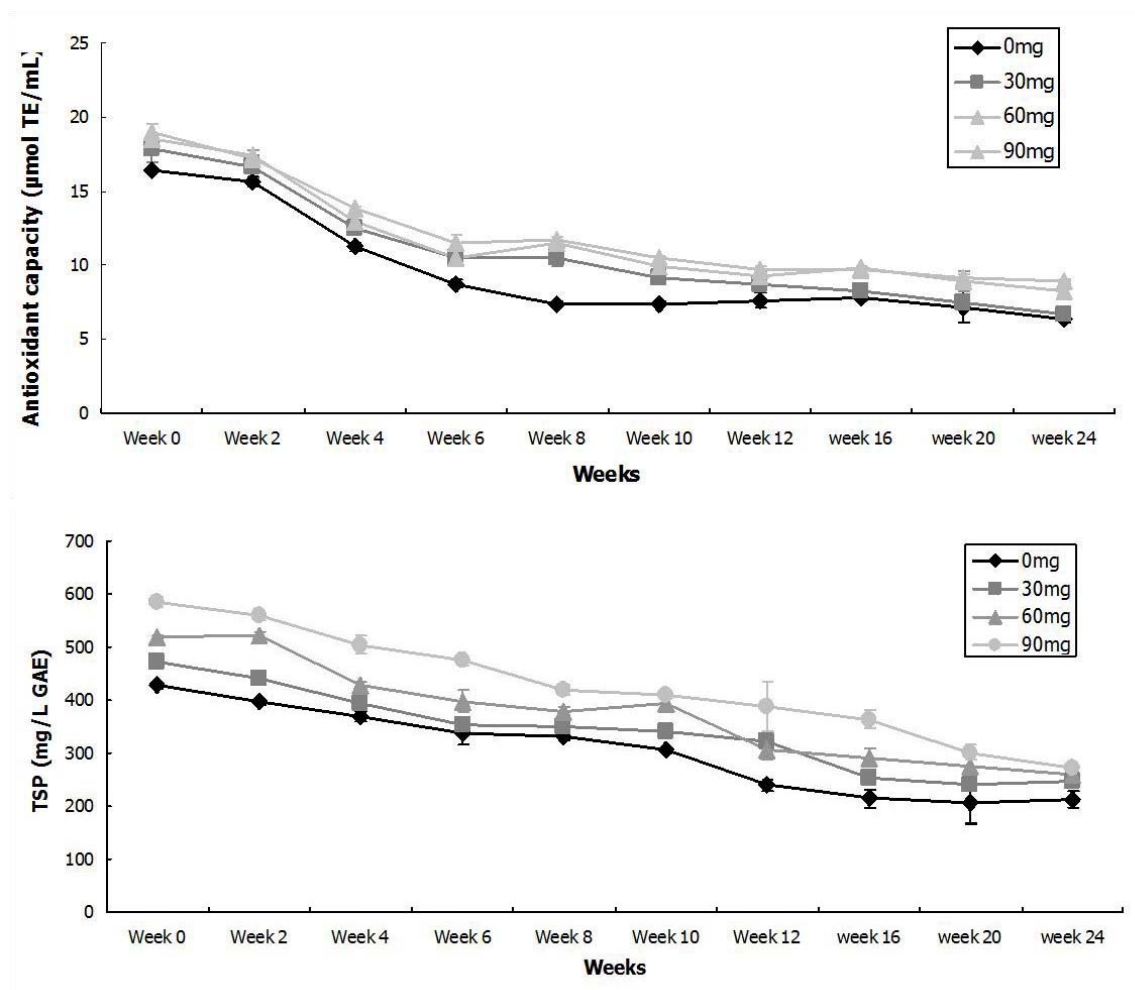


**Figure 4-3.** Change in total flavonol glycoside (mg/L) from myricetin 3-glycoside, quercetin 3-rutinoside, quercetin 3-glycoside, kaempferol 3-glycoside, and kaempferol 3-rutinoside affected by 30, 60, and 90 mg of ascorbic acid addition to 240 mL of green tea infusion during 24 week storage. Data was expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

***Influence of ascorbic acid addition on the changes in antioxidant capacity and total soluble phenolics in green tea infusion during storage***

Antioxidant capacity of green tea infusion was higher retained in ascorbic acid added green tea infusions during 24 week storage. As the result of ascorbic acid addition into the tea infusion, antioxidant capacity at 30, 60, and 90 mg initially increased by 9, 12, and 14 %, respectively compared to control (16.43  $\mu$ mol Trolox equivalent/mL) (**Figure 4-4**). Antioxidant capacity at 90 mg was higher during 16 week storage than control but no difference was observed between week 16 and 20. Moreover, higher concentration of ascorbic acid (90 mg) was more effective to maintain antioxidant

capacity in green tea infusion than lower concentrations (30 and 60 mg) for the first 10 weeks as observed in tea catechin changes.



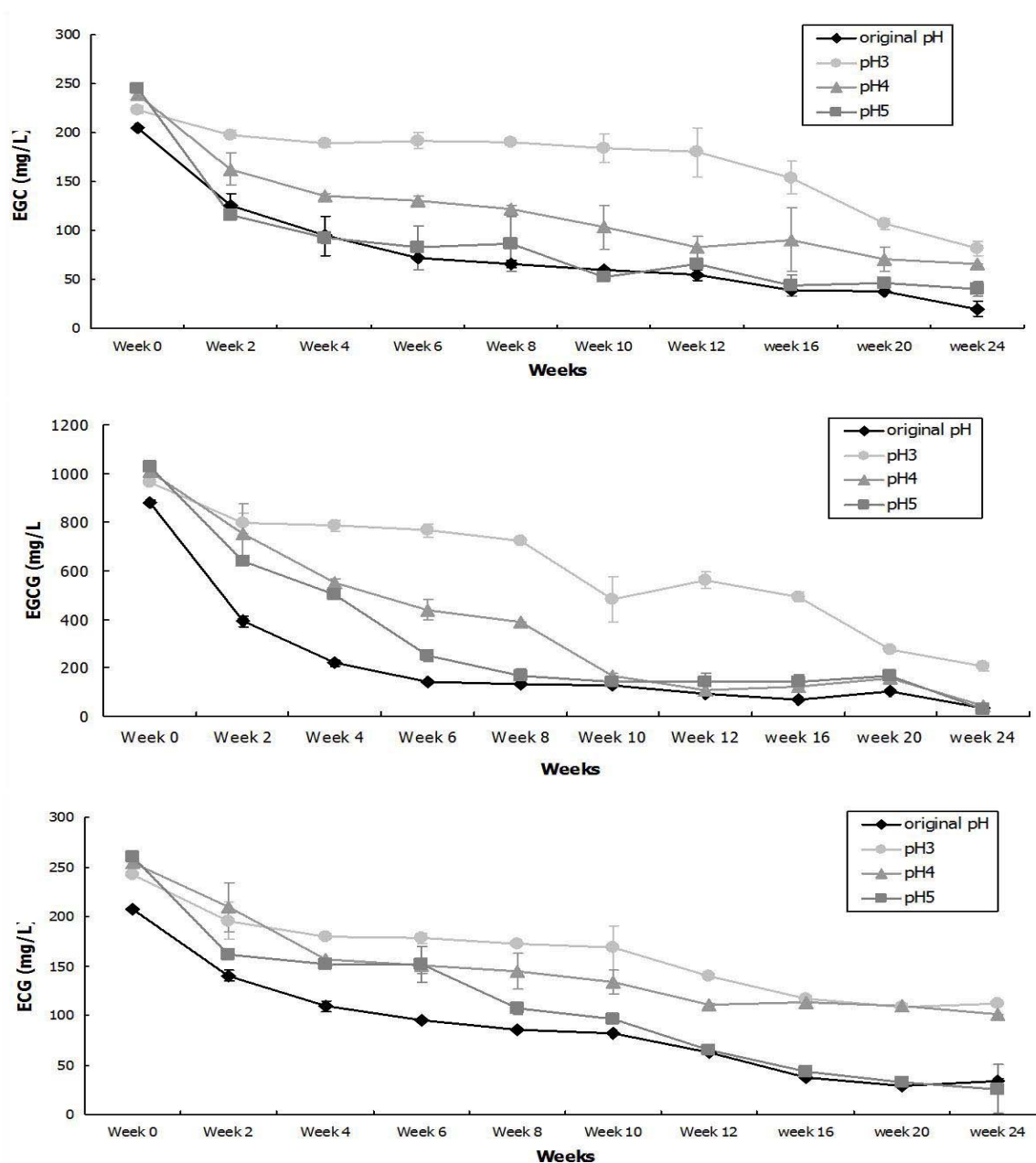
**Figure 4-4.** Changes in antioxidant capacity (top,  $\mu\text{mol TE/mL}$ ) and total soluble phenolics (TSP) (bottom,  $\text{mg/L GAE}$ ) influenced by 0, 30, 60, and 90 mg of ascorbic acid addition to 240 mL of green tea infusion during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean ( $n=3$ ).

Folin-Ciocalteu assay was conducted to measure total soluble phenolics with the purpose to quantify the total reducing capacity of phenolic compounds present in tea infusions including contribution from ascorbic acid. By adding ascorbic acid to the infusion, initial concentration of total soluble phenolics significantly increased by 10, 18 and 27 % at 30, 60, and 90 mg compared to the control (427.25 mg/L GAE). Total soluble phenolics in green tea infusions fortified with ascorbic acid was also higher retained until the end of 24 week storage and the higher addition of ascorbic acid showed higher maintenance of total soluble phenolics due to higher retained polyphenolic in green tea infusion.

***Influence of pH conversion on the stability of polyphenolics and caffeine in green tea infusion during storage***

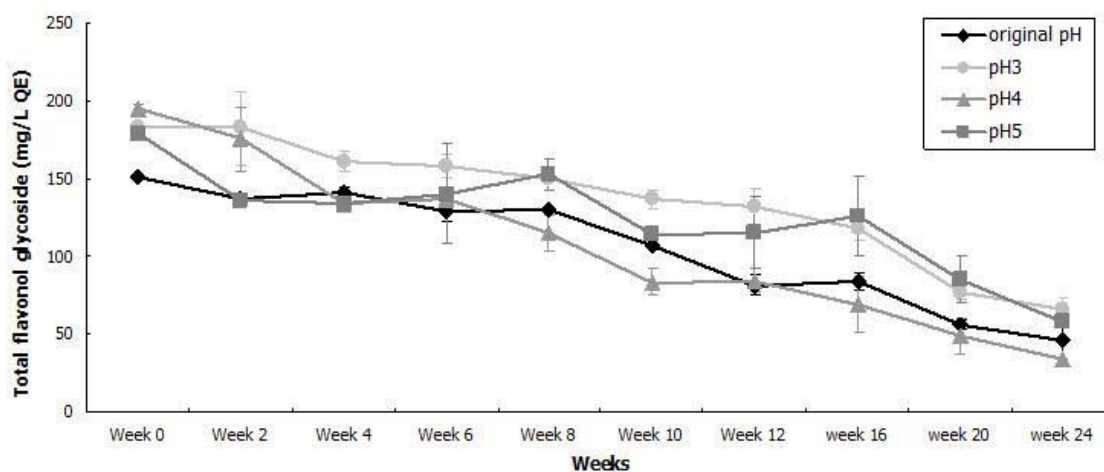
The stability of phenolic compounds is highly pH dependent and varied by structural property. Flavan-3-ols shows high storage stability under acidic conditions but are very unstable in neutral pH (33, 43, 67, 168, 169). Likewise, in the present study, both all of the tea catechins (**Figure 4-5**) and the flavonol glycosides (**Figure 4-6**) were higher retained as the pH was lower during 24 week storage. Since the pH of green tea infusion (5.09) was slightly higher than pH 5, infusions at pH 5 were not different from the control or showed only minor effect. Data was shown only for the three predominant catechins such as EGC, EGCG, and ECG and total flavonol glycoside. In green tea, changing pH affected the rate of hydrogen peroxide. According to Akagawa et al. (163),





**Figure 4-5.** Changes in concentrations of EGC, EGCG and, ECG (mg/L) (from the top to the bottom, respectively) affected by pH conversion of green tea infusion to pH 3, 4, and 5 during 24 week storage. Error bars represent standard error of the mean (n=3). Abbreviation used: EGC. (-)-epigallocatechin, EGCG. (-)-epigallocatechin gallate, ECG. (-)-epicatechin gallate.

when pH of green tea infusion was lowered, the production rate of hydrogen peroxide and superoxide was significantly reduced. Moreover, ascorbic acid is more stable at lower pH, indicating the protective effect on polyphenolics is higher at lower pH (170). These explain why polyphenolics in green tea was higher retained at lower pH.

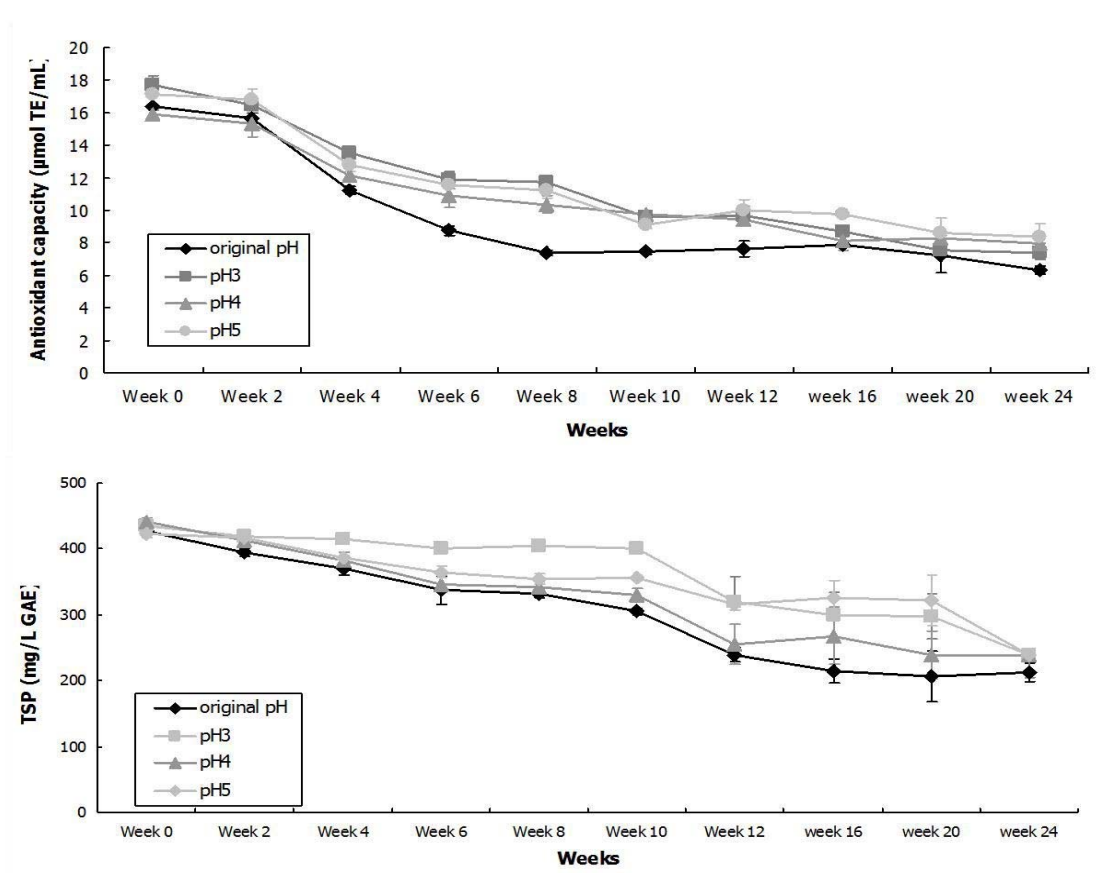


**Figure 4-6.** Change in total flavonol glycoside (mg/L) from myricetin-3-glycoside, quercetin-3-rutinoside, quercetin-3-glycoside, kaempferol-3-glycoside, and kaempferol-3-rutinoside affected by pH conversion of green tea infusion to pH 3, 4, and 5 during 24 week storage. Data was expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

***Influence of pH conversion on the changes in antioxidant capacity and total soluble phenolics in green tea infusion during storage***

Antioxidant capacity was higher retained at lowered pH during 16 week storage and it was highest at pH 3 until the end of storage (**Figure 4-7**). Changes in the antioxidant capacity of green tea infusion were directly related to the changes in

antioxidant tea catechins that showed considerably higher stability at lower pH. Ascorbic acid was also less stable as pH increases in aqueous solution (170, 171). Lowered pH did not influence the initial concentration of ascorbic acid but it may degrade during storage due to its unstable nature at higher pH (neutral or basic). Degraded ascorbic acid concentration during storage also influenced lower antioxidant capacity at higher pH.



**Figure 4-7.** Changes in antioxidant capacity (top, μmol TE/mL) and total soluble phenolics (TSP) (bottom, mg/L GAE) influenced by pH conversion to 3, 4, and 5 in green tea infusion during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean (n=3).

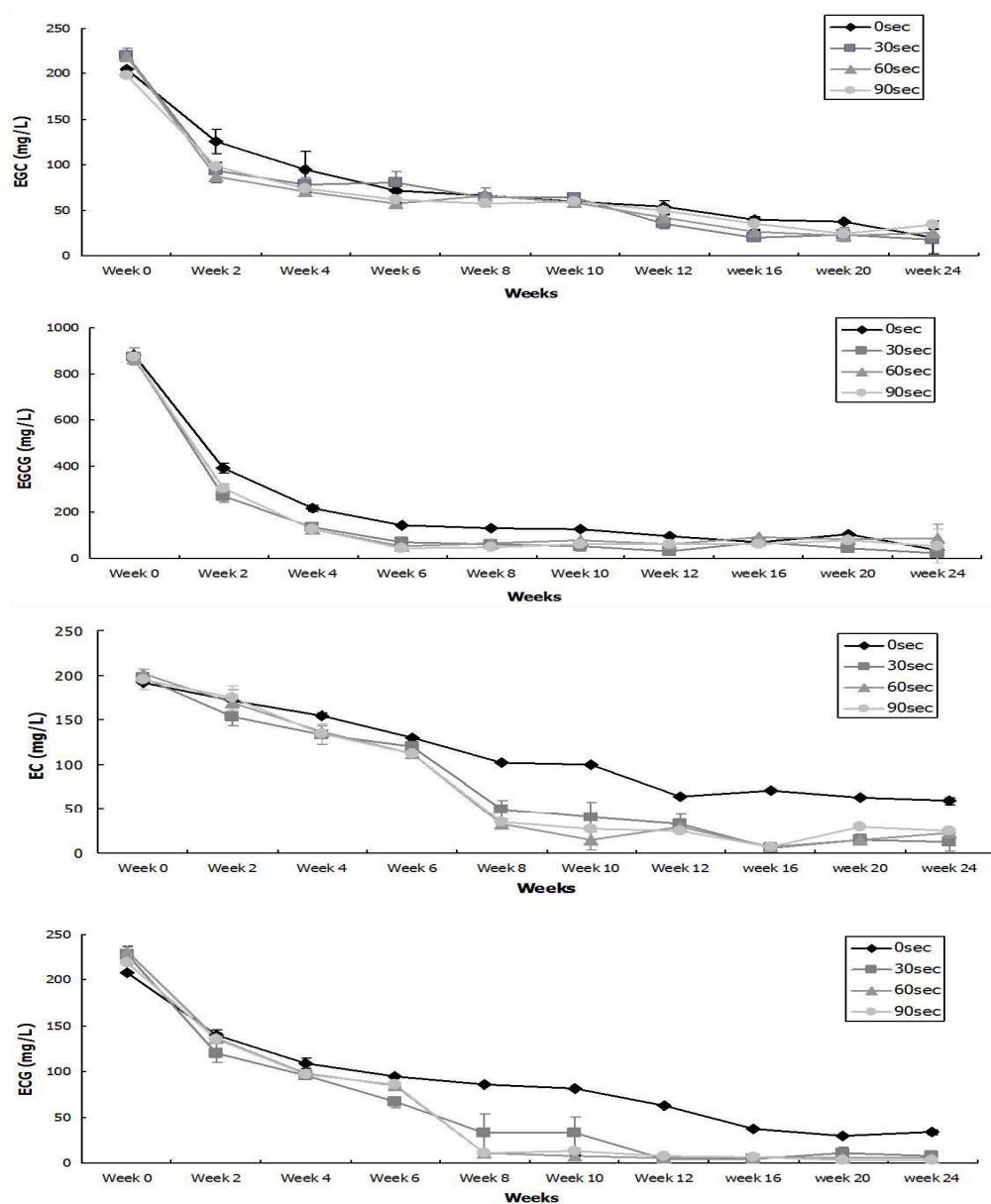
Total soluble phenolics showed the similar result with antioxidant capacity changes during storage. It was also higher at lower pH and the higher storage stability was observed as pH decreased. As observed in antioxidant capacity, this trend is due to tea catechin and ascorbic acid changes by pH conversion.

***Influence of heat processing on the stability of polyphenolics and caffeine in green tea infusion during storage***

Heat pasteurization is currently utilized to sterilize the spores of thermophilic anaerobes and aerobic spoilers present in RTD tea products. Since the heat processing is known to change the functional properties in the RTD teas such as color, flavor, and taste, minimization of those changes during manufacturing process is important to keep tea's characteristic appealing properties. So far, many studies have focused on changes in such sensorial qualities by heating (65, 66, 172), but only limited information is available on phytochemical stability of tea products during long term storage, especially on botanical teas besides teas from the *camellia sinensis* tea plant. In the present study, the impact of heat processing on phytochemical stability of various botanical teas was determined by comparing to that of green tea from *camellia sinensis* tea plant during storage.

Heat processing did not have affect caffeine stability during 24 week storage. Caffeine was reduced by 14 % at 0 sec (control) and the final concentration of caffeine (395.96 mg/L) at 0 sec was not significantly different from heat treated infusions (Data not shown). Heat processing with different durations at 95 °C did not have an effect on

the initial concentration of tea catechins of EGC, EGCG, EC, and ECG but significantly influenced the stability of these compounds during 24 week storage. ECG was higher retained at 0 sec than 30, 60, and 120 sec for the first 4 weeks but no difference was found between control and heat treated infusions after Week 4 and until the end of 24 week storage (**Figure 4-8**). EGCG significantly decreased for the first 2 week storage but the degradation rate became slower until the end of storage. EGCG at 0 sec was higher retained than all heat treated infusions at 30, 60, and 120 sec during the first 12 week storage. The EGCG concentration in the infusions at 30, 60, and 90 sec was not different throughout 24 week storage. The concentration of EC at 0 sec showed a higher stability than those at 30, 60, and 120 sec especially after Week 6 even though EC was higher retained after Week 4. As observed in previous tea catechin changes, the storage stability of ECG was also affected by heat treatment. No initial difference was observed with all the tea infusions regardless of heat treatment but the concentration of ECG was significantly higher at 0 sec until the end of storage. The heat treatment decreased the storage stability of all tea catechins present in green tea infusion and the duration of heating was not a factor in polyphenolic stability. Kim et al. (65) suggested that mild heat pasteurization temperature (85 °C) kept green tea's characteristic color and flavor better than higher temperatures (95, 110, and 120 °C), which induced unpleasant odor by increasing indole (animal-like) and  $\alpha$ -terpineol (faint ammoniacal). Likewise, in the present study, high temperature (95 °C) also induced a negative effect by lowering



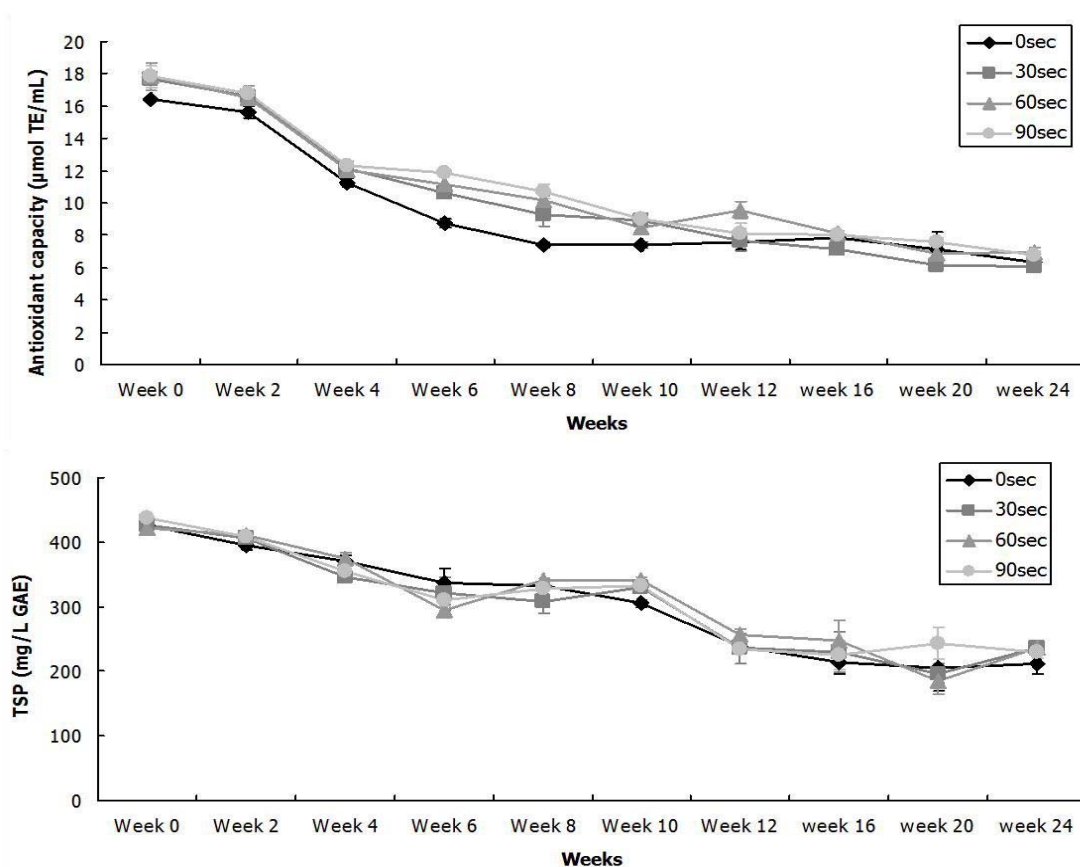
**Figure 4-8.** Changes in EGC, EGCG, EC, ECG and total flavonol glycoside from myricetin-3-glycoside, quercetin-3-rutinoside, quercetin-3-glycoside, kaempferol-3-glycoside, and kaempferol-3-rutinoside (mg/L) (from the top to the bottom, respectively) in green tea infusion affected by heat processing for 0, 30, 60, and 120 sec during 24 week storage. Total flavonol glycoside was expressed as quercetin equivalents. Error bars represent standard error of the mean (n=3). Abbreviation used: EGC. (-)-epigallocatechin, EGCG. (-)-epigallocatechin gallate, ECG. (-)-epicatechin gallate.

polyphenolic stability during storage regardless of time duration. The lower phytochemical stability of green tea may be caused by loss of ascorbic acid due to heat. Since ascorbic acid is very unstable by heat treatment (173), its protective effect on polyphenolics was reduced. Therefore, using mild heating temperature for pasteurization of green tea infusion is recommended for not only its sensorial quality but also its storage stability.

***Influence of heat treatment on the changes in antioxidant capacity and total soluble phenolics in green tea infusion during storage***

Antioxidants present in many foodstuffs are lost by heat processing such as sterilization, pasteurization, and dehydration and also during storage. In some cases, processing induces the formation of the compounds, which have novel antioxidant activity resulting in maintaining or even increasing antioxidant potential of various foods (174, 175). Antioxidant capacity of green tea infusion was higher for the first 12 weeks at all the heat treated samples and this was inversely proportional to the changes of tea catechins (**Figure 4-9**). During heat treatment, about 7, 8, and 8 % of antioxidant capacity was gained from 16.42  $\mu\text{mol}$  Trolox equivalent/mL (control) as a result of 30, 60, and 90 sec of durations, respectively. This trend was somewhat unexpected since the antioxidant capacity of green tea infusion was directly related to tea catechin changes when ascorbic acid was added and pH of the infusion was lowered. The changes in antioxidants in foods by heat processing and during storage have been studied without considering the chemical reactions, which can generate new compounds having

antioxidant capacity (175). Forced oxygenation to increase the oxidation rate induced a rapid increase of the chain breaking activity in aqueous tea infusion in the study conducted by Manzocco et al. (174). The antioxidant capacity in green tea infusion was enhanced due to the formation of other antioxidants by heat treatment and during storage even though there was loss of tea catechins during the processing and storage.



**Figure 4-9.** Changes in antioxidant capacity (top, μmol TE/mL) and total soluble phenolics (TSP) (bottom, mg/L GAE) influenced by heat treatment for 0, 30, 60, and 120 sec of green tea infusion during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean (n=3).



Total soluble phenolics was not influenced by heat processing with different durations and also no difference was observed between control and heat treated samples during 24 week storage. Since tea catechins were more retained in the untreated control than in the heat treated tea infusions during 24 week storage, no difference of total soluble phenolics between unheated and heated samples were not expected. The difference between phenolic concentration determined by HPLC and total soluble phenolics measured using Folin-Ciocalteu assay is not often correlated. The Folin-Ciocalteu assay is widely used to estimate the total amount of phenolics in many applications although it was intended for the analysis of proteins using the activity of the Folin-Ciocalteu reagent toward protein tyrosine residue which contains a phenol group (176, 177). This assay became widely used for determination of total phenols after Sigleton et al. (178) adapted this method to analyze the amount of total phenols in wine. However, it was early pointed out by Swain and Hillis, (155) who illustrated that Folin reagent is not appropriate for the analysis of phenolic containing samples because the absorbance of the reagents is affected by phenolic's structural variation and interfering metabolite is present in the samples. Additionally, the Folin-Ciocalteu reagent is not specific to phenolic compounds since it could be reduced by other non-phenolic compounds such as ascorbic acid and metal ions (177). Even though Folin-Ciocalteu assay is an easy way to estimate total soluble phenolics in various samples by measuring reducing power of phenolic compounds while providing a relatively low variance among the samples with high  $R^2$  of standard curves (179), it would be hard to say it accurately

measures the exact amount of total phenolic compounds due to the limitations and barriers described above.

***Influence of ascorbic acid addition on the stability of phenolic compounds in yaupon holly infusion during storage***

Yaupon holly has totally different phenolic profile compared to green tea which is originally from East Asian countries such as Korea, Japan, and China. A significant amount of chlorogenic acid and its isomers in yaupon holly extract was by Palumbo et al. (48) who identified and quantified the compounds by conducting HPLC analysis and also the identification was in agreement with the present study (**Table 4-3**).

**Table 4-3.** Initial concentrations of polyphenolics and caffeine present in yaupon holly infusion determined by HPLC analysis.

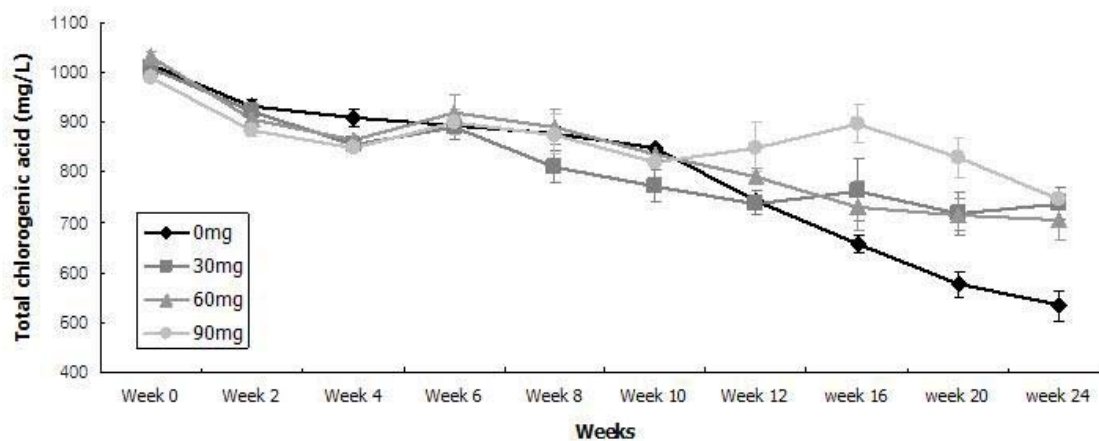
No.	Compounds	Initial content (mg/L)
1	3-caffeoylquinic acid	255.15 ± 8.57 <sup>a</sup>
2	5-caffeoylquinic acid	514.50 ± 14.59
3	4-caffeoylquinic acid	248.29 ± 8.51
4	3,4-dicaffeoylquinic acid	43.30 ± 2.24
5	3,5-dicaffeoylquinic acid	196.08 ± 14.72
6	Quercetin-3-rutinoside (rutin)	701.57 ± 30.32 <sup>b</sup>
7	Keampferol-3-rutinoside	54.00 ± 7.46

a. Data was expressed as mean concentration (mg/L) ± standard deviation of n=9.

<sup>b</sup>. The values between no.6 and 7 were expressed as quercetin equivalent.

Total chlorogenic acid (sum of 3, 4, and 5-caffeoylquinic acids) in yaupon holly infusion was more stable than tea catechins in green tea during 24 week storage. Total chlorogenic acid decreased by 48 % while total concentration of tea catechins from EC, EGC, EGCG, GCG, and ECG was reduced by 88 % during 24 week storage at 25 °C. Higher stability of chlorogenic acid and its isomers have been reviewed by many studies, which illustrated that chlorogenic acid and its isomers were stable in many kinds of beverages and plants during long-term storage with various conditions compared to tea catechins (67, 77, 180).

The storage stability of total chlorogenic acid was affected by ascorbic acid addition when yaupon holly infusions were stored longer than 10 weeks. No difference between control and ascorbic acid fortified samples during the first 10 weeks was observed except the total chlorogenic acid was lower at 30 mg in week 8 and 10 (**Figure 4-10**). At Week 12, total chlorogenic acid was higher by 7 and 13 % at 60 and 90 mg, respectively, while no difference was observed in the samples between control and 30 mg. After Week 12, total chlorogenic acid in control was reduced by 29 % during 12 week storage, whereas those in 30, 60, and 90 mg showed significantly lower reductions by 1, 7, and 13 % and became indifferent at the end of 24 week storage. Even though the overall reduction was highest at 90 mg among ascorbic acid fortified tea infusions between Week 12 and 24, total chlorogenic acid was higher at 90 mg between Week 10 and 24, indicating higher addition of ascorbic acid to yaupon holly infusions resulted in higher maintenance of total chlorogenic acid for long term storage.

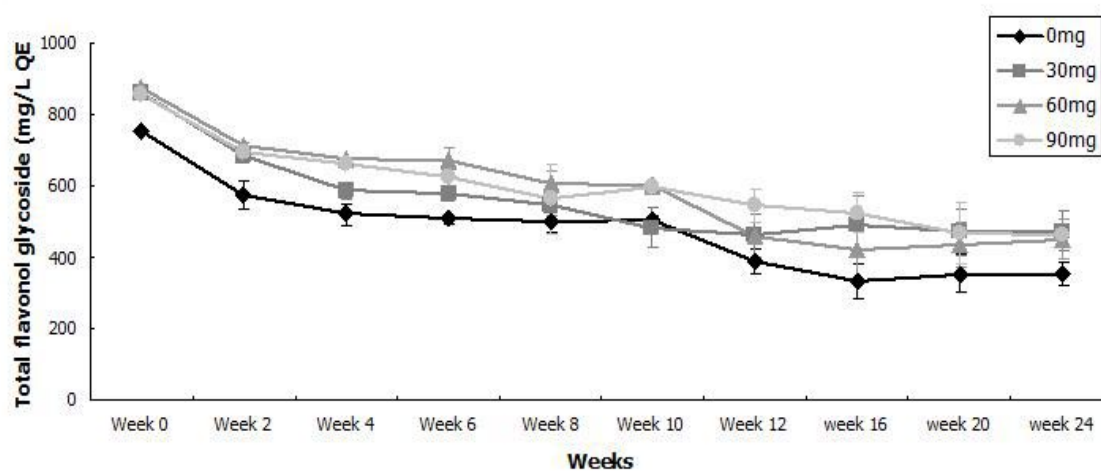


**Figure 4-10.** Changes in total chlorogenic acid (mg/L) from 3, 4, and 5-caffeoylquinic acid affected by 30, 60, and 90 mg of ascorbic acid addition to 240 mL of yaupon holly infusion during 24 week storage. Error bars represent standard error of the mean (n=3).

Ascorbic acid is an important agent in food system in terms of protection of polyphenolics against oxidative degradation by lowering the reduction rate during storage (32, 62). More specifically, ascorbic acid prevents the oxidation of chlorogenic acid and it was confirmed by Richard-Forget et al. (181) who reported that oxidative degradation catalyzed by PPO was inhibited by adding stopping solution containing ascorbic acid. The authors claimed that the oxidative degradation of chlorogenic acid may have been inhibited by lowered pH of the model solution. This suggestion was in agreement with the work by Friedman and Jügens, (180) who explained that chlorogenic acid is more stable as the pH of aqueous solution decreased. Unlike tea catechins in green tea infusion, chlorogenic acid stability was more pH sensitive since tea catechins such as C (catechin) and EGC could resist against pH induced degradations more than chlorogenic acid (180). As observed in tea catechin changes, ascorbic acid was effective

to prevent the oxidative degradation from oxidizing radicals such as  $H_2O_2$  and superoxide produced present in tea infusions and/or the degradation was inhibited by lowered pH as a result of ascorbic acid addition.

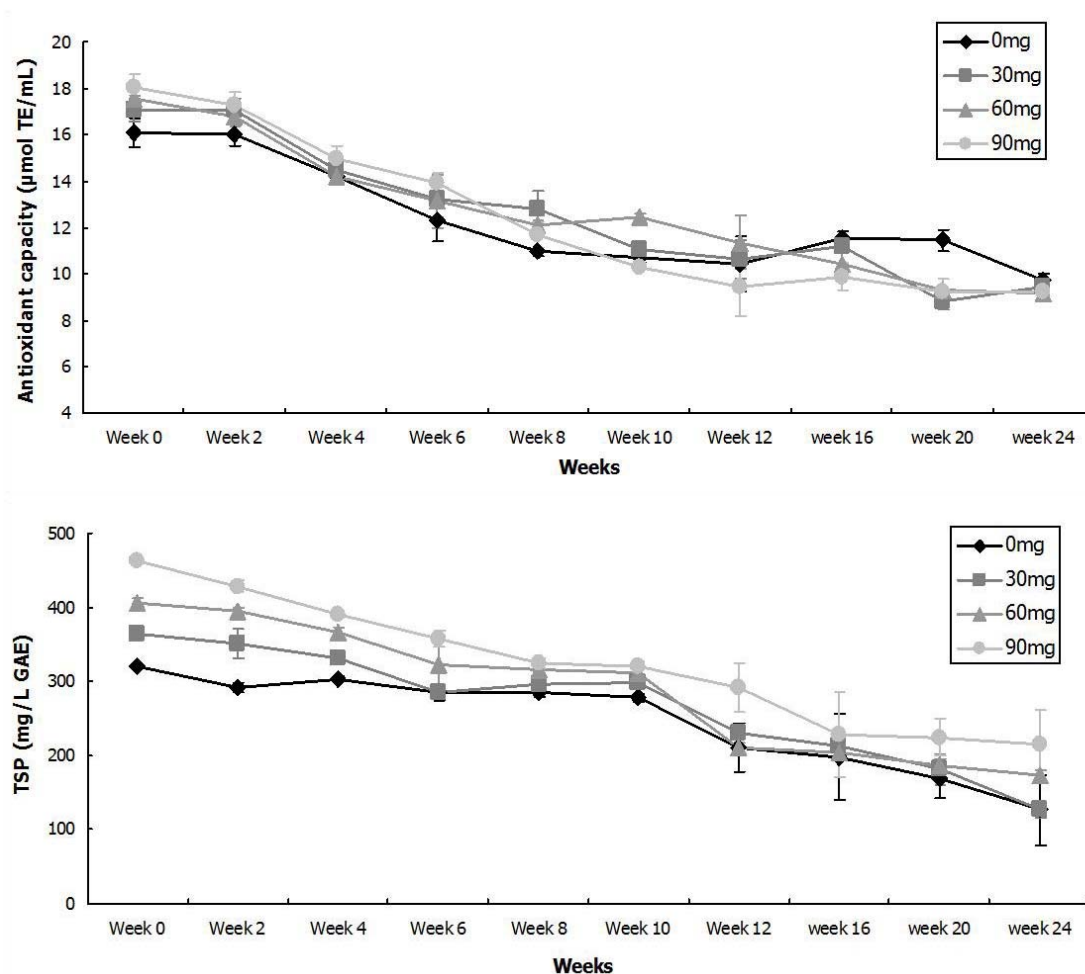
Total flavonol glycoside from quercetin-3-rutinoside (rutin) and kaempferol-3-rutinoside present in yaupon holly infusion was found at the concentration of 755.57 mg/L out of 2012.89 mg/L of total phenolic compounds. Total flavonol glycosides were reduced by 53 % during 24 week storage (**Figure 4-11**). Total flavonol glycosides were higher in all ascorbic acid fortified samples at the end of storage since the initial concentration was higher in the ascorbic acid fortified samples. During storage, the total loss of flavonol glycosides were 384, 423, and 395 mg/L at 30, 60, and 90 mg, respectively, while loss of total flavonol glycosides in control was 404 mg/L. This tendency in yaupon holly infusion was similar with that of green tea, which showed no effect on total flavonol glycosides by ascorbic acid addition.



**Figure 4-11.** Changes in total flavonol glycosides (mg/L) from quercetin-3-rutinoside, and kaempferol-3-rutinoside affected by 30, 60, and 90 mg of ascorbic acid addition to 240 mL of yaupon holly infusion during 24 week storage. Data was expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

***Influence of ascorbic acid addition on the changes in antioxidant capacity and total soluble phenolics in yaupon holly infusion during storage***

There was an impact on initial antioxidant capacity of yaupon holly infusion by fortifying ascorbic acid but no difference was observed at the end of 24 week storage. The initial change of antioxidant capacity by ascorbic acid addition was 17.06, 17.52, and 18.08  $\mu\text{mol Trolox equivalent/mL}$  at 30, 60, and 90 mg, respectively, when the control contained 16.08  $\mu\text{mol Trolox equivalent/mL}$ . The addition of ascorbic acid showed higher antioxidant capacity for the first 8 weeks at 30 and 90 mg but the tea infusion with 60 mg of ascorbic acid retained higher antioxidant capacity until Week 12 as compared to the control (**Figure 4-12**). However, total chlorogenic acid changes,



**Figure 4-12.** Changes in antioxidant capacity (top,  $\mu\text{mol TE/mL}$ ) and total soluble phenolics (TSP) (bottom,  $\text{mg/L GAE}$ ) influenced by 0, 30, 60, and 90 mg of ascorbic acid to 240 mL of yaupon holly infusion during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean ( $n=3$ ).

which showed no difference until Week 10 and 12 but higher concentrations was observed after Week 12 in all ascorbic acid fortified infusions. This indicates that even though ascorbic acid was effective at protecting chlorogenic acid from oxidative

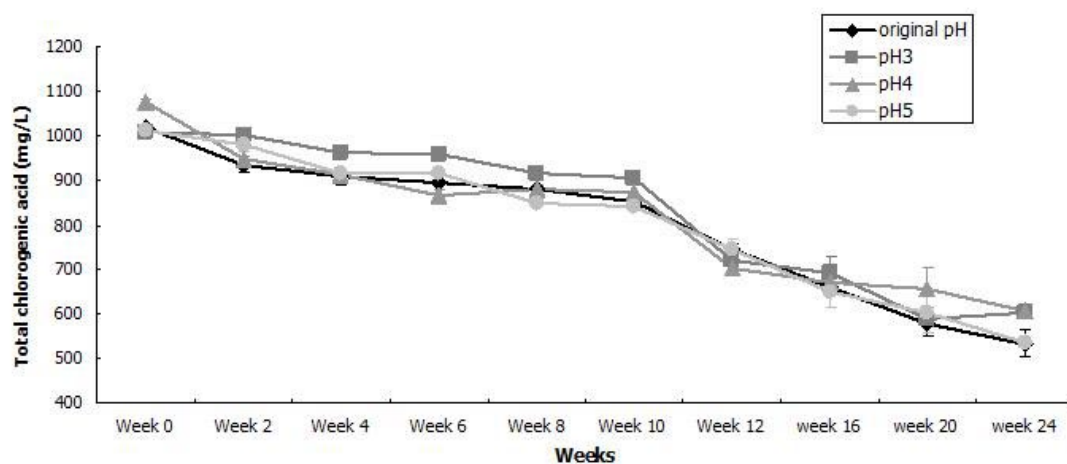
degradation only when the storage was longer than 12 week, antioxidant capacity stability was not affected by that change due to the activity of other strong antioxidants in yaupon holly infusion. Since EC, EGC, ECG, and EGCG in green tea show about 2.5 to 3 fold higher in antioxidant capacity compared to chlorogenic acid and than any other polyphenolics except procyanidins (182), the antioxidant capacity changes directly affected by tea catechin concentration could be explained. Unlike green tea catechins, chlorogenic acid is categorized as weak antioxidant containing only 1.45 mmol trolox equivalent/mL when determined using TEAC (trolox equivalent Antioxidant Capacity) assay, which is almost equal value with ascorbic acid (1.42 mmol trolox/L) (182), indicating there may be a presence of other strong antioxidants in yaupon holly infusion. Significant amounts of saponins that also show free radical scavenging activity (183) were also found in yaupon holly infusion in chapter VII and it may contribute to antioxidant capacity in yaupon holly infusion.

As observed in changes of total soluble phenolics in green tea infusion, the concentration was directly affected by ascorbic acid addition. The initial total soluble phenolics was elevated by 12, 21, and 31 % at 30, 60, and 90 mg compared to 319 mg/L Gallic acid equivalent in control. During 24 week storage, total soluble phenolics at 90 mg was consistently higher than that in control while those at 30 and 90 mg were higher until 6 weeks then showed insignificant difference from the control. However, total soluble phenolics at 60 mg were significantly higher at the end of 24 week storage.



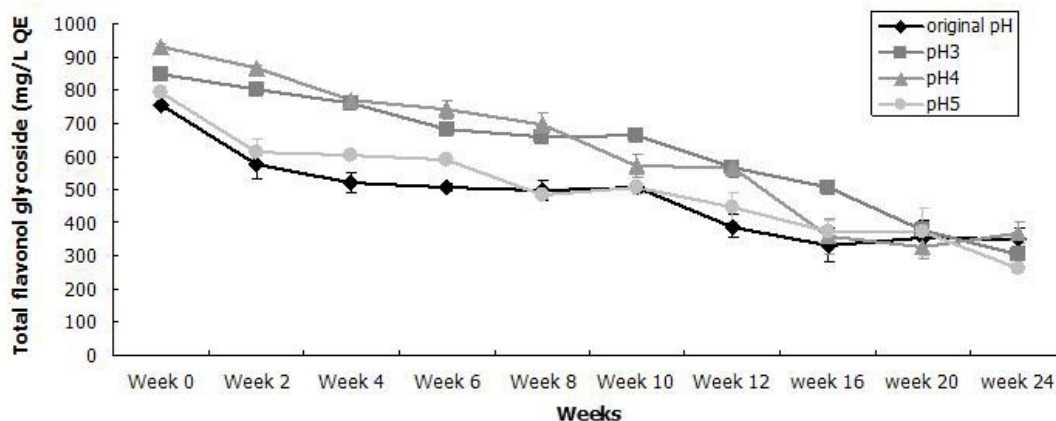
***Influence of pH conversion on the stability of phenolic compounds in yaupon holly infusion during storage***

Total chlorogenic acid was higher at pH 3 until Week 10 while the concentrations at pH 4 and 5 were not significantly different from control (pH 4.94) until 16 week of storage (**Figure 4-13**). At the end of 24 week storage, the infusions at pH 3 and 4 retained higher concentration of total chlorogenic acid than at pH 5 and control. As observed in tea catechin changes in green tea infusion, samples at lower pH retained higher concentration of phenolic compounds in yaupon holly infusion and the stability of chlorogenic acid was also dependent on pH change. The pH induced stability changes of chlorogenic acid were discussed by Friedman and Jügens, (180) who illustrated chlorogenic acid is stable at acidic pH but not stable at basic pH according to the result from ultraviolet spectroscopy. In case of green tea, the pH of tea infusion was lowered before bottling in order to increase stability and improve flavor by adding ascorbic acid or organic acids such as citric and malic acid to mimic citrus flavor. Even though yerba maté, which is in the same *Ilex* genus and has very similar polyphenolic profile, is currently being manufactured as a RTD tea (184) and ascorbic acid or organic acids are also infused to the infusion, no studies has reported the effect on phytochemical stability of yerba maté as influenced by low pH. As a result of ascorbic acid addition and pH change studies with yaupon holly tea, it was proved that these processing methods were effective to slower the reduction of predominant compounds (chlorogenic acid and its isomers), which were caused by oxidative degradation. This information would be helpful in yerba maté manufacturing.



**Figure 4-13.** Changes in total chlorogenic acid from 3, 4, and 5-caffeoylquinic acids (mg/L, top) and total flavonol glycoside from quercetin-3-rutinoside and kaempferol-3-rutinoside (mg/L, the bottom) in yaupon holly infusion affected by pH conversion to 3, 4, and 5 during 24 week storage. Total flavonol glycoside was expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

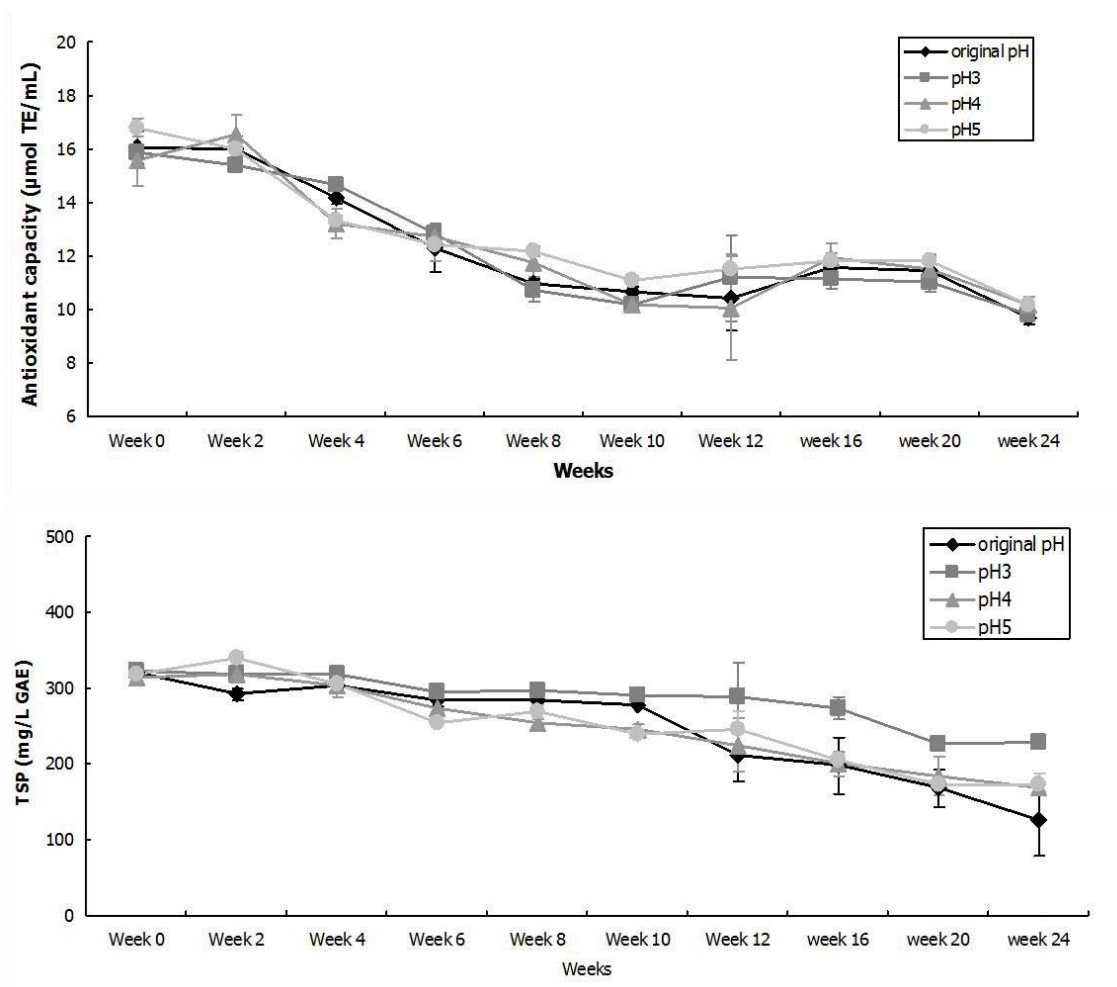
The stability of total flavonol glycosides were also affected by pH conversion and it was more stable at lower pH during 24 week storage. Yaupon holly infusion at pH 3 and 4 retained higher total flavonol glycosides than control and pH 5 until week 14 and 20, respectively (**Figure 4-14**). The overall concentration was not different between control and pH 5 but it was higher at pH 5 in Week 4, 6, and 12 than control. This trend was observed in total flavonol changes in green tea, which showed higher storage stability at lower pH.



**Figure 4-14.** Changes in total flavonol glycoside (mg/L) from quercetin 3-rutinoside, and kaempferol 3-rutinoside in yaupon holly infusion affected by pH conversion to 3, 4, and 5 during 24 week storage. Data was expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

***Influence of pH conversion on the changes in antioxidant capacity and total soluble phenolics in yaupon holly infusion during storage***

No significant difference in antioxidant capacity changes between control and pH adjusted samples was observed in the yaupon holly infusion during 24 week storage. The antioxidant capacity decreased by 40, 39, 35, and 40 % at control, pH 3, 4, and 5, respectively, but no difference was observed at the end of 24 week storage (**Figure 4-15**). Similar result was previously observed in antioxidant changes by ascorbic acid addition, which displayed weak correlation between phenolic compounds and antioxidant changes.



**Figure 4-15.** Changes in antioxidant capacity (top,  $\mu\text{mol TE/mL}$ ) and total soluble phenolics (TSP) (bottom,  $\text{mg/L GAE}$ ) influenced by pH conversion to 3, 4, and 5 in yaupon holly infusion during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean ( $n=3$ ).

Total soluble phenolics was higher at pH 3 between Week 4 and 24 while those at pH 4 and 5 did not show significant difference until week 20 but those were higher at pH 4 and 5 in week 24 compared to control. Total soluble phenolics decreased by 61, 30,

47, and 46 % at control, pH 3, 4, and 5, respectively, and the largest difference between pH 3 and others formed after Week 10. This result was also in agreement with a previous ascorbic acid addition study, which illustrated the direct relationship between phenolic compounds and total soluble phenolics changes during storage. Even though other compounds such as reducing sugar, ascorbic acid, and even protein contribute to total soluble phenolics besides phenolic compounds, total soluble phenolics in yaupon holly infusion better reflected the changes in phenolic compounds than antioxidant capacity.

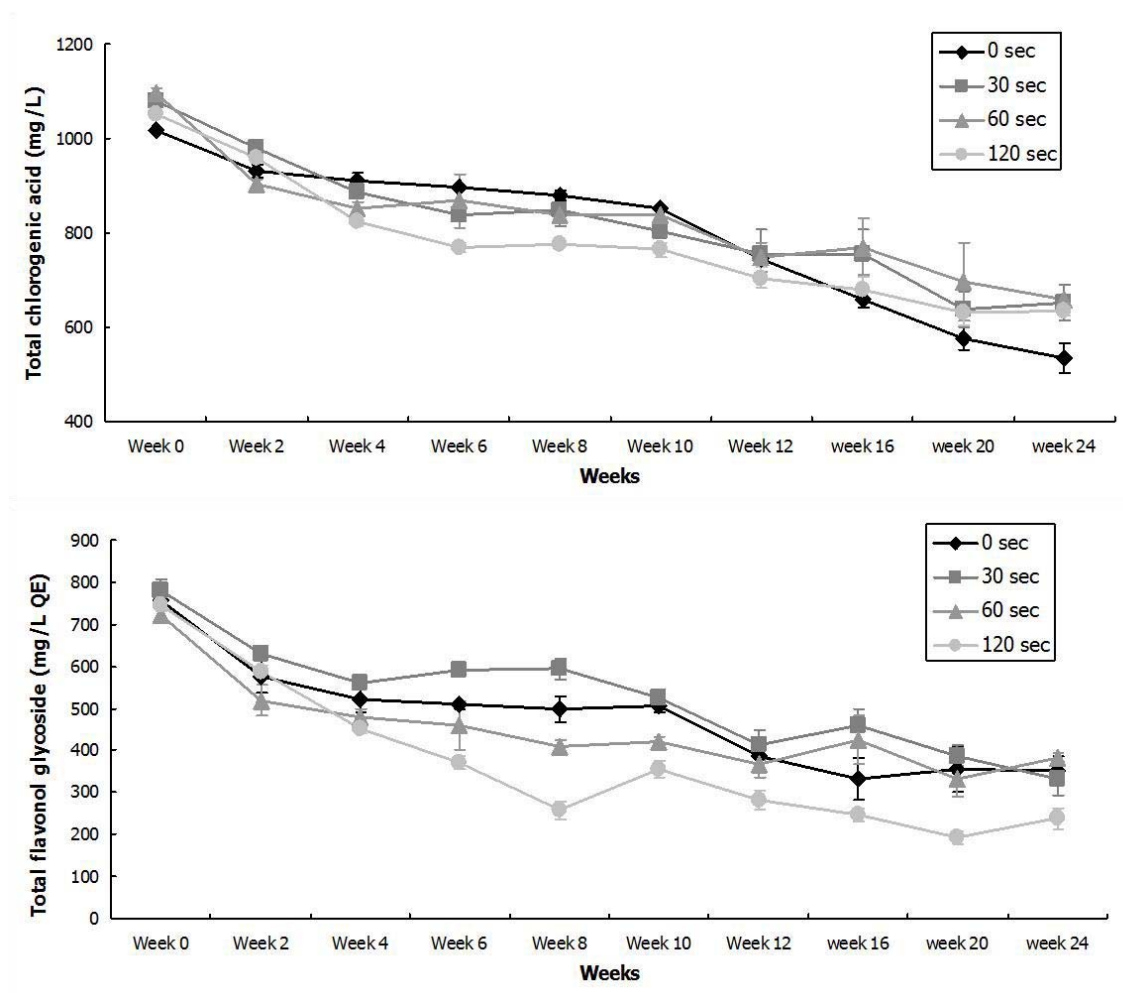
***Influence of heat treatment on the stability of phenolic compounds present in yaupon holly infusion during storage***

Polyphenolics present in diverse foods are generally consumed after exposure to heat by cooking or pasteurization. Since the reaction from heat treatment may vary depends on the type of foods, many studies have focused on antioxidant phytochemical changes as a result of heat processing with many different foods. For example, the radical scavenging activity of some vegetables increased during cooking due to the inactivation of PPO and ascorbate oxidase (185) while polyphenolics in some fruits lose their antioxidant capacity due to the adverse effect of heat treatment during processing (186). Even though some information is available about the impact of heat treatment on antioxidant phytochemical changes in green tea, no studies have reported changes in polyphenolics and resultant antioxidant capacity in botanical teas such as yaupon holly and mamaki, which contain totally different polyphenolic profiles and phytochemical

stability changes during storage. Thus, this study has illustrated heat treatment impact on phytochemical stability and antioxidant capacity changes during long term storage.

During 24 week storage at 25 °C, 48, 40, 39, and 40 % of total chlorogenic acid was found at 0, 30, 60, and 120 sec, respectively from the initial concentration of 1017.94 mg/L (control) (**Figure 4-16**). The initial change by heat treatment was a negligible since the gain of total chlorogenic acid was only 60.65, 79.72, and 34.10 mg/L at 30, 60, and 120 sec, respectively. During the first 10 week storage, yaupon holly infusion without heat treatment retained higher total chlorogenic acid while the infusion at 120 sec showed the lowest stability of total chlorogenic acid until Week 12. After Week 12, infusions at 30 and 60 sec started showing higher concentration than at control and 120 sec and the infusion at 120 sec was higher between Week 16 and 24 than control. Heat treatment retained least amount of total chlorogenic acid for the first 12 weeks, but the stability of all heat treated infusions was higher for the last 12 weeks than control.

The initial concentration of total flavonol glycoside was not affected by heat treatment and the initial concentration gains or losses were 23.85, -33.35, and -8.96 mg/L at 30, 60, and 90 sec, respectively compared to that of control (755.57 mg/L). During 24 week storage, 55, 58, 47, and 69 % of total flavonol glycosides were degraded. Total flavonol glycosides were lowest at 120 sec between Week 4 and 24 while the concentrations were not significantly different at the end of storage between control and less heated infusions (30 and 60 sec) even though there were some differences for the first 12 weeks (higher at 30 sec and lower at 60 sec than control).



**Figure 4-16.** Changes in total chlorogenic acid from 3, 4, and 5-caffeoylquinic acids (mg/L, top) and total flavonol glycoside from quercetin-3-rutinoside and kaempferol-3-rutinoside (mg/L, the bottom) in yaupon holly infusion affected by heat treatments for 0, 30, 60, and 120 sec during 24 week storage. Total flavonol glycoside was expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

The heat impact on chlorogenic acid concentration in various foods was reported in a few studies but no information is available on heat treatment effect on phytochemical stability changes in yaupon holly infusion during storage. According to

Murakami et al. (187), chlorogenic acid present in model system was decomposed by heat treatment at 180 °C for 60 to 360 min and longer treatment time induced more significant reduction in chlorogenic acid concentration while almost no degradation was observed when treating chlorogenic acid at 100 °C. Chen et al. (22) additionally confirmed this tendency by showing chlorogenic acid reduction by heat treatment at 98 °C for 10 min in burdock root. The authors also illustrated that when the authors expanded the heat treatment time (5, 10, 20, and 30 min) to determine the kinetics of chlorogenic acid degradation, higher degrees of decomposition in chlorogenic acid concentration were observed. In the present study, no initial impact on total chlorogenic acid by heat treatment was observed due to the less degree of heat treatment with tea infusions. The heat impact on initial changes on chlorogenic acid is important to determine the optimal treatment conditions to prevent chlorogenic acid loss during processing. Chlorogenic acid was not initially degraded by heat pasteurization but the treatment displayed a negative effect on chlorogenic stability for relatively short term storage (12 weeks), whereas it showed higher stability for relatively long term storage (24 weeks). Therefore, a shorter treatment time was desirable when pasteurizing tea infusion containing high amount of chlorogenic acid such as yaupon holly infusion since less difference was found between shorter treated tea infusions and control for the short term storage but the concentration was higher for the long term storage.

Rutin in buckwheat grit cake was degraded at higher rate as treatment time (5 to 8 sec) and temperature (240 to 258 °C) increased (188). Additionally, the authors illustrated that heat treatment time contributed to changes in rutin concentration more

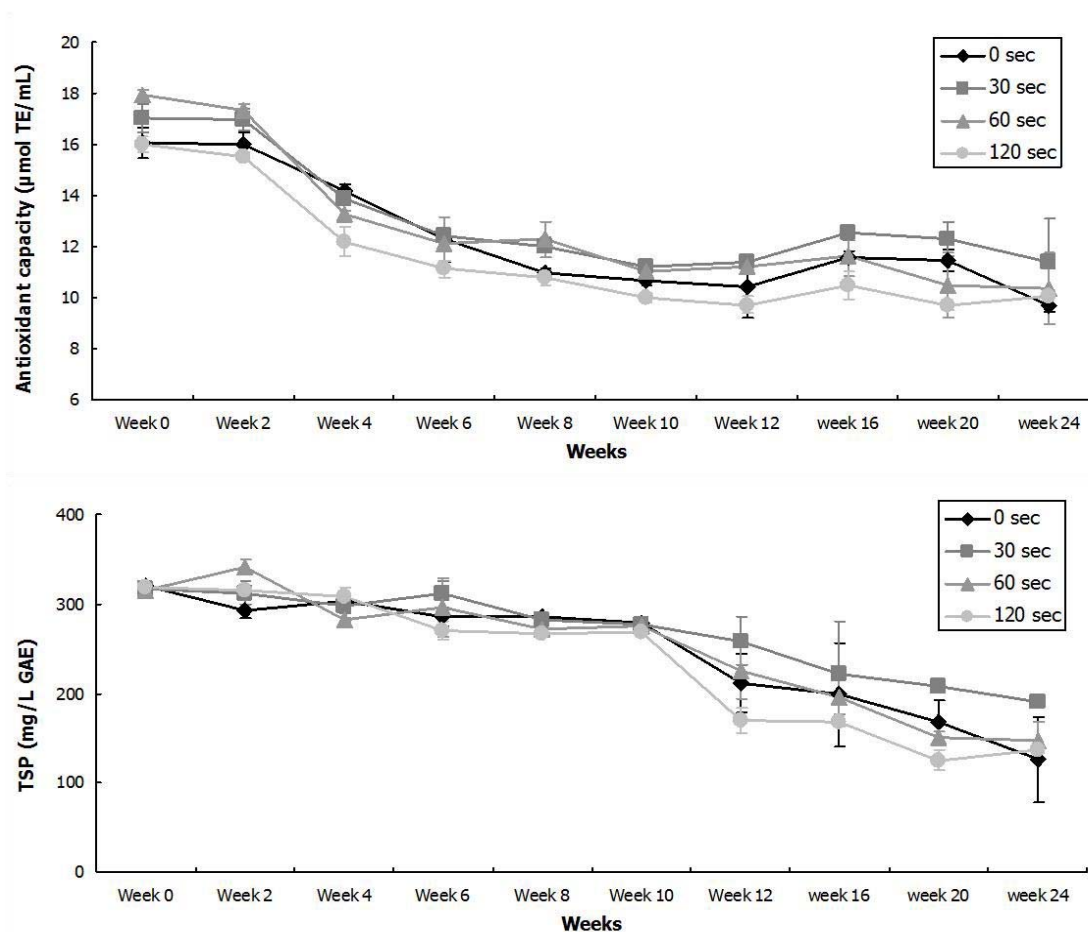


than heating temperature. In the present study, only slight initial changes were observed in rutin concentration due to lower treatment temperature but longer treatment time reduced storage stability of rutin. However, rutin concentration at 30 sec showed higher storage stability than control and other treatment durations for 20 week storage, indicating mild heat treatment increased stability of rutin. Therefore, mild heat treatment (especially 30 sec) was highly recommended to pasteurize yaupon holly infusion in terms of phytochemical stability of all phenolic compounds in the infusion.

***Influence of heat treatment on the changes in antioxidant capacity and total soluble phenolics in yaupon holly infusion during storage***

Antioxidant capacity was reduced in all tea infusions regardless of heat treatment during 24 week storage. The reduction of antioxidant capacity was 40, 42, 38, and 34 % in 0, 30, 60, and 120 sec, respectively for 24 week (**Figure 4-17**). The reduction in antioxidant capacity of yaupon holly infusion was lower than that of total chlorogenic acid except at 120 sec and also lower than that of total flavonol glycoside. This result was in agreement with Murakami et al. (187) who reported the radical scavenging activity was more stable than chlorogenic acid and rutin in model system against heating. This indicates that other antioxidants present in yaupon holly infusion also contributed to total antioxidant capacity such as saponins, ascorbic acid, and caffeine. As observed in the reduction trend of total chlorogenic acid and flavonol glycosides changes, antioxidant capacity at 120 sec was lower until week 20 than in any other samples. Since saponins are thermal sensitive (189) and the storage stability of ascorbic

acid (173) and caffeine (190) are also affected by various combinations of temperature and time duration, their changes likely affected the changes in the stability of antioxidant capacity besides phenolic compounds in yaupon holly infusion even though there were no changes in initial ascorbic acid concentration with heat treatment in this study.



**Figure 4-17.** Changes in antioxidant capacity (top, μmol TE/mL) and total soluble phenolics (TSP) (bottom, mg/L GAE) influenced by heat treatments for 0, 30, 60, and 120 sec in yaupon holly infusion during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean (n=3).

Total soluble phenolics was not changed during 10 week storage but started degrading after Week 10 and decreased until the end of 24 week storage. The overall reduction of total soluble phenolics for entire storage period was 193.69, 125.53, 168.98, and 180.41 mg/L Gallic acid equivalent. As observed in total chlorogenic acid and flavonol glycoside changes, total soluble phenolics was highest at 30 sec.

***Influence of ascorbic acid addition on the stability of phenolic compounds in mamaki infusion during storage***

In mamaki infusion, seven phenolic compounds were identified and quantified using HPLC analysis and similar phenolic profile was observed with that of yaupon holly. Three phenolic acids such as 3, 4, and 5-caffeoylquinic acids and two flavonol glycosides such as quercetin-3-rutinoside and kaempferol-3-rutinoside were found in both yaupon holly and mamaki infusions. Mamaki contains 47 % of total chlorogenic acid and 53 % of total flavonol glycoside out of 1419.26 mg/L of total phenolic compound, whereas total chlorogenic acid was the predominant compound in yaupon holly infusion (**Table 4-4**).

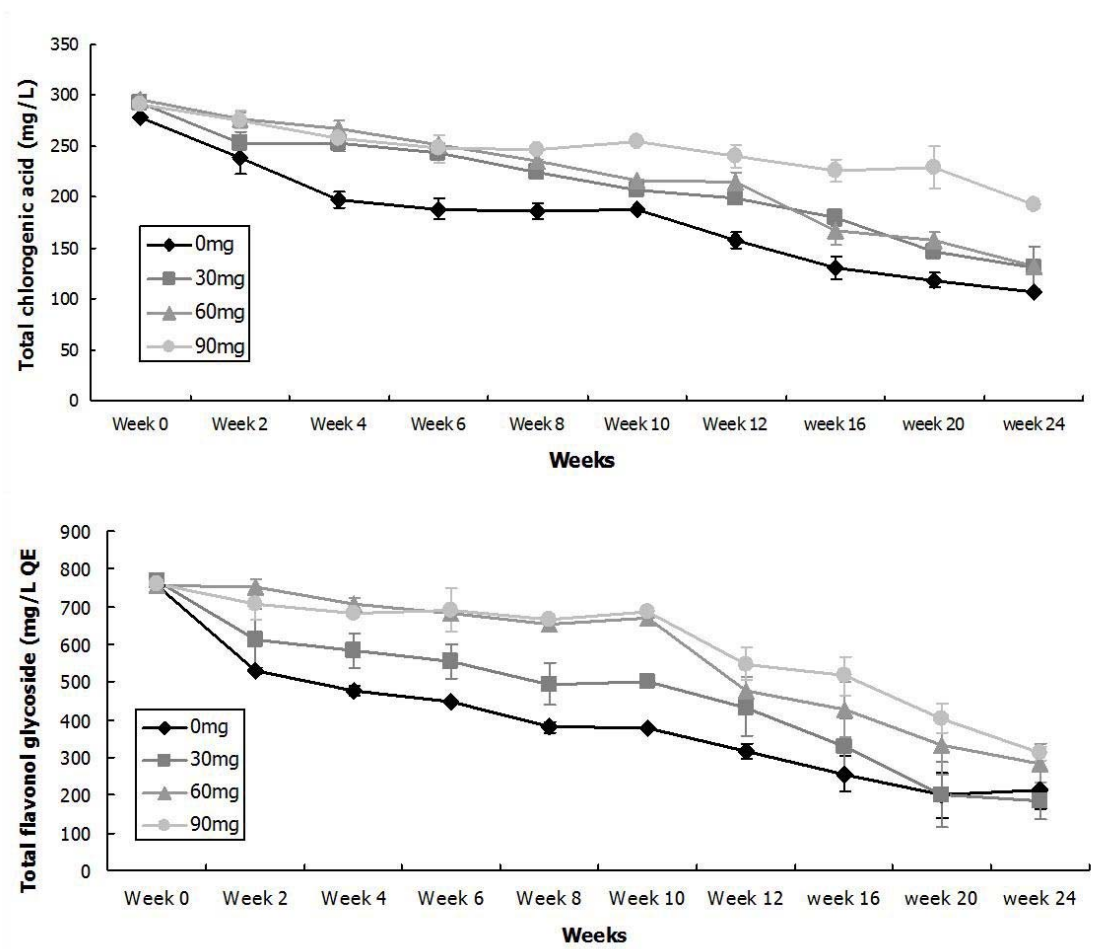
**Table 4-4.** Initial concentrations of polyphenolics and caffeine present in mamaki infusion determined by HPLC analysis.

No.	Compounds	Initial content (mg/L)
1	3-caffeoylquinic acid	172.67 ± 12.26 <sup>a</sup>
2	5-caffeoylquinic acid	248.44 ± 2.97
3	4-caffeoylquinic acid	242.57 ± 3.09
4	Apigenin 3-glucoside	105.46 ± 3.14 <sup>b</sup>
5	Apigenin 3-glucoside	152.89 ± 12.56
6	Quercetin-3-rutinoside (rutin)	276.22 ± 10.68
7	Keampferol-3-rutinoside	221.01 ± 18.64

<sup>a</sup>. Data was expressed as mean concentration (mg/L) ± standard deviation (n=9).

<sup>b</sup>. Mean values from no.4 to 7 were expressed as quercetin equivalent.

Total chlorogenic acid declined in all mamaki infusions without respect to ascorbic acid addition during 24 week storage. The reduction in total chlorogenic acid was 62, 55, 55, and 40 % in the control (0 mg), 30, 60, and 90 mg, respectively, and the infusion fortified with 90 mg of ascorbic acid retained higher concentration of total chlorogenic acid than others during storage between Week 6 and 24 (**Figure 4-18**). Total chlorogenic acid in the control was lower than those at 60 and 90 mg throughout storage and also lower than that in 30 mg between Week 2 and 24. The concentrations at 30 and 60 mg were not significantly different until the end of 24 week only except Week 2. Total flavonol glycosides were also degraded by 72, 76, 62, and 59 % in the control, 30, 60, and 90 mg of ascorbic acid addition during storage (**Figure 4-18**). The



**Figure 4-18.** Changes in total chlorogenic acid from 3, 4, and 5-caffeoylquinic acids (mg/L, top) and total flavonol glycoside from two apigenin-3-glycosides, quercetin-3-rutinoside and kaempferol-3-rutinoside (mg/L, the bottom) affected by 30, 60, and 90 mg of ascorbic acid addition to 240 mL of mamaki infusion during 24 week storage. Total flavonol glycosides were expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

concentration was lowest in the control than ascorbic acid fortified infusions until Week 20 but no difference was observed between control and 30 mg for the last 4 weeks. The higher additions of ascorbic acid (60 and 90 mg) were more effective at retaining higher

concentrations of total flavonol glycosides throughout storage and the highest addition of ascorbic acid (90 mg) delayed the reduction in the total concentration more than the 60 mg addition after Week 10. As observed in total chlorogenic acid changes by ascorbic acid addition, the storage stability of total flavonol glycosides was highly influenced by ascorbic acid addition and higher amount of ascorbic acid retained higher concentration of total flavonol glycosides during 24 week storage.

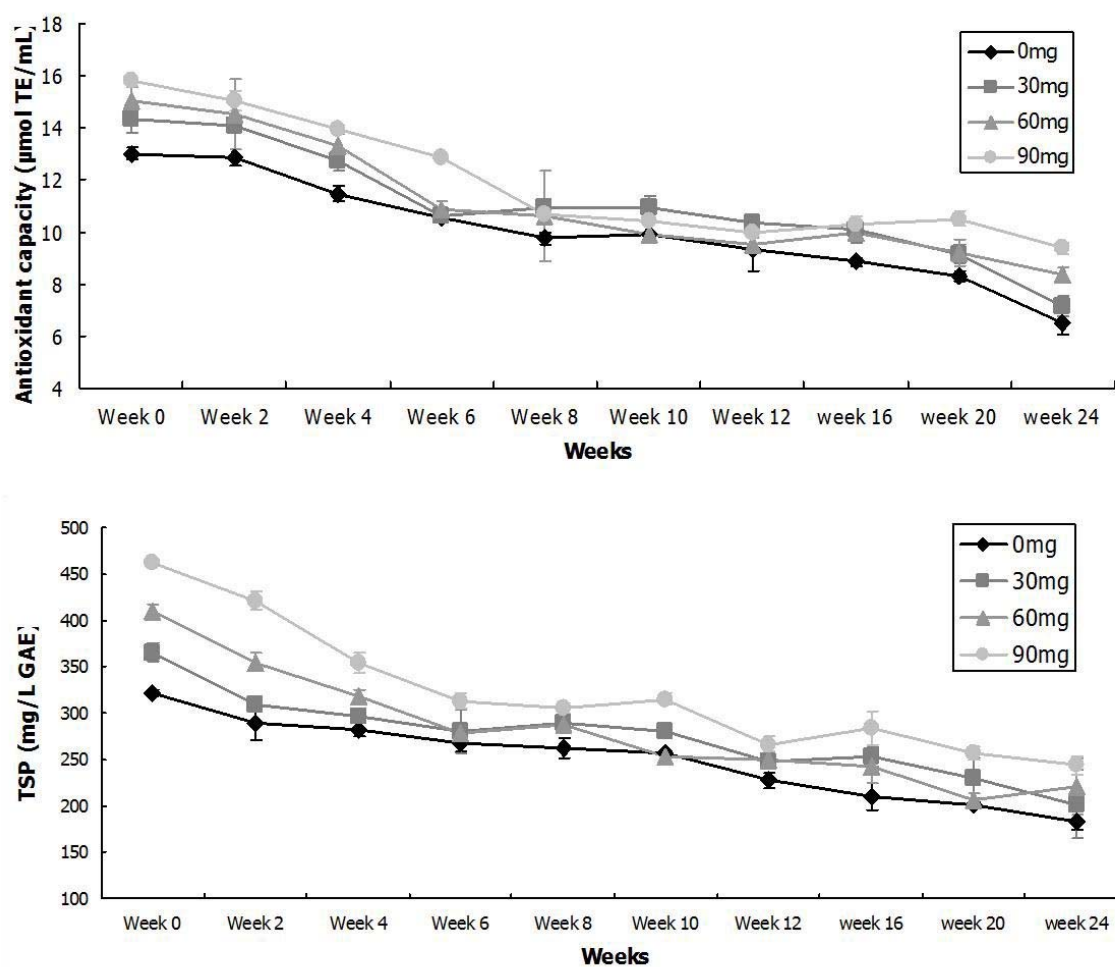
Even though ascorbic acid addition to yaupon holly infusion was effective at delaying the reduction of phenolic compounds during storage, the effectiveness with ascorbic acid fortification in regards to phytochemical stability was even higher in mamaki infusion. As previously discussed, ascorbic acid postponed the reduction of phenolic compounds by delaying oxidation in yaupon holly infusion. Besides that, another factor is also involved in delaying the reduction rate especially in mamaki infusion by adding ascorbic acid to tea infusions. In the tea beverage industry, a diversity of organic acids such as citric acid, malic acid, succinic acid, fumaric acid, L-glutamic acid, L-aspartic acid as well as ascorbic acid are fortified into tea infusions to improve storage stability and flavor of green tea (60). Addition of these chemical agent for stability may reduce the amount of reactive oxygen species (ROS) especially including  $H_2O_2$ , superoxide and  $OH\cdot$  due to lowered pH of green tea infusion (60). The pH effect on total chlorogenic acid was already observed in yaupon holly infusion, which the infusions at lower pH (especially at pH 3) retained higher concentration of both total chlorogenic acid and flavonol glycosides during 24 week storage. Since pH of mamaki infusion (pH 6.99) was significantly higher than that of yaupon holly (pH 4.94) and it

was nearly neutral pH which makes most of phenolic compounds unstable, addition of ascorbic acid was more effective in holding more phenolic compounds in mamaki infusion by the combination of two reactions (reducing the amount of ROS and lowering pH).

***Influence of ascorbic acid addition to mamaki infusion on the changes in antioxidant capacity and total soluble phenolics during storage***

The antioxidant capacity of mamaki infusion (13.00  $\mu\text{mol Trolox equivalent/mL}$ ) was lower than green tea and yaupon holly (16.43 and 16.08  $\mu\text{mol Trolox equivalent/mL}$ , respectively) due to lower concentrations of total phenolic compounds. The initial antioxidant capacity was elevated by adding ascorbic acid by 9, 14, and 18 % at 30, 60, and 90 mg, respectively and antioxidant capacity was higher retained in the infusions with higher ascorbic acid fortification (60 and 90 mg) (**Figure 4-19**). The remaining % of antioxidant capacity was 50, 50, 55, and 59 % in the control, 30, 60, and 90 mg when determined at the end of 24 week storage. Even though only mamaki infusion with 90 mg retained higher antioxidant capacity throughout storage, the infusions with 30 and 60 mg also showed higher antioxidant capacity during storage except Week 6 for 30 mg and Week 6, 10, and 12 for 60 mg. Antioxidant capacity did not reflect the changes in phenolic compounds in yaupon holly infusion, but it better reflected phenolic changes in mamaki infusion. This may be because other antioxidants present in mamaki infusion were lower than those of yaupon holly such as saponins and

contain no caffeine in the infusion. This may mean phenolic compounds in mamaki infusion predominantly affected the changes of antioxidant capacity.



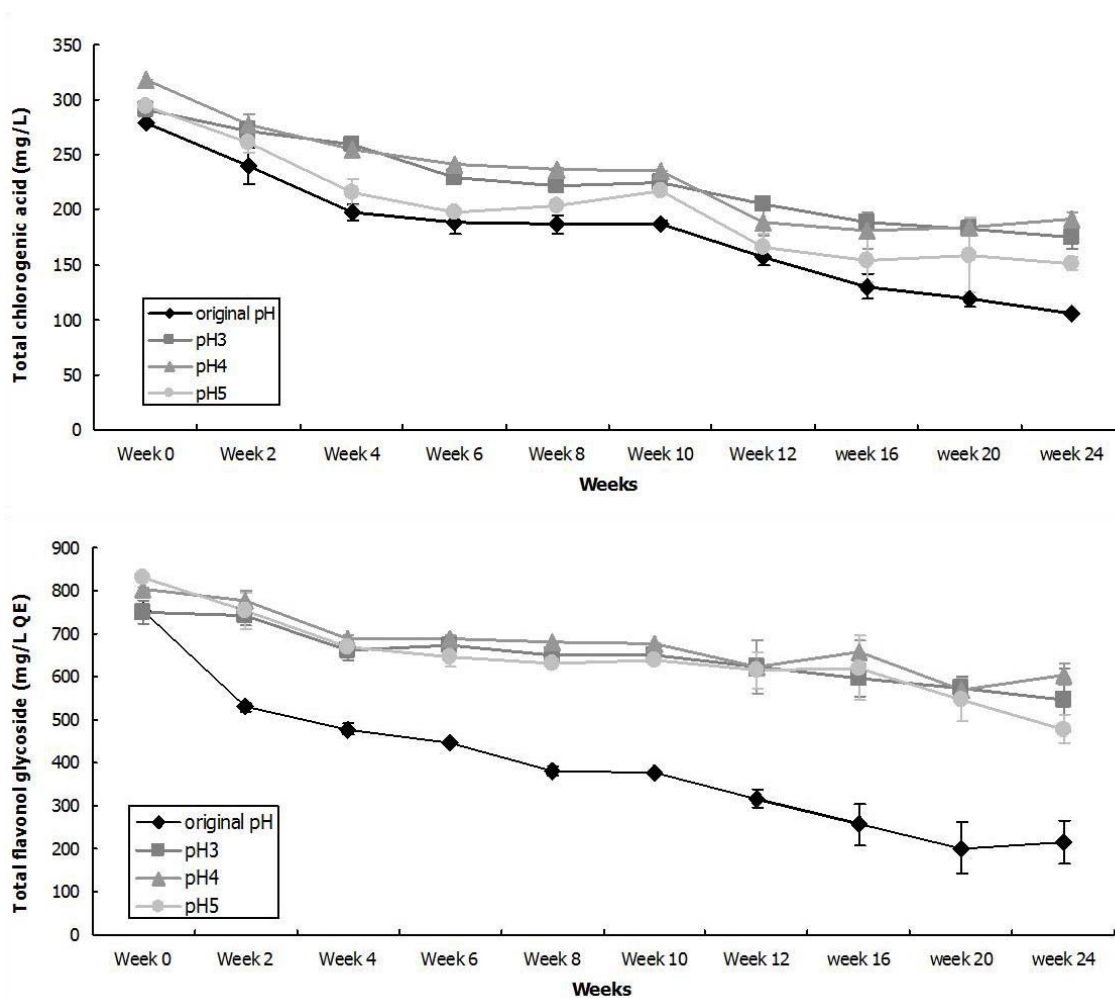
**Figure 4-19.** Changes in antioxidant capacity (top,  $\mu\text{mol TE/mL}$ ) and total soluble phenolics (TSP) (bottom,  $\text{mg/L GAE}$ ) influenced by 0, 30, 60, and 90 mg of ascorbic acid addition infusion to 240 mL of mamaki infusion during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean ( $n=3$ ).



The initial concentration of total soluble phenolics in mamaki infusion was also influenced by ascorbic acid addition and the reduction rate was not significantly different between control and ascorbic acid fortified infusions. The initial gain in total soluble phenolic by ascorbic acid additions of 30, 60, and 90 mg was 12, 22, and 41 %, respectively, due to the contribution from reducing capacity of ascorbic acid. During 24 week storage, the remaining % of total soluble phenolics was 56, 55, 54, and 53 % in the control, 30, 60, and 90 mg, respectively, at the end of 24 week storage. Even though the final concentration of total soluble phenolics in ascorbic acid fortified infusions was higher than that of control, it was due to the initial gain by the addition not due to better retained concentration. Mamaki has similar phenolic profile with yaupon holly but it contains significantly higher other constituents contributing total soluble phenolics such as protein and metal (40 and 39 % higher in mamaki, respectively, as determined in chapter VII) due to their reducing capacity. These might interrupt the estimate of the amount of soluble phenolic by Folin-Ciocalteu assay in mamaki infusion.

***Influence of pH conversion on the stability of phenolic compounds in mamaki infusion during storage***

As anticipated in total chlorogenic acid and flavonol glycosides changes due to ascorbic acid addition, the reduction rate of both concentrations significantly decreased by lowering pH of mamaki infusion. The remaining % of total chlorogenic acid was 38, 60, 60, and 51 % at original pH (6.99, control) and pH 3, 4, and 5, respectively during 24 week storage (**Figure 4-20**). Total chlorogenic acid at original infusion was lowest



**Figure 4-20.** Changes in total chlorogenic acid from 3, 4, and 5-caffeoylquinic acids (mg/L, top) and total flavonol glycosides from two apigenin-3-glycosides, quercetin-3-rutinoside and kaempferol-3-rutinoside (mg/L, the bottom) in mamaki infusion affected by pH conversion to 3, 4, and 5 during 24 week storage. Total flavonol glycosides were expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

throughout the storage and mamaki infusion at pH 3 and 4 retained higher concentration of total chlorogenic acid than at pH 5 except Week 2 and 10. Total flavonol glycosides

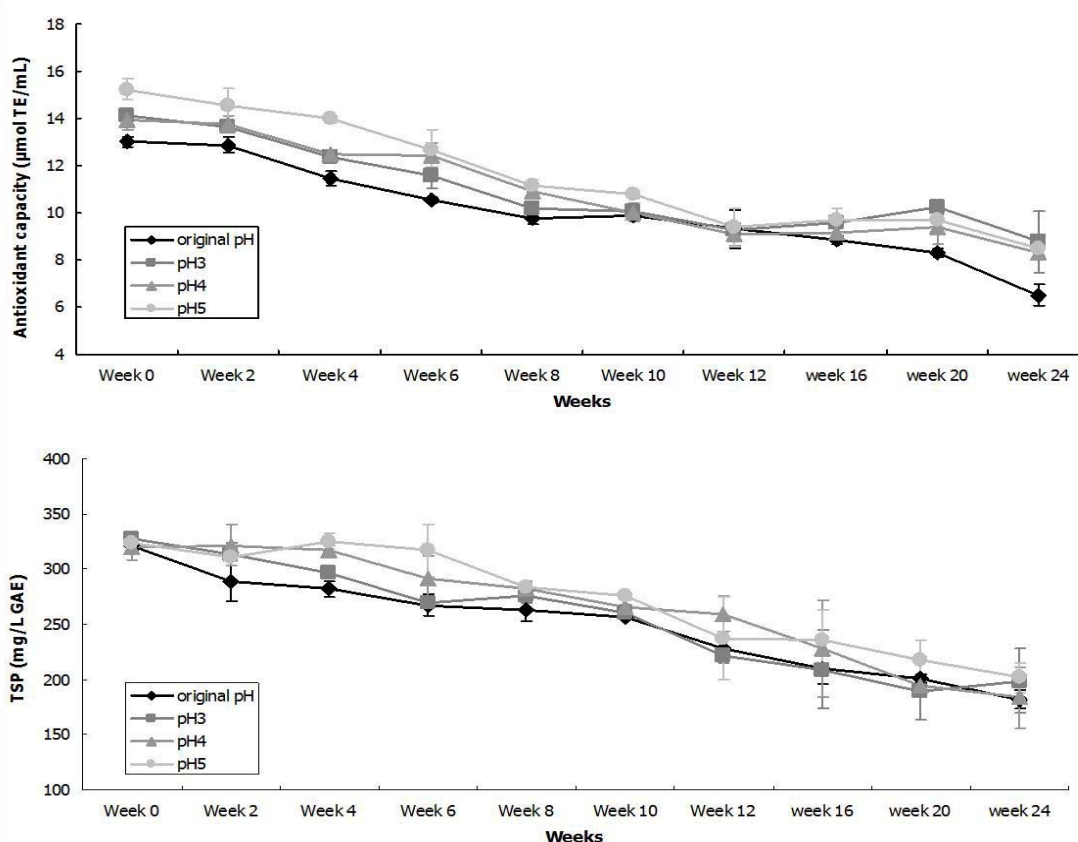
were more highly influenced by pH conversion. The remaining % of total flavonol glycosides were 28, 73, 75, and 58% in the control and pH 3, 4, and 5, respectively, during 24 week storage and no significant difference was observed in between samples at lowered pH until Week 20. At the end of storage, the concentrations at pH 3 and 4 were higher than at pH 5. Even though a similar phenolic profile was found in yaupon holly and mamaki, the pH effect on phytochemical stability was higher in mamaki infusion due to naturally high pH that induced high degradation of polyphenolics including chlorogenic acid (180).

***Influence of pH conversion on antioxidant capacity and total soluble phenolics in mamaki infusion during storage***

Antioxidant capacity of mamaki infusion was reduced by 50, 38, 41, and 56 % in the control and pH 3, 4, and 5, respectively during 24 week storage (**Figure 4-21**). Antioxidant capacity was higher at pH 3 during 4 week storage and then no difference was observed with those at pH 3 and 4 only except Week 10 but antioxidant capacity was higher in the infusions with lowered pH than in the control that was lowest all of the time except between Week 10 and 16. Lowered pH of mamaki infusion was effective to retain higher antioxidant capacity as observed in changes in phenolic compounds in the mamaki infusion.

No significant difference was found in changes of total soluble phenolics during 24 week storage. Total soluble phenolic at control decreased at slightly higher rate than those at reduced pH but no significant difference was found throughout the storage

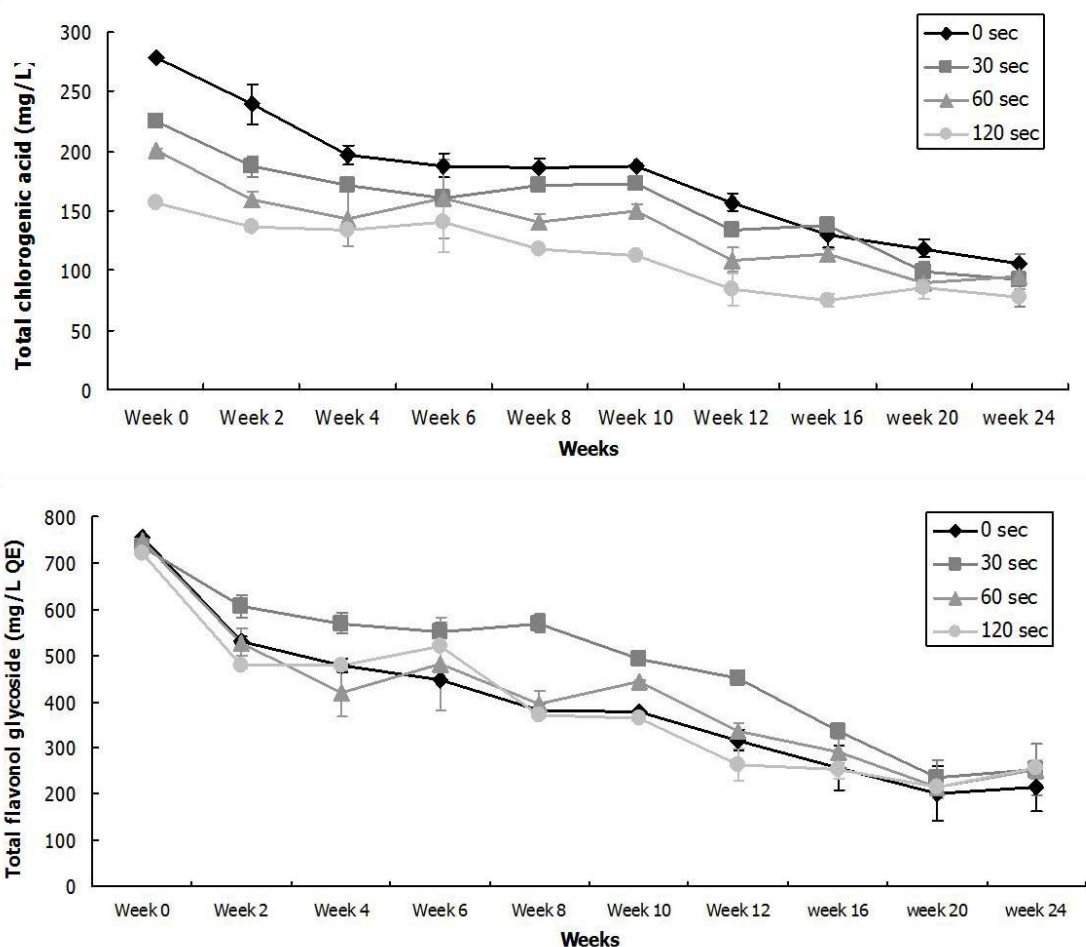
(Figure 4-21). Since significantly higher concentrations of phenolic compounds in acidified mamaki infusion were observed, no significant difference was somewhat unexpected. However, this trend was previously found in ascorbic acid addition study with mamaki infusion. As discussed above, total soluble phenolics are not a good method to estimate total amount of phenolic compounds present in mamaki infusion.



**Figure 4-21.** Changes in antioxidant capacity (top, μmol TE/mL) and total soluble phenolics (TSP) (bottom, mg/L GAE) influenced by pH conversion of mamaki infusion to 3, 4, and 5 during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean (n=3).

***Influence of heat treatment on the stability of phenolic compounds present in mamaki infusion during storage***

The total chlorogenic acid was influenced by heat treatment and the infusion without heat treatment retained higher concentration than heat treated samples. The remaining % of total chlorogenic acid was 38, 41, 47, and 50 % from the initial concentration of 278.57 mg/L at 0, 30, 60, and 120 sec, respectively, indicating the infusions at 60 and 120 sec showed higher reduction than those at 0 and 30 sec (**Figure 4-22**). The initial concentration was significantly reduced by heat treatments and the initial reduction was 53.12, 77.56, and 121.42 mg/L at 30, 60, and 90 sec, respectively subtracted from the initial concentration of control. This result was not in agreement with the previous observation of total chlorogenic acid changes of yaupon holly, which showed no initial impact from heat treatment. Even though the heat treatment conditions used in the present study (30 to 120 sec at 95 °C) were not critical for the impact on the initial concentration of total chlorogenic acid present in yaupon holly infusion, there was a significant reduction of total chlorogenic acid in mamaki infusion. This indicates that when chlorogenic acid was heat treated at high pH (neutral), the reduction occurred due to the synergistic effect. Since no difference between 0 and 30 sec treatments were observed in stability of chlorogenic acid while higher reduction rate was found in 60 and 120 sec, less degree of heat treatment was desirable for mamaki infusion.



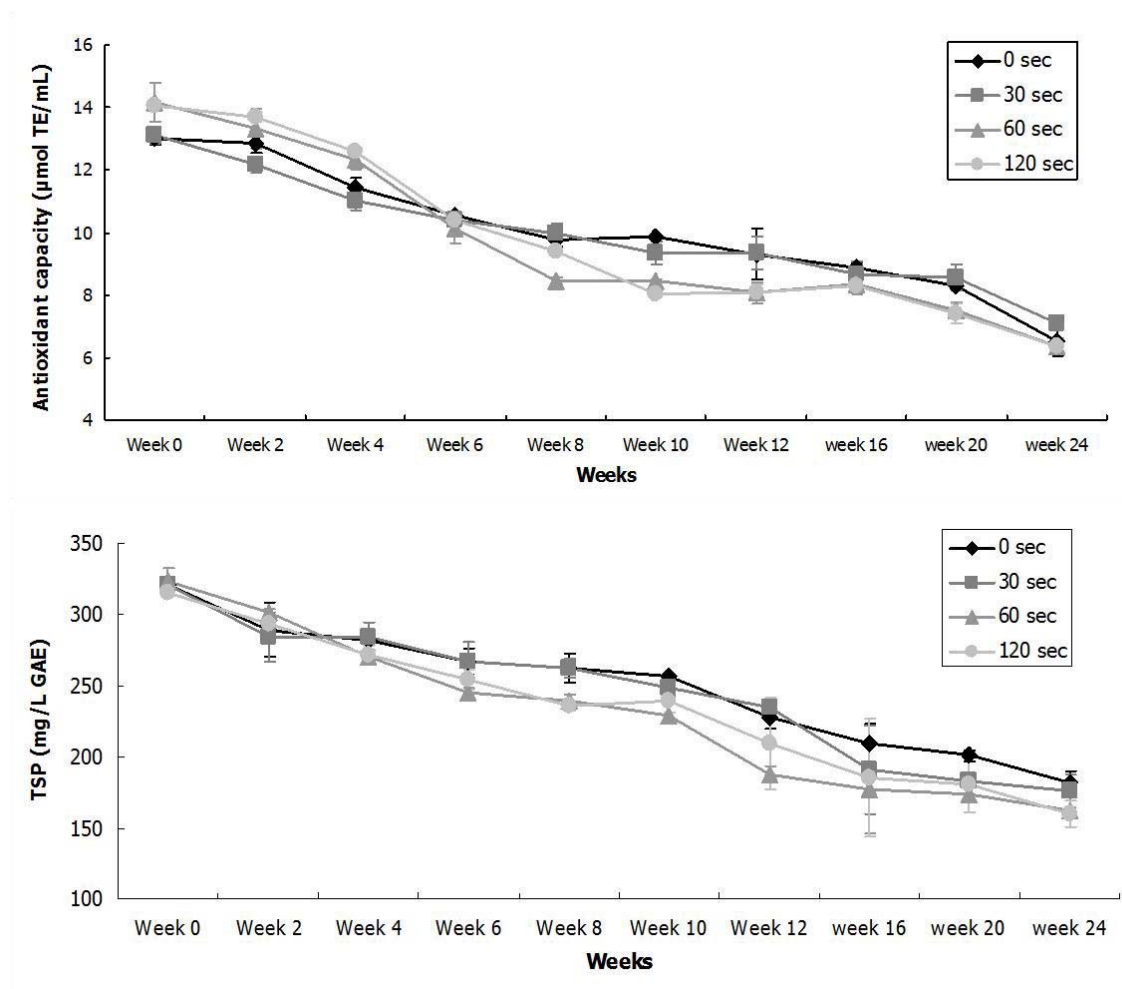
**Figure 4-22.** Changes in total chlorogenic acid from 3, 4, and 5-caffeoylquinic acids (mg/L, top) and total flavonol glycoside from two apigenin-3-glycosides, quercetin-3-rutinoside and kaempferol-3-rutinoside (mg/L, the bottom) in mamaki infusion affected by heat treatment for 0, 30, 60, and 120 sec during 24 week storage. Total flavonol glycoside was expressed as quercetin equivalent (QE). Error bars represent standard error of the mean (n=3).

Total flavonol glycosides were not initially impacted by heat treatment but mamaki infusion heated for 30 sec showed higher stability compared to 0, 60, and 120 sec during 16 week storage. Even though the overall degradation was higher in all heated

infusions at the end of 24 week storage (72, 66, 66, and 64 % reduction at 0, 30, 60, and 120 sec), it was significantly lower at 30 sec until 16 week storage. According to Murakami et al. (187), when chlorogenic acid and rutin (the most prevalent flavonol glycoside in mamaki) were present in the same medium, the degradation rate of rutin due to heating was significantly lower than that of rutin alone due to the protective effect from chlorogenic acid when heated. This tendency was not observed in yaupon holly infusion due to lower pH than the pH of distilled water used in Murakami's study (pH 5.6 to 7.0) (EPA 2007). In mamaki infusion, heating 30 sec was optimal for total chlorogenic acid and flavonol glycosides at a given temperature (95 °C).

***Influence of heat processing on antioxidant capacity and total soluble phenolics in mamaki infusion during storage***

Antioxidant capacity of mamaki infusion was reduced regardless of degree of heat treatment during 24 week storage. The remaining % of antioxidant capacity was 50, 54, 45, and 45 % at 0, 30, 60, and 120 sec, respectively and the smallest reduction in antioxidant capacity was observed in the infusion heated for 30 sec (**Figure 4-23**). The initial antioxidant capacity was elevated by 8 % at 60 and 120 sec and the concentrations were higher for the first 4 week storage while no difference between 0 and 30 sec was observed at the same period. Mamaki infusion heated for 0 and 30 sec retained higher antioxidant capacity between Week 8 and 20 than those at 60 and 120 sec only except Week 16. Especially, antioxidant capacity was higher at 30 sec than those in all other infusions at the end of 24 week storage. Heating was responsible for an increase or



**Figure 4-23.** Changes in antioxidant capacity (top, mol TE/mL) and total soluble phenolics (TSP) (bottom, mg/L GAE) influenced by heat treatment for 0, 30, 60, and 120 sec of mamaki infusion during 24 week storage. Antioxidant capacity and total soluble phenolics was expressed as Trolox equivalent (TE) and Gallic acid equivalent (GAE), respectively. Error bars represent standard error of the mean (n=3).

retain of the antioxidant capacity from Week 0 to 4 while lower degree of heating (30 sec) or no heating (0 sec) decreased the reduction rate of antioxidant capacity. The higher antioxidant capacity at 0 and 30 sec was anticipated due to higher concentrations



of total chlorogenic acid and flavonol glycoside but initially higher antioxidant capacity at 60 and 120 sec was not expected.

Total soluble phenolics was reduced by 43, 55, 50, and 49 % at 0, 30, 60, and 90 sec, respectively during 24 week storage and higher concentration was observed at 0 and 30 sec between Week 2 and 12. Especially, total soluble phenolics was more retained in the control than those at 60 and 120 sec between Week 4 and 24, indicating higher degree of heat treatment significantly influenced total soluble phenolics. Otherwise, total soluble phenolics at 30 sec were not different with control except between Week 20 and 24. As observed phenolic compounds changes in mamaki infusion, total soluble phenolics was also more stable when no heat or lower degree of heat was applied to the mamaki infusion.

**CHAPTER V**  
**THE IMPACT OF PACKAGING MATERIALS ON THE ANTIOXIDANT**  
**PHYTOCHEMICAL STABILITY OF THREE TEA VARIETIES**

**Introduction**

Ready-to-drink (RTD) tea is commonly sold in glass, plastic, steel, or aluminum containers with little regard to the potential effects that these packages may have on phytochemical stability or product quality. In fruit juices, these packages can affect quality by influencing browning, flavor, and nutrient losses during storage (68, 69), yet no studies have reported the phytochemical stability changes by various packaging materials. Furthermore, since commercial beverages readily deteriorate due to browning, taste and flavor changes, and loss of ascorbic acid, packaging materials need to be considered to protect the beverage (69). The chemical changes from various packagings are mostly caused by oxygen contact and light transmission through the package (191). Therefore, these studies were aimed at evaluating the phytochemical stability and quality changes of two botanical tea infusions of yaupon holly and mamaki in comparison to green tea. All were evaluated over time in glass, polyethylene terephthalate (PET) and a retortable pouch (RP) made from polypropylene and polyester each with different oxygen permeability. RP was chosen for this study due to its excellent mechanical properties (microwavable, lightweight, and durable), ease of sterilization (high heat resistant), and economical advantage (inexpensive). The stability of antioxidant polyphenolics provides an understanding of the impact of packaging materials that lead

to retention of those compounds responsible for the quality and potential health benefits of these products.

## **Materials and Methods**

### ***Tea preparation and packagings***

Green tea from Hwagae, Korea and yaupon holly leaves harvested in Texas were used in the present study. Mamaki leaf was kindly donated from the University of Hawaii. The three teas were used and brewed as described in chapter III. Briefly, each tea leaf was powdered and then brewed with hot water at 90 °C by directly pouring purified water using Mille-Q water system (Billerica, MA) onto the leaves (tea leaf : water = 1:100, w/v) with constant stirring for 10 min and then this tea infusion was separated into the triplicates.

Each infusion was prepared independently in triplicate and subsequently divided into three equal portions for transfer to the different packaging materials (glass, PET, and RP) for storage at 3 °C. Prior to filling, each package was sterilized with 50 mg/L (50 pap) chlorine for 30 sec at 25 °C and extensively washed with sterile water to remove chlorine residues prior to air dry. The fill volumes for glass and PET were maximized to minimize headspace while the RP were flushed with nitrogen immediately prior to heat-sealing. The cooled tea infusions were filled to capacity in the package to minimize a production of acetaldehyde that causes undesirable odor and altered taste (192). Tea infusions were stored for 12 weeks in the dark and analyzed biweekly for

polyphenolics and antioxidant capacity. Samples were taken from each package for chemical analysis through a rubber septum using a sterilized needle to prevent microbial contamination or air intake to the containers.

### ***Phytochemical and color analyses***

Individual polyphenolics were analyzed by HPLC as described in chapter III. The authentic standards used in this study were purchased from Sigma chemical Co., (St.Louis, MO). Antioxidant capacity was determined by the method described by Talcott et al. (109) on a 96-well Molecular Devices fmax<sup>®</sup> fluorescent microplate reader (Sunnyvale, CA) against a standard of Trolox with data expressed in  $\mu\text{mol Trolox/g}$ . Total soluble phenolics was determined as described in chapter IV. The color of the tea infusions were measured using a Gardner Colorgard system/05 colorimeter and recorded as CIE color values ( $L^*$ ,  $a^*$  and  $b^*$ ). Hue angles and chroma values were calculated as described by López and Gómez, (193).

### ***Statistical analysis***

Data was analyzed as a 3x7 full factorial including three packagings at seven sampling times for each tea. Data represent the mean triplicate analysis using ANOVA (analysis of variance) with JMP 5 statistical software (110). Mean separation was conducted using the LSD test ( $P < 0.05$ ).

## Results and Discussion

### *Phytochemical changes in different packaging materials containing green tea infusion*

Initial concentrations of 11 polyphenolics and caffeine in green tea determined by HPLC were reported in **Table 5-1** and the identifications of each polyphenolic compound were based on the result by LC/ESI/MS<sup>n</sup> analysis conducted in chapter III and retention time and UV spectral similarities to external authentic standards of EGC, EGCG, ECG, GCG, EC, C, kaempferol 3-rutinoside (rutin), caffeine, kaempferol, and quercetin. The most predominant compounds were flavan-3-ols (EGC, EGCG, ECG, GCG, and EC) with 91 % and flavonol glycosides with 9 % of total polyphenolics in green tea infusion. The only phenolic acid found in green tea was gallic acid and its concentration was less than 1 %.

EGCG, EGC, and ECG together accounted for 82 % (1385 mg/L) of the total phenolics in green tea sharing a common chemical structure of 2-phenylchromen-4-one (2-phenyl-1,4-benzopyrone). These three flavan-3-ols were previously reported to contain the highest antioxidant capacity among polyphenolics present in green tea (67, 122, 182). During 12 weeks of storage at 3 °C, concentrations of EGCG were reduced by 26, 44, and 80 % in glass, PET and RP, respectively whereas EGC (38, 43, and 57 %) and ECG (11, 34, and 79 %) followed a similar trend as oxidation progressed with storage (**Figure 5-1**). Their oxidative degradation was not observed for the first 6 weeks storage ( $P < 0.05$ ), yet their subsequent degradation progressed rapidly for RP and PET compared to glass.

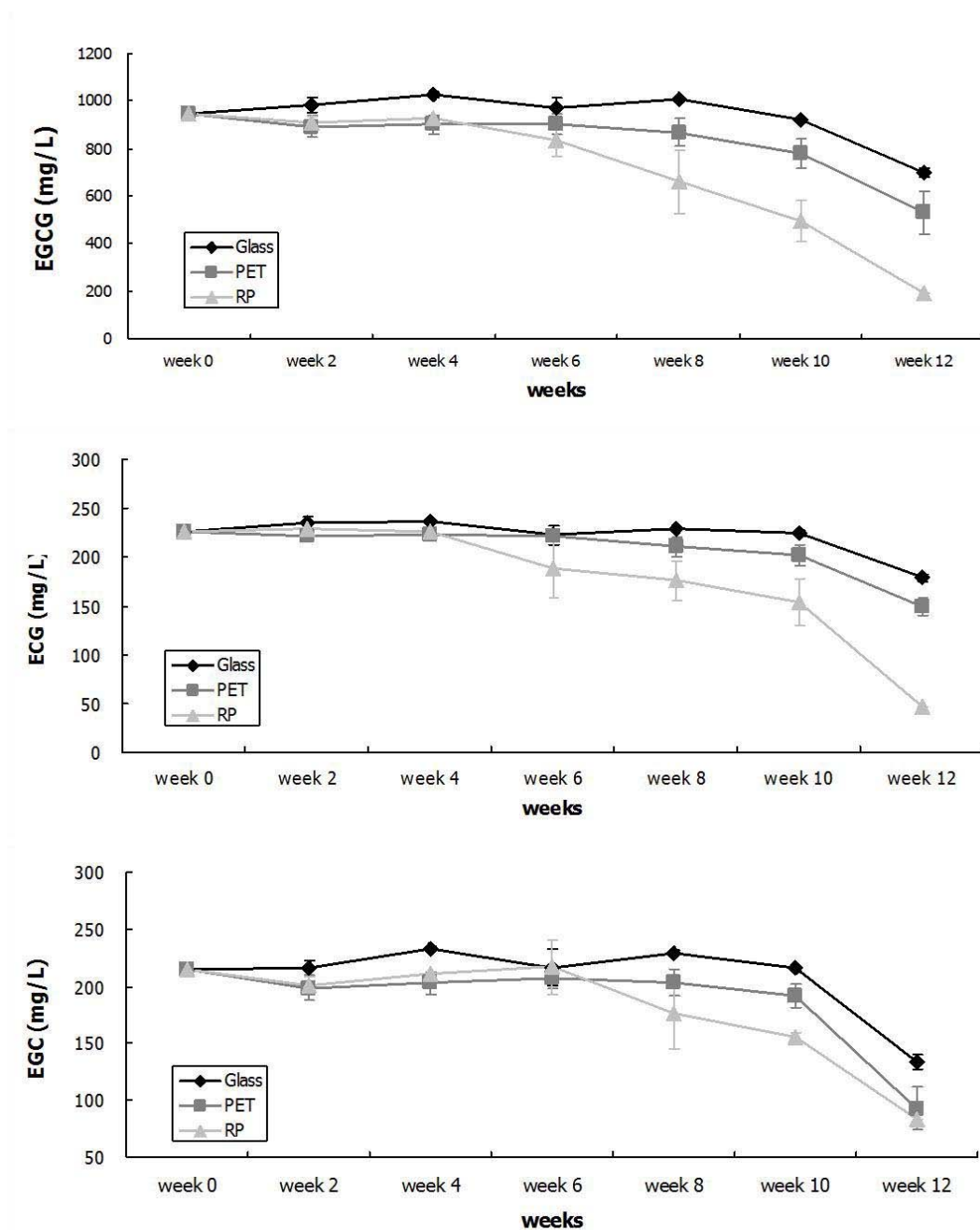
**Table 5-1.** Initial concentrations (mg/L) and  $\lambda_{\max}$  of polyphenolics in green tea infusion identified by HPLC\_PDA<sup>a</sup> at 280 nm.

No.	Compounds	$\lambda_{\max}$	Initial content (mg/L)
1	Gallic acid	277.5	1.62 ± 0.12 <sup>b</sup>
2	(-)-epigallocatechin	272.8	214.81 ± 7.84
3	Caffeine	272.8	445.19 ± 10.1
4	(-)-epigallocatechin gallate	277.5	946.75 ± 29.2
5	(-)-epicatechin	277.5	46.23 ± 1.60
6	(-)-gallocatechin gallate	277.5	96.74 ± 3.78
7	(-)-epicatechin gallate	277.5	225.94 ± 4.73
8	Myricetin 3-glycoside	263.4, 357.5	20.7 ± 0.66
9	Quercetin 3-rutinoside (rutin)	258.6, 357.5	78.93 ± 2.21
10	Quercetin 3-glycoside	258.6, 357.5	23.25 ± 1.78
11	Kaempferol 3-glycoside	268.1, 348.7	13.14 ± 0.69
12	Kaempferol 3-rutinoside	268.1, 349.7	18.39 ± 1.00

<sup>a</sup>. Photodiode Array Detector

<sup>b</sup>. Data are expressed as mean ± standard deviation of n=9.

Packaging green tea infusions into gas-impervious glass bottles was critical to decelerate polyphenolic oxidation in relation to RP and PET. The oxygen transmission rate of PET is 0.02 mL O<sub>2</sub>/pkg/day whereas those of polypropylene and polystyrene, the two plastic polymers of RP, are 0.68 and 2.29 mL O<sub>2</sub>/pkg/day at 6 °C (194). The stability of antioxidant tea catechins in green tea infusion was inversely proportional to the oxygen transmission rates of the respective packaging materials.



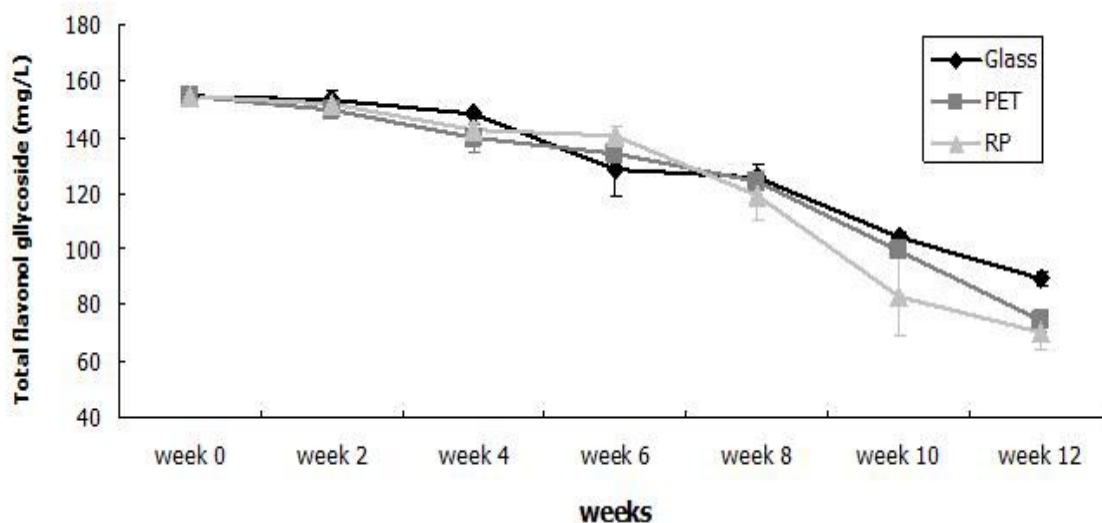
**Figure 5-1.** Changes in epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and epigallocatechin (EGC) (mg/L) contents in green tea infusion in glass, PET and RP without exposure to light at 3 °C during 12 week storage. Error bars represent standard error of the mean (n=3).

The semiquinone radical is formed more readily on the ring possessing three hydroxyl groups as opposed to two hydroxyl groups and this fact makes EGCG the least stable flavan-3-ols present in green tea since EGCG has two moieties such as gallyl group (B ring of flavan-3-ols) and galloyl (gallic acid group attached to C ring of flavan-3-ols) moiety possessing three hydroxyl group (162). More susceptibility to semiquinone free radical formation when compared to other catechins makes EGCG an excellent compound to monitor for green tea quality and stability. Likewise, in the present study, EGCG was more rapidly degraded than ECG (only two hydroxyl groups on its B ring) in PET and EGC (no galloyl group) in RP during 12 week storage. The overall reduction rate of EGC and ECG in PET and RP was lower than EC due to presence of only two hydroxyl groups and no galloyl moiety on its B ring of EC (data not shown). Not only oxidative degradation was observed in PET and RP but also it was found in non-gas permeable glass bottles. Dissolved oxygen in tea infusion may react with flavan-3-ols even though there was no oxygen intake into the glass bottle during storage. This is supported by studies indicated dissolved oxygen accelerated destruction of antioxidants and flavor compounds such as phenolic compounds in various beverages (195, 196, 197). However, there may be low concentration of oxygen present in tea infusions since the water used for brewing tea was previously heated, indicating it may or may not be the main reason to cause oxidative degradation. In tea infusion, hydrogen peroxide ( $H_2O_2$ ) was quickly generated from phenolic compounds with air contact containing dioxygen ( $O_2$ ) under the presence of transition metals (163, 198), which induces generation of free radicals and oxidative degradation as discussed in chapter IV. Therefore, dissolved



oxygen and/or air contact during treatment likely induced oxidative degradation in green tea infusions even when no air intake was allowed into packaging.

Total flavonol glycoside (sum of five flavonol glycosides) decreased by 42, 52 and 55 % in glass, PET and RP, respectively during 12 week storage (**Figure 5-2**) and the presence of flavonol glycosides in green tea infusion is in agreement with Atoui et al. (111) and Del Rio et al. (112). The concentration of total flavonol glycoside was about 10 % of total phenolic compounds in tea infusion, which is significantly lower compared to the concentrations of total flavan-3-ols (90 % of total phenolic compounds). For the first 4 week period, total flavonol glycoside in all the packaging materials did not decrease until Week 4 compared to the concentration in Week 0 but the concentration decreased after Week 4. As observed in changes of flavan-3-ols, total flavonol concentration was significantly higher in glass bottles by 17 and 21 % than that in PET and RP, respectively at the end of 12 week storage. The reduction rate of flavonol glycosides was higher than that of flavan-3-ols in the present study regardless of packaging materials.



**Figure 5-2.** Changes in total concentration of five flavonol glycosides present in green tea (myricetin 3-glycoside, quercetin 3-rutinoside, quercetin 3-glycoside, kaempferol 3-glycoside, and kaempferol 3-rutinoside) during 12 week storage at 3 °C.

Rutin which is the most predominant flavonol glycoside of quercetin (49 % of total flavanol glycoside at 154.41 mg/L) was degraded by oxidation. Oxidative degradation of rutin and its aglycone quercetin was previously reported by Makris and Rossiter, (199). Oxidative degradation of flavonols occurred due to their special structure. Flavonol contains an *ortho*-dihydroxy structure on the B ring (also known as catechol structure), which donates protons and/or chelates metal ions (200).

Additionally, the 2, 3-double bond with respect to the 4-keto function and the 3-hydroxyl group on the C ring contributes to the formation of a *para*-quinoic structure that causes electron delocalization and stabilization of the radicals (200). Since the flavonols show the antioxidant activity from its characteristic structure by donating protons, oxidative degradation may be responsible for lowered antioxidant capacity of

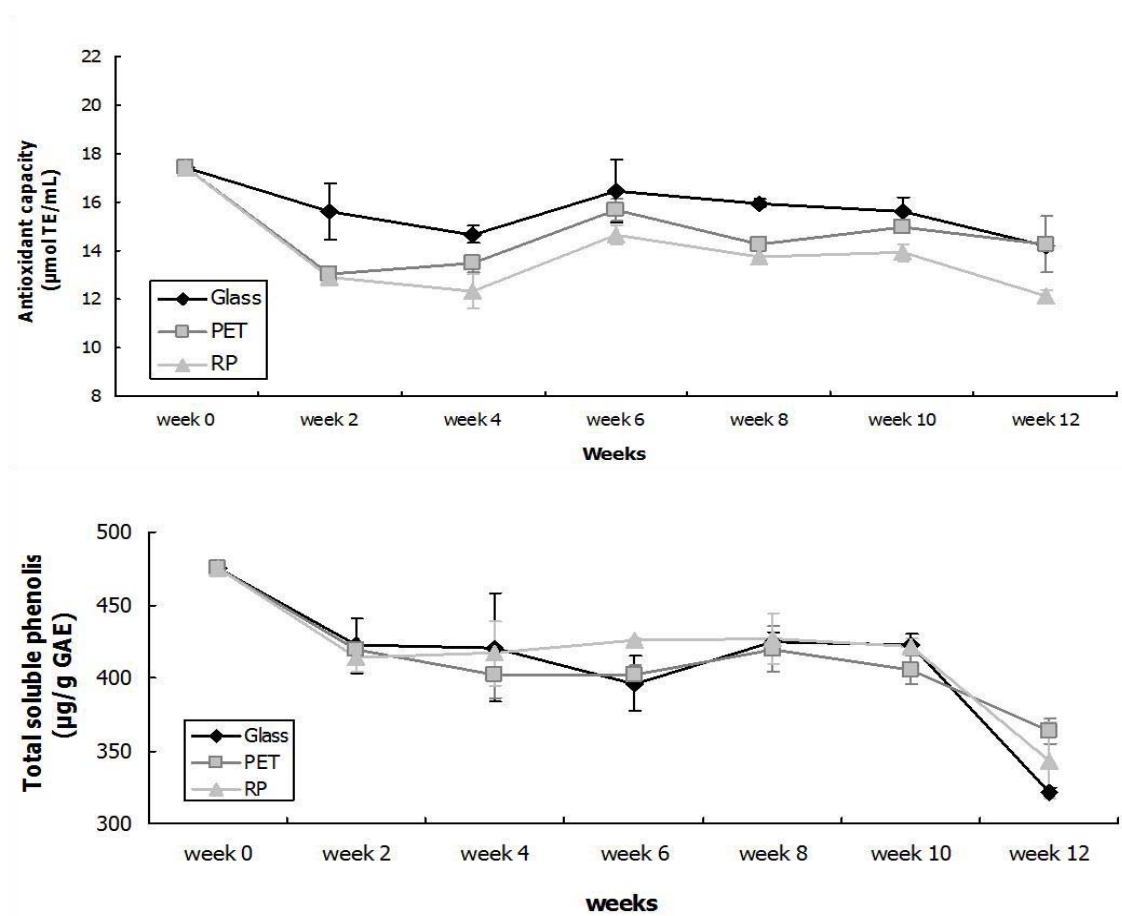
green tea.

Caffeine (1,3,7-trimethylxanthine) was stable for 12 weeks in all types of packaging materials and no difference was observed between packaging materials during 12 week storage (data not shown). Caffeine is known to be stable in under ordinary conditions (not extreme pH and temperature) during storage because it has a stable crystalline structure with a boiling point of 178 °C. Since there is a significant amount of caffeine in green tea (2.5 to 3.5 % of dry weight) and tea quality and health properties are related to caffeine, maintaining caffeine content in tea beverages during storage should be considered a key factor to increase health benefits from tea (40, 86). As a result of this study, packaging was not a factor that influenced the caffeine concentration in green tea.

***Changes in antioxidant capacity and total soluble phenolics of green tea infusion during storage***

Antioxidant capacity of green tea infusions was measured based on peroxy radical scavenging activity of phenolic compounds and found to significantly decrease by 19, 19, and 30 % in glass, PET and RP respectively during 12 weeks storage (**Figure 5-3**). Antioxidant capacity was higher in glass than in PET for the first 8 weeks except Week 6 but no different was observed between Week 8 and 12 between packagings ( $P < 0.05$ ). Antioxidant capacity in RP was significantly lower than in glass throughout storage and it was also lower than PET after 8 weeks of storage. As observed in EGC, EGCG, and ECG changes, antioxidant capacity was higher in glass than in RP

throughout storage but the difference was not significant between glass and PET. This indicates that not only the polyphenolic changes during storage influenced antioxidant capacity of green tea but also other antioxidant compounds contributed to antioxidant capacity changes such as ascorbic acid, and saponins which were not evaluated in this study but the present in green tea was reported by Cabrera et al. (145) and Matsui et al. (201).



**Figure 5-3.** Changes in antioxidant capacity (top) and total soluble phenolics (bottom) of mamaki infusion in glass, PET and RP without exposure to light at 3 °C during 12 week storage. Error bars represent standard error of the mean (n=3). Abbreviation used. TE, Trolox equivalent, GAE. Gallic acid equivalent.

Total soluble phenolics of green tea infusion determined by Folin-Ciocalteu method was also reduced in all packaging materials during 12 week storage. The most reduction in total soluble phenolics occurred between Week 0 and 2 and Week 10 and 12 while no changes were observed in all the packaging materials during storage between Week 2 and 10. No difference of total soluble phenolics was found between packaging materials until Week 10 but it was higher in PET than glass at Week 12. As observed in antioxidant capacity changes, the changes of tea catechins and total soluble phenolics was not directly connected. Total soluble phenolics did not reflect changes of phenolic compounds due to the contribution from ascorbic acid, reducing sugars and some soluble proteins. The presence of ascorbic acid and protein in green tea was confirmed in chapter VII. Moreover, total soluble phenolics was not well correlated with antioxidant capacity ( $r = 0.51$ ).

Folin-Ciocalteu assay used for total soluble phenolics is an ET (electron transfer) based assay which quantifies the reducing capacity of antioxidants in samples based on measuring color changes when oxidants are reduced (177, 202). Since Folin-Ciocalteu assay was developed for protein analysis (176) and later modified for measuring total phenol in wine (178), the assay is not limited to measuring “total soluble phenolics” but can also measure total reducing capacity of any compounds that has reducing power in a sample (202). This explains why total soluble phenolics did not reflect the concentration changes of polyphenolics in green tea that contains other reducing compounds. The correlation between total soluble phenolics and polyphenolics is highly dependent on the type of samples and constituents in a sample. The assays for

measurement of antioxidant capacity of a sample are generally divided into two categories based on their principle chemistry. ET-based assay generally includes a redox reaction using an oxidant probe to monitor the reaction to an endpoint and to indicate the endpoint of the reaction (177). The ET-based assays include Folin-Ciocalteu assay, TEAC (Trolox equivalent antioxidant capacity), FRAP (ferric ion reducing antioxidant parameter) and DPPH (diphenyl-1-picrylhydrazyl copper (II) reduction capacity) (202). Another basic assay used to measure antioxidant capacity is HAT (hydrogen atom transfer) which operates on the basis of measuring the inhibitory capacity of antioxidants against thermally generated peroxy free radicals by hydrogen donation (202, 203). The antioxidant assays sharing this mechanism are ORAC (oxygen radical absorbance capacity), TRAP (total radical trapping antioxidant parameters), Crocin bleaching assay, IOU (inhibited oxygen intake), inhibition of linoleic acid oxidation, and inhibition of LDL oxidation (177). In the present study, the results from both assays were not well correlated and it may be due to the difference in chemistry of these two assays.

### ***Phytochemical changes in different packaging materials containing yaupon holly infusion***

As an American holly species in the *Ilex* genus, yaupon holly was previously reported to have a similar phytochemical composition to the South American *Ilex paraguariensis* commonly known as yerba maté (48, 117). Chlorogenic acid and its isomeric forms (3 and 4-caffeoylquinic acids) were the predominant phenolic compounds in yaupon holly infusions accounting for 67 % of total polyphenolics present

followed by two dicaffeoylquinic acids at 16 % and flavonol glycosides at 14 % of the total phenolic concentration (1,596 mg/L) (**Table 5-2**). This phenolic profile is similar to the polyphenolics reported in *Ilex paraguariensis* and included chlorogenic acid (5-caffeoylquinic acid), chlorogenic acid isomers (3 and 4-caffeoylquinic acids), 3, 4 and 3, 5-dicaffeoylquinic acids, and quercetin glycosides (117, 204).

**Table 5-2.** Initial concentrations (mg/L) and  $\lambda_{\text{max}}$  of polyphenolics in yaupon holly infusion identified by HPLC\_PDA<sup>a</sup> at 280 nm.

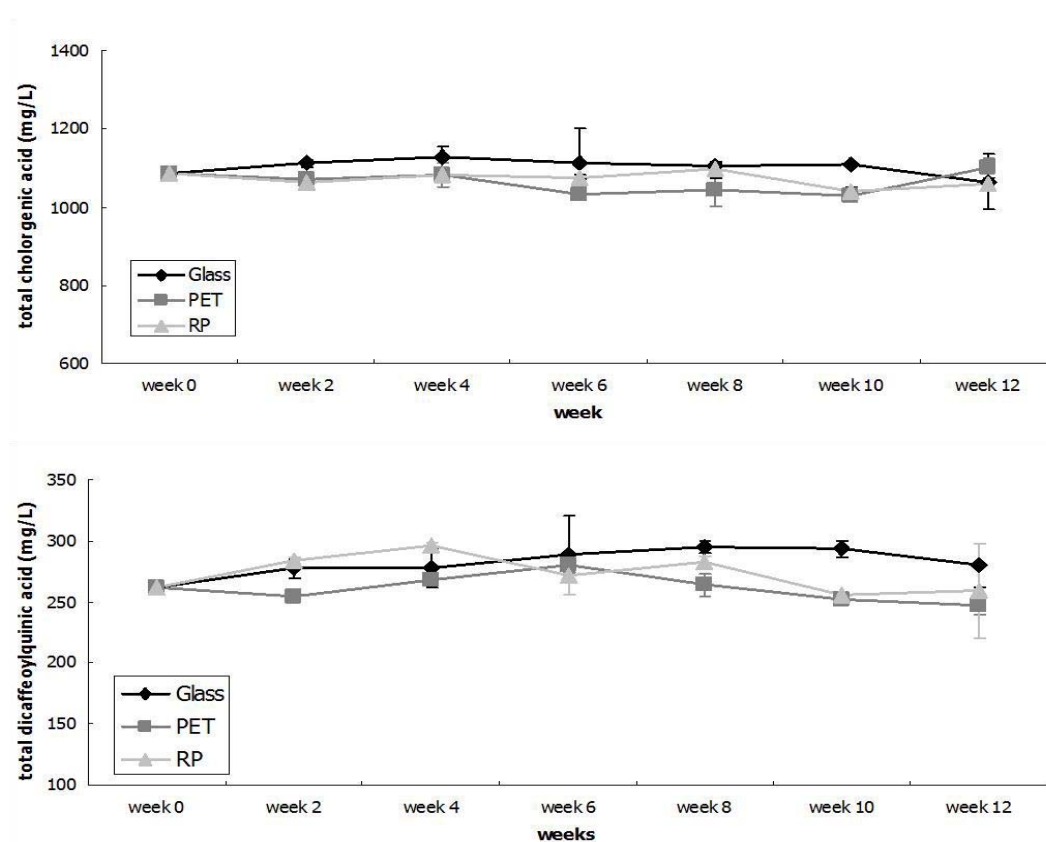
No.	Compounds	$\lambda_{\text{max}}$	Initial content (mg/L)
1	3-caffeoylquinic acid	324.9	256.18 ± 9.5 <sup>b</sup>
2	5-caffeoylquinic acid	329.6	541.62 ± 16.9
3	4-caffeoylquinic acid	329.6	279.36 ± 10.2
4	3,4-dicaffeoylquinic acid	329.6	45.10 ± 3.7
5	3,5-dicaffeoylquinic acid	329.6	216.85 ± 15.2
6	Quercetin-3-rutinoside (rutin)	256.4, 363.8	237.44 ± 13.8
7	Keampferol-3-rutinoside	268.1, 348.7	19.93 ± 3.8

<sup>a</sup>. Photodiode Array Detector

<sup>b</sup>. Data are expressed as mean concentration (mg/L) ± standard deviation of n=9

During the first 4 weeks of storage, the concentration of total chlorogenic acid (sum of chlorogenic acid and its isomers) did not appreciably change in any of the packaging materials ( $P < 0.05$ ) (**Figure 5-4**). The stability of chlorogenic acid was in agreement with several studies where certain polyphenols such as chlorogenic acid were stable in beverages and plants during long-term storage (67, 180, 205). Additionally, in

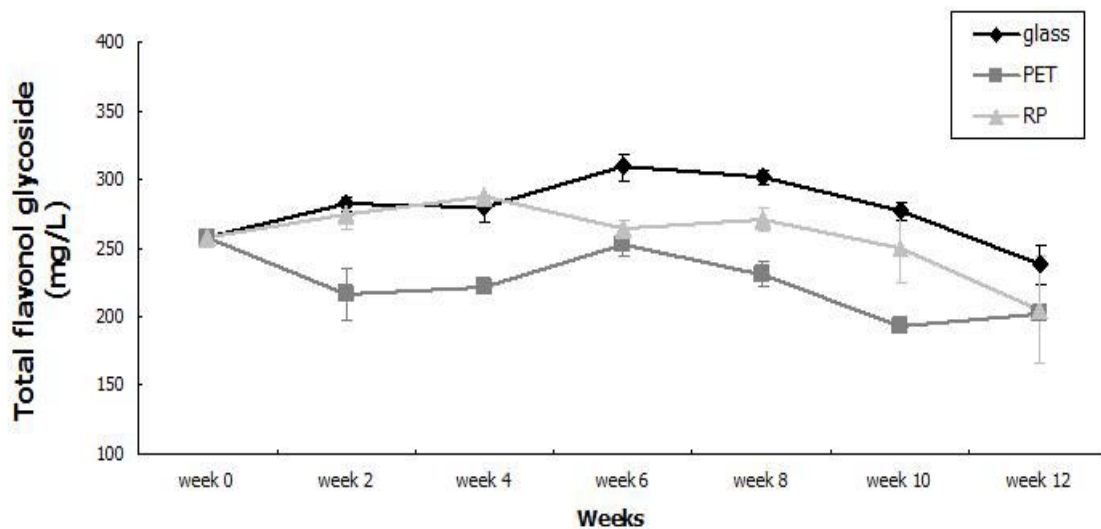
terms of oxidation, it was earlier reported that chlorogenic acid was not degraded even during 12 weeks of dark storage at 4 °C even though the sample fruit drink was stored in gas-permeable polyethylene packaging as observed in the present study (77). A similar tendency was observed for total dicaffeoylquinic acid (sum of 3,4 and 3,5-dicaffeoylquinic acid) in yaupon holly infusions where no significant concentration change was observed during storage in all packaging materials.



**Figure 5-4.** Changes in total chlorogenic acid (3,4, and 5-caffeoylquinic acid, top) and total dicaffeoylquinic acid (3,4 and 3,5-dicaffeoylquinic acid, bottom) (mg/L) contents in yaupon holly infusion in glass, PET and RP without exposure to light at 3 °C during 12 week storage. Error bars represent standard error of the mean (n=3).



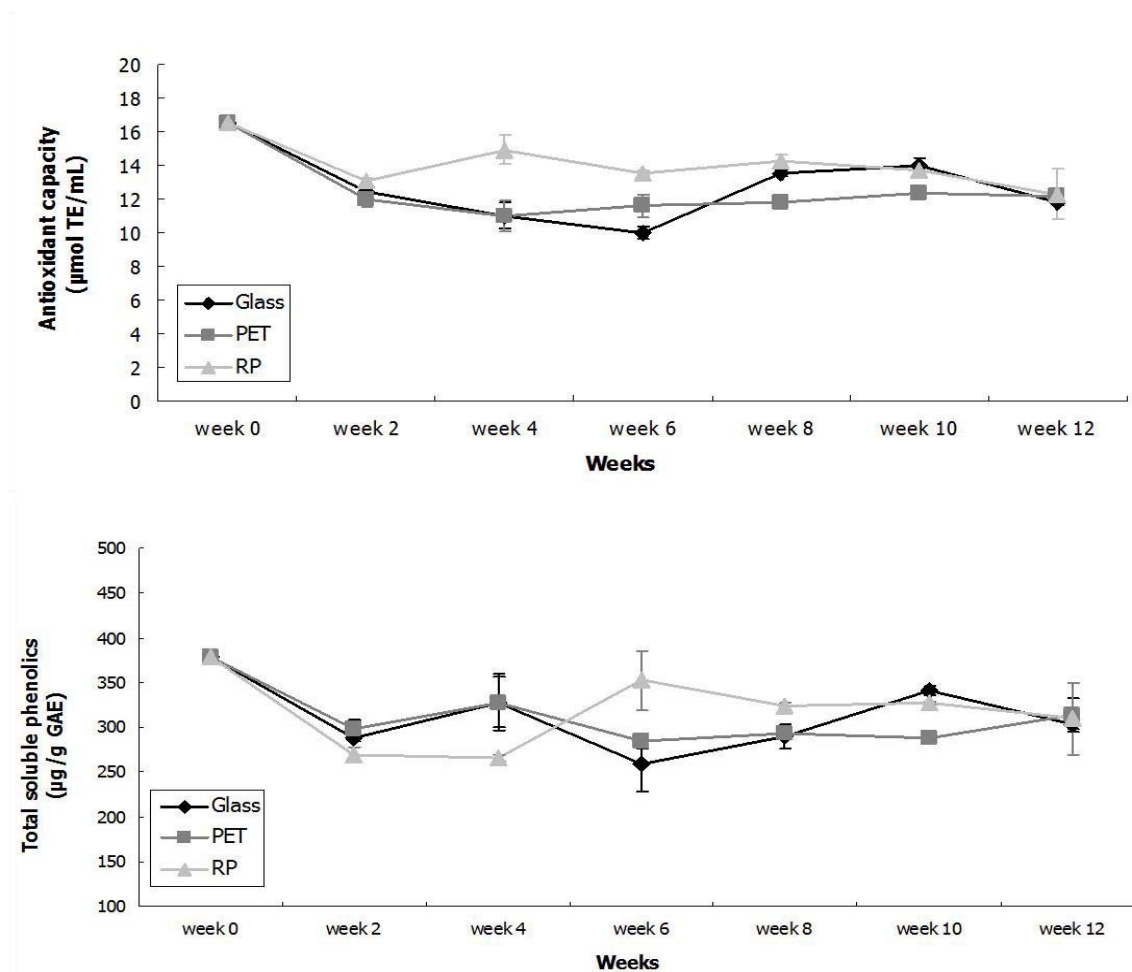
Flavonols have been recognized as potent antioxidants due to the keto group conjugated to a double bond in the C ring and the presence of hydroxyl groups in the B ring (142). The total concentration of two flavonol glycosides was reduced by 22 and 21 % in PET and RP, respectively, while no change was observed in glass during 12 week storage (**Figure 5-5**). Even though a difference in total flavonol content was found in between non gas-permeable packaging (glass) and gas-permeable packagings (PET and RP) as observed in green tea infusion, the concentration in glass was not reduced while there was significant reduction in glass of green tea infusion. Since all the flavonol glycosides in green tea and yaupon holly were degraded without respect to the packaging materials, it was somewhat unexpected that no degradation of flavonol glycosides was noted in glass packaged yaupon holly infusions. Saponins, which were previously reported present in the *Ilex* genus, were also detected in yaupon holly infusion. In the study conducted in chapter VII, saponins were found at the highest concentration in yaupon holly infusion as compared to green tea and mamaki infusions. Saponins are degraded by many factors such as processing, cooking, and storage (206, 207). The reaction between polyphenols and saponins have been reviewed by Argentieri et al. (208) and San Martin and Magnunacelaya, (209) who described a higher biological effect when those compounds are present together *in vitro*. It was hypothesized that higher amount of saponins in yaupon holly infusion stored in glass bottles may cause protective effect on flavonols resulting in higher stability throughout the storage.



**Figure 5-5.** Changes in total flavonol glycosides (mg/L) present in yaupon holly infusion in glass, PET and RP without exposure to light at 3°C during 12 week storage. Error bars represent standard error of the mean (n=3).

***Changes in antioxidant capacity and total soluble phenolics of yaupon holly infusion during storage***

The antioxidant capacity of yaupon holly infusion was reduced by 29, 27, and 26 % in glass, PET and RP, respectively during 12 week storage (**Figure 5-6**). No difference was found in antioxidant capacity in all packagings at the end of storage but it was higher in glass between Week 2 and 6, whereas there was no difference between PET and RP during storage. This study reports new data regarding to packaging impact on antioxidant capacity changes and confirms that antioxidant capacity of yaupon holly infusion was influenced by type of packaging materials for only short term storage. Total soluble phenolics of yaupon holly infusion were also reduced within 2 weeks of storage



**Figure 5-6.** Changes in antioxidant capacity (top) and total soluble phenolics (bottom) of yaupon holly infusion in glass, PET and RP without exposure to light at 3 °C during 12 week storage. Error bars represent standard error of the mean (n=3). Abbreviation used. TE, Trolox equivalent, GAE. Gallic acid equivalent.

and no changes were observed for the rest of storage as observed in antioxidant capacity changes. No packaging effect was observed between packaging materials except total soluble phenolics was higher in glass between Week 4 and 6. Antioxidant capacity and total soluble phenolics did not reflect the changes of polyphenolics present in green tea and yaupon holly infusions due to the presence of the compounds contributing to their

values.

***Phytochemical changes in different packaging materials containing mamaki infusion***

Chlorogenic acid and its isomers and four flavonol glycosides were identified by comparing with authentic standard based on retention time and similarity of spectra. The most predominant compounds in mamaki infusion were chlorogenic acid and its isomers (77 %) followed by flavonol glycosides (23 %) where the total phenolic concentration of mamaki was 806.5 mg/L (**Table 5-3**). The total phenolic concentration of mamaki was lower by 49 % than that of yaupon holly and the major phenolic compounds (chlorogenic acid and its isomers) were also significantly lower by 43 %. Besides chlorogenic acid and its isomers, quercetin-3-rutinoside (rutin) was the most predominant flavonol glycoside in mamaki infusion as observed in yaupon holly infusion, however, the concentration was higher in mamaki infusion.

**Table 5-3.** Initial concentrations (mg/L) and  $\lambda_{\max}$  of polyphenolics in mamaki infusion identified by HPLC\_PDA<sup>a</sup> at 280 nm.

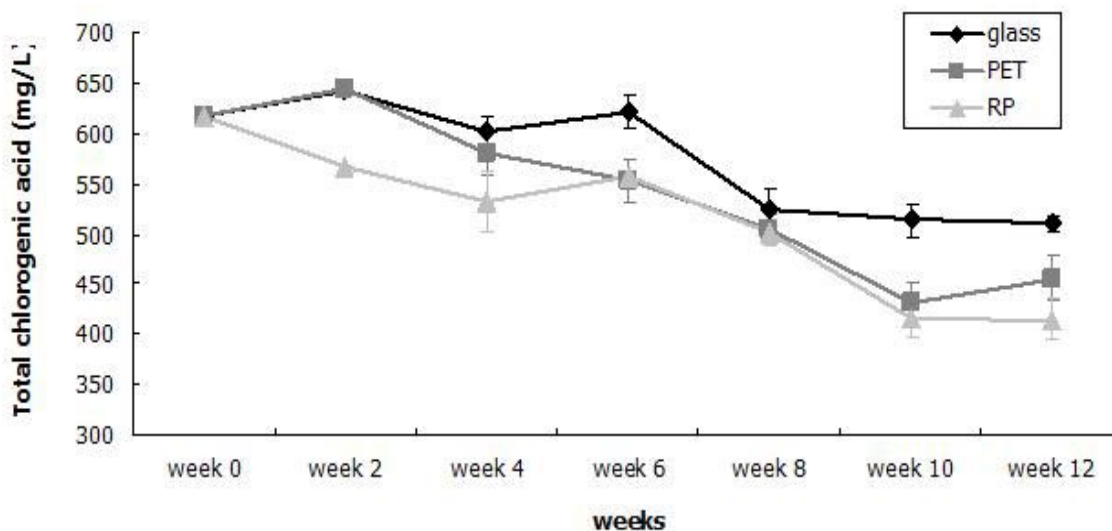
No.	Compound	$\lambda_{\max}$	Initial concentration (mg/L)
1	3-caffeoylquinic acid	324.9	210.63 ± 25.50 <sup>b</sup>
2	5-caffeoylquinic acid	329.6	203.85 ± 17.10
3	4-caffeoylquinic acid	329.6	203.31 ± 7.57
4	Apigenin 3-glucoside	268.1, 339.2	19.43 ± 0.80
5	Apigenin 3-glucoside	272.8, 339.2	25.78 ± 2.34
6	Quercetin-3-rutinoside (rutin)	258.6, 357.5	123.12 ± 11.50
7	Keampferol-3-rutinoside	268.1, 348.7	20.34 ± 1.67

<sup>a</sup>. Photodiode array detector

<sup>b</sup>. Data was expressed as mean concentration (mg/L) ± standard deviation of n=3.

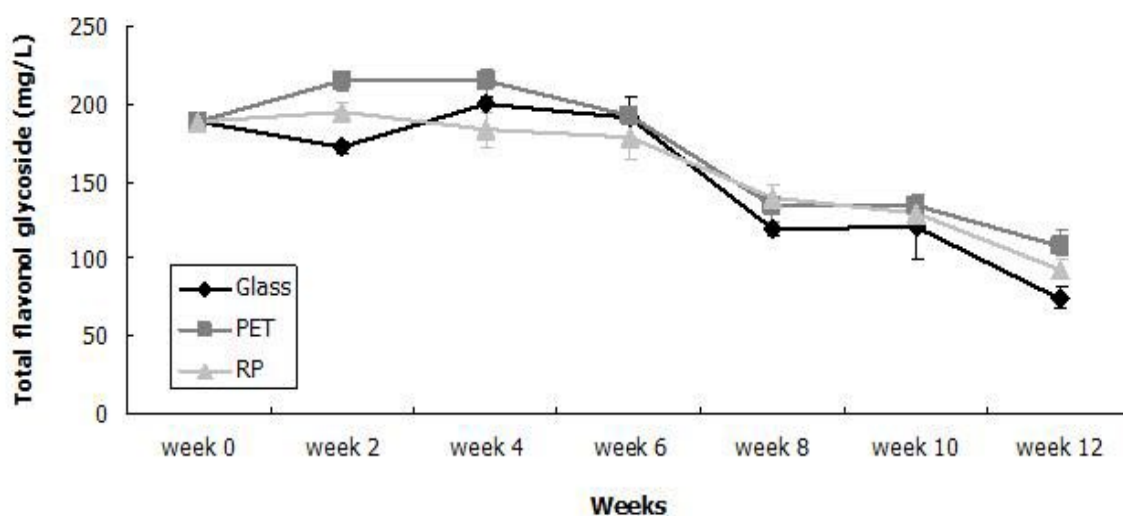
Total chlorogenic acid (sum of chlorogenic acid and its isomers) decreased by 18, 27 and 33 % in glass, PET, and RP, respectively, during 12 week storage (**Figure 5-7**). This tendency was not observed in total chlorogenic acid in yaupon holly infusion. Since chlorogenic acid is generally stable during storage for most beverages (210), significant reduction of chlorogenic acid in mamaki infusion was not predicted. However, it is known that the stability of polyphenolic compounds is highly pH dependent. When pH increases, the stability of polyphenolics, including phenolic compounds present in tea such as gallic acid, flavan-3-ols, chlorogenic acid and rutin, is inversely associated with elevated pH (33, 180, 211) while higher stability is observed under acidic environment (212, 213). The pH of each tea was 5.09, 4.94 and 7.50 in green tea, yaupon holly, and

mamaki, respectively. This naturally high pH of mamaki infusion unlike other teas was a factor to reduce chlorogenic acid and its isomers in mamaki infusion in all packaging materials. The reduction of total chlorogenic acid was higher in oxygen permeable packaging materials than non-oxygen permeable packaging (RP > PET > glass). According to Oszmianski and Lee, (214), the oxidation of chlorogenic acid was significantly higher at pH 6.5 than pH 3.5 when phenolic oxidation was determined using PPO (polyphenol oxidase). Chlorogenic acid and its isomers were degraded at neutral pH and the reduction rate was higher when the neutral pH tea infusion was stored in oxygen permeable packaging materials.



**Figure 5-7.** Changes in total chlorogenic acid (3, 4, and 5-caffeoylquinic acid, top) (mg/L) in mamaki infusion in glass, PET and RP without exposure to light at 3 °C during 12 week storage. Error bars represent standard error of the mean (n=3).

In mamaki infusion, the flavonol glycosides were present at higher concentration (30 % of total phenolics in mamaki) compared to green tea (9 %) and yaupon holly (16 %) infusions. The total concentration of four flavonol glycosides was significantly reduced by 73, 65, and 68 % in glass, PET and RP, respectively during 12 week storage (**Figure 5-8**). No impact of packaging materials was found on total flavonol glycoside content in the mamaki infusion. The total flavonol glycosides in mamaki infusion were lower than that of yaupon holly by 27 % while it was higher than that of green tea by 19 %.



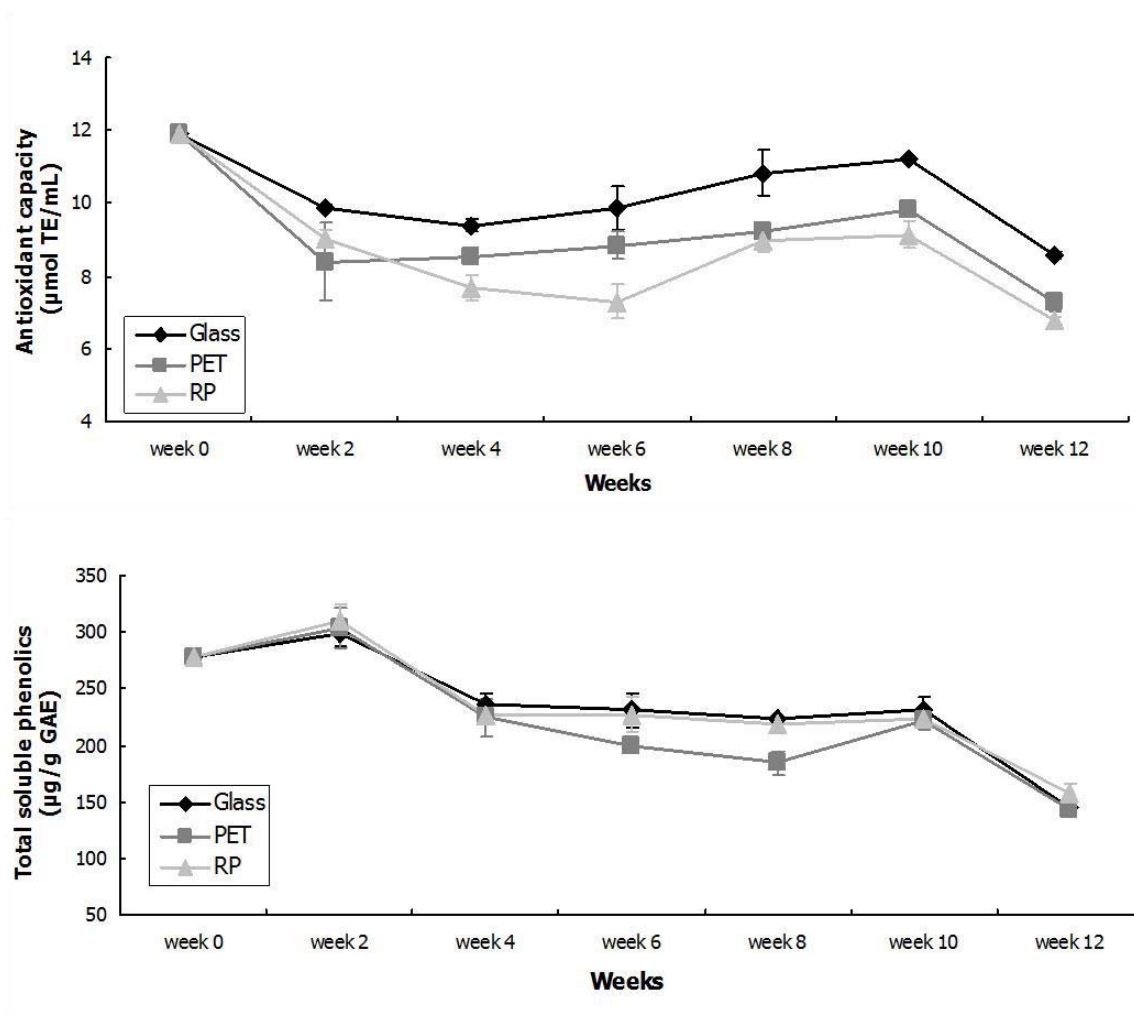
**Figure 5-8.** Changes in total flavonol glycosides (mg/L) in mamaki infusion in glass, PET and RP without exposure to light at 3 °C during 12 week storage. Error bars represent standard error of the mean (n=3).

***Changes in antioxidant capacity and total soluble phenolics of mamaki infusion during storage***

Antioxidant capacity of mamaki infusion was higher in glass bottles than in PET

and RP and the reduction rate was 28, 39 and 43 % in glass, PET and RP, respectively during 12 week storage (**Figure 5-9**). Even though polyphenolics present in mamaki tea infusion was reduced during 12 week storage, antioxidant capacity was significantly higher in glass bottles throughout storage. Also, it was higher in PET than RP during storage except Week 2, 8, and 12, indicating antioxidant capacity was influenced by oxidative degradation of chlorogenic acid and its isomers present in mamaki. Since significantly less saponin was found than green tea and yaupon holly as determined in chapter VII and no caffeine that has antioxidant property is present in mamaki infusion, antioxidant capacity was more influenced by phenolic compound changes compared to previous tea infusions. Total soluble phenolics was reduced during 12 week storage in all packaging materials and the reduction rate was 48, 48 and 45 % in glass, PET and RP, respectively. Packaging had no impact on total soluble phenolics during storage only except for lower levels in PET between Week 6 and 8. Since mamaki infusion contains a higher concentration of non-polyphenolic compounds that have reducing capacity, such as protein and metal ions, total soluble phenolics did not reflect the changes of phenolic compounds in mamaki infusion.





**Figure 5-9.** Changes in antioxidant capacity (top) and total soluble phenolics (bottom) of mamaki infusion in glass, PET and RP without exposure to light at 3 °C during 12 week storage. Error bars represent standard error of the mean (n=3). Abbreviation used. TE, Trolox equivalent, GAE. Gallic acid equivalent.

### *Color changes in green tea, yaupon holly, and mamaki during storage*

L\* value (relative darkness or lightness) of green tea did not significantly change yet slightly increased in the three different packagings throughout 12 week storage (Table 5-4). During tea storage, the unique color of green tea (bright green) changes to

olive green (dark green) and then it continues to change to brownish green (83). However, in this study, independent of the packaging material, the lightness of green tea ( $L^*$ ) only slightly increased from greenish to brownish, indicating either there was no significant changes in color compounds including chlorophyll or the color compounds were present at very low concentration. It is known that color deterioration of green tea is caused by conversions from chlorophyll a and b (greenish) to pheophytin a and b (olive-brown), however, since chlorophyll is not a water soluble compound, only insignificant amounts are present in green tea infusion (83). Hue angle and chroma were numerically reported to describe yellow ( $90^\circ$ ) or green ( $180^\circ$ ) if  $a^* > 0$  and  $b^* < 0$  and vividness to dullness (high to low) of tea color, respectively (193, 215). Hue angle in

**Table 5-4.** Changes in  $L^*$ , hue angle, and chroma values of green tea infusion in three packaging materials at  $3^\circ\text{C}$  during 12 week storage.<sup>a</sup>

Packaging	$L^*$			Hue angle ( $^\circ$ )			Chroma		
	Glass	PET <sup>c</sup>	RP <sup>d</sup>	Glass	PET	RP	Glass	PET	RP
<b>Week 0</b>	18.3b <sup>b</sup>	18.3b	18.3a	151.7a	151.7ab	151.7b	7.72b	7.72c	7.72c
<b>Week 2</b>	20.9a	21.9a	20.8a	151.5a	139.8d	146.2bc	8.97ab	8.63bc	9.22bc
<b>Week 4</b>	20.6ab	17.5b	19.5a	147.4a	147.6bc	150.5b	9.44a	9.22b	9.01bc
<b>Week 6</b>	20.5ab	21.0a	19.8a	151.5a	146.1d	151.6b	9.78a	8.85bc	9.81b
<b>Week 8</b>	21.9a	22.4a	21.4a	154.6a	157.6a	164.1a	9.50a	10.85a	12.01a
<b>Week 10</b>	21.3a	20.6a	19.2a	152.7a	154.8a	147.9bc	9.18ab	9.06bc	9.76b
<b>Week 12</b>	19.9ab	18.4b	19.9a	150.4a*	142.9cd	140.6c	9.70a	8.76bc	8.58bc

<sup>a</sup>. Data are expressed as mean of  $n=3$ .

<sup>b</sup>. Means with similar letters within columns are not significantly different during 12 week storage (LSD test,  $P < 0.05$ ).

<sup>c</sup>. Polyethylene terephthalate

<sup>d</sup>. Retortable pouch

glass bottle did not change during 12 week storage while hue angle was significantly reduced in PET and RP. This is in agreement with the work by Alper and Acar, (197) where the hue angle of fruit juice was reduced after oxidation. Losing the characteristic green color due to the oxidation leads to quality degradation of green tea. Chroma value of green tea in glass packaging was also significantly elevated whereas the values in PET and RP did not change throughout tea beverage. Increase in chroma indicates that purity of tea infusion was improved but lowered chroma represents colors existing in tea infusion were mixed, which may cause consumer rejection for the tea product (193). L\* values of yaupon holly during storage did not significantly changes in all packaging materials during 12 week storage (**Table 5-5**). Also, only minute differences were found among L\* values of packaging materials as observed in green tea infusion. Hue angle which is an indicator in showing the degree of greenish and yellowish of objects were also not altered in glass and RP but reduced in PET bottles. The hue angle change indicates that the characteristic light brown color of yaupon holly infusion was not altered in glass and RP but it became darker in PET. Chroma values of yaupon holly significantly increased in all packaging materials resulting in no changes in purity of yaupon holly infusion during 12 week storage.

**Table 5-5.** Changes in L\*, hue angle and chroma values of yaupon holly infusion in three packaging materials at 3°C during 12 week storage.<sup>a</sup>

Packaging	L*			Hue angle (°)			Chroma		
	Glass	PET <sup>c</sup>	RP <sup>d</sup>	Glass	PET	RP	Glass	PET	RP
<b>Week 0</b>	17.1cd <sup>b</sup>	17.1a	17.1b	152a	152a	152a	7.28c	7.28d	7.28d
<b>Week 2</b>	19.4ab	17.9ab	18.7ab	144b	143b	152a	8.37bc	9.00bc	8.73bcd
<b>Week 4</b>	20.1a	16.0b	16.5b	144b	140bc	145b	9.26ab	10.4a	10.1ab
<b>Week 6</b>	19.3ab	17.9ab	17.7ab	146ab	140bc	149ab	9.00ab	9.14bc	9.31abc
<b>Week 8</b>	17.3cd	16.1ab	17.9ab	149ab	139c	147ab	10.2a	10.1ab	10.2a
<b>Week 10</b>	18.2bc	18.9a	20.4a	146ab	138c	146ab	9.05ab	8.69c	8.41cd
<b>Week 12</b>	16.5d	16.3ab	17.8ab	150ab	137c	147ab	8.90ab	9.14bc	8.83abc

<sup>a</sup>. Data are expressed as mean of n=3.

<sup>b</sup>. Means with similar letters within columns are not significantly different during 12 week storage (LSD test, P < 0.05).

<sup>c</sup>. Polyethylene terephthalate

<sup>d</sup>. Retortable pouch

Lightness of mamaki tea infusion (L\*) also did not change as observed in color changes of previous two tea infusions in all packaging materials during storage (**Table 5-6**). However, hue angle increased in glass bottles while hue angles in other packaging materials were not altered during storage. This represents that the mamaki infusion's characteristic light brown color changed to greenish brown. Chroma values in each tea all increased regardless of packaging materials during 12 week storage. By development of several color compounds in mamaki, the tea lost its own color, so dullness (mixed color) of each tea color was elevated but the packagings did not affect those changes.

**Table 5-6.** Changes in L\*, hue angle, and chroma values of mamaki infusion in three packaging materials at 3°C during 12 week storage.<sup>a</sup>

Packaging	L*			Hue angle (°)			Chroma		
	Glass	PET <sup>c</sup>	RP <sup>d</sup>	Glass	PET	RP	Glass	PET	RP
<b>Week 0</b>	15.2ab <sup>b</sup>	15.2ab	15.2ab	101bc	101abc	101ab	8.34d	8.34d	8.34d
<b>Week 2</b>	15.5a	16.0a	16.5a	99.3c	102ab	99.9b	10.8bc	9.76c	9.90cd
<b>Week 4</b>	12.2c	15.2ab	13.0b	105ab	98.7c	101ab	12.4ab	11.2ab	13.3a
<b>Week 6</b>	13.4abc	15.0ab	13.7ab	108a	100bc	98.9b	10.6bc	9.74c	11.7ab
<b>Week 8</b>	15.6a	11.6c	12.8b	100c	102ab	99.9b	10.3c	11.2ab	11.8ab
<b>Week 10</b>	13.0bc	15.1ab	14.4ab	104abc	102abc	106a	11.6bc	10.3bc	10.9bc
<b>Week 12</b>	14.3abc	13.5bc	15.4ab	108a	104a	103ab	14.0a	12.3a	12.0ab

<sup>a</sup>. Data are expressed as mean of n=3.

<sup>b</sup>. Means with similar letters within columns are not significantly different during 12 week storage (LSD test, P < 0.05).

<sup>c</sup>. Polyethylene terephthalate

<sup>d</sup>. Retortable pouch

**CHAPTER VI**

**DETERMINATION OF THE INFLUENCE OF VARIABLE STORAGE  
CONDITIONS ON THE QUALITY AND PHYTOCHEMICAL STABILITY OF  
THREE TEA VARIETIES**

**Introduction**

Ready-to-drink (RTD) teas are exposed to potentially deleterious conditions during handling, transportation, and retail storage that may range from a few days to a few years of storage. In today's market, RTD teas are gaining in popularity due to their convenience and perceptions of health and wellness. However, chemical compounds in RTD tea products naturally deteriorate during storage. According to Yamanish (83), deterioration in tea quality during storage appears in several ways such as a reduction of ascorbic acid, color changes, aroma transformations, and taste loss of astringency and/or bitterness characteristic of a particular tea. These reactions are caused and accelerated by oxygen, light, and temperature (192). The loss of aroma of tea is caused by decrease of several volatile compounds during storage. The major contributor to the characteristic aroma in tea, (z)-3-Hexenyl hexanoate, naturally decreases while the other contributor, 2,4-heptadienal increases. This combination is the main mechanism to alter the aroma of tea. Other unsaturated carbonyl compounds such as 2, 6, 6-trimethyl-2-hydroxycyclohexanone,  $\beta$ -cyclocitral,  $\alpha$ -ionone,  $\beta$ -ionone, 5, 6-epoxy- $\beta$ -ionone, and dihydroactinidiolide also increases and affects aroma of tea during storage. Otherwise,

the color of tea is decided upon the amount of flavonoids, oxidation, and/or condensation products present as the major compounds that give tea its characteristic color (41, 216).

Changes related to antioxidant phytochemical stability in botanical teas held under various storage conditions are scarcely reported in the literature. Since teas are widely claimed to show health benefits such as anti-stress, immunomodulation, anti-aging, anti-inflammatory, anti-depressant, anti-diabetic, and as a stimulant (217) based on their high polyphenolic contents. Understanding their changes during storage are critical such that efforts to maintain the stability and determining the most appropriate storage conditions can be assessed. In the present study, three tea varieties such as green tea, yaupon holly, and mamaki were chosen for determination of storage stability variable conditions of light and temperature and evaluated for phytochemical stability and resultant antioxidant capacity.

## **Materials and Methods**

### ***Tea preparation and storage conditions***

Three tea infusions from green tea, yaupon holly, and mamaki were prepared by the method described in chapter III and transferred to previously sterilized glass bottles (120 mL) to prevent air intake during storage. Headspace was minimized by filling tea infusion to bottle capacity (6 bottles each) and evaluated in a storage chamber held at 3, 12, and 25 °C. In each storage chamber, half of tea samples were shielded from light by aluminum foil (in the dark) while the other half were exposed to fluorescent light at 725

lumens placed 50 cm away from the sample bottles (in the light). Infusions were stored for 12 weeks and analyzed biweekly for chemical and color analysis. Samples were taken from the bottles through the caps fitted with a rubber septum and sampled using a sterilized needle in attempts to prevent microbial contamination and air intake during sampling.

### *Chemical analyses*

Individual polyphenolics were determined by HPLC and total soluble phenolics measured by the Folin-Ciocalteu assay as described in chapter IV. Antioxidant capacity was determined by the method described in chapter III.

### *Statistical analysis*

Data was analyzed as a 2 x 3 x 7 full factorial including three temperatures with or without exposure to fluorescent light at seven sampling times for each tea. Data represent the mean triplicate analysis using ANOVA (analysis of variance) with JMP 5 statistical software (110). Mean separation was conducted using the LSD test ( $P < 0.05$ ).

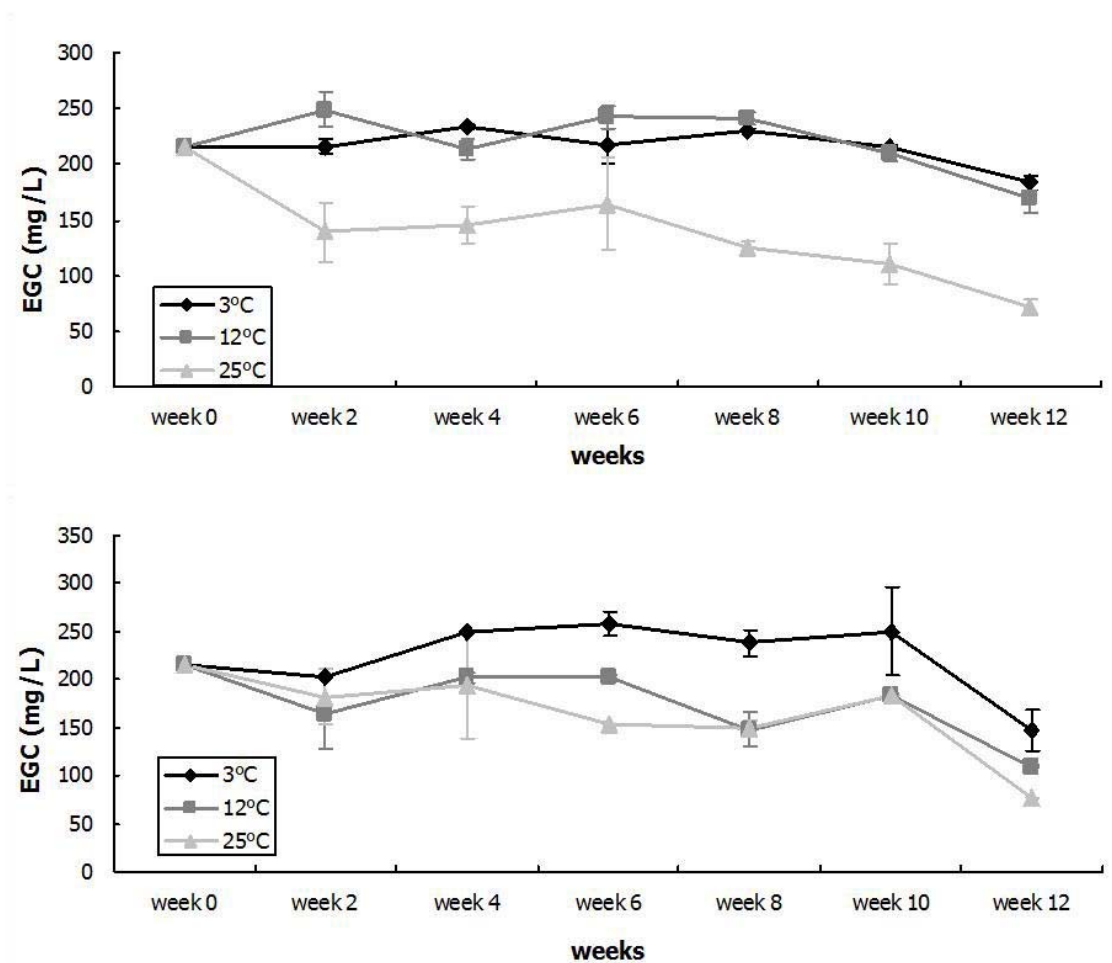


## Results and Discussion

### *Changes in polyphenolics present in green tea infusion by different storage temperatures*

Storage conditions (temperature and light exposure) influenced on phytochemical stability during 12 week storage and in general, when green tea infusion was stored under the lower temperature in the dark, polyphenolics were higher retained. Total polyphenolics in green tea infusion determined using HPLC was 1691 mg/L and the most predominant compounds were flavan 3-ols (EGC, EGCG, EC, GCG, and ECG) at the concentration of 1530 mg/L (90 % of total polyphenolics). Among flavan 3-ols, EGCG concentration was highest at 946.8 mg/L followed by ECG (225.9 mg/L) and EGC (214.8 mg/L). Flavonol glycosides were present at the concentration of 154.4 mg/L (10 % of total polyphenolics) and only 6.5 mg/L (less than 1 %) of gallic acid was found in the infusion.

During 12 week storage under various conditions, the three major flavan 3-ols (EGCG, ECG, and EGC) were reduced in all storage conditions and lower storage temperature was more effective to retain their concentrations than higher temperatures. ECG which was found as the third most major flavan 3-ols in green tea infusion was degraded by 15, 21, and 67 % at 3, 12, and 25 °C, respectively in the dark while 31, 34, and 64 % reduction was observed in the light (**Figure 6-1**). When green tea infusion was stored in the dark, EGC concentration was significantly higher at lower temperatures (3 and 12 °C) compared to storage at 25C, while higher concentration of EGC was

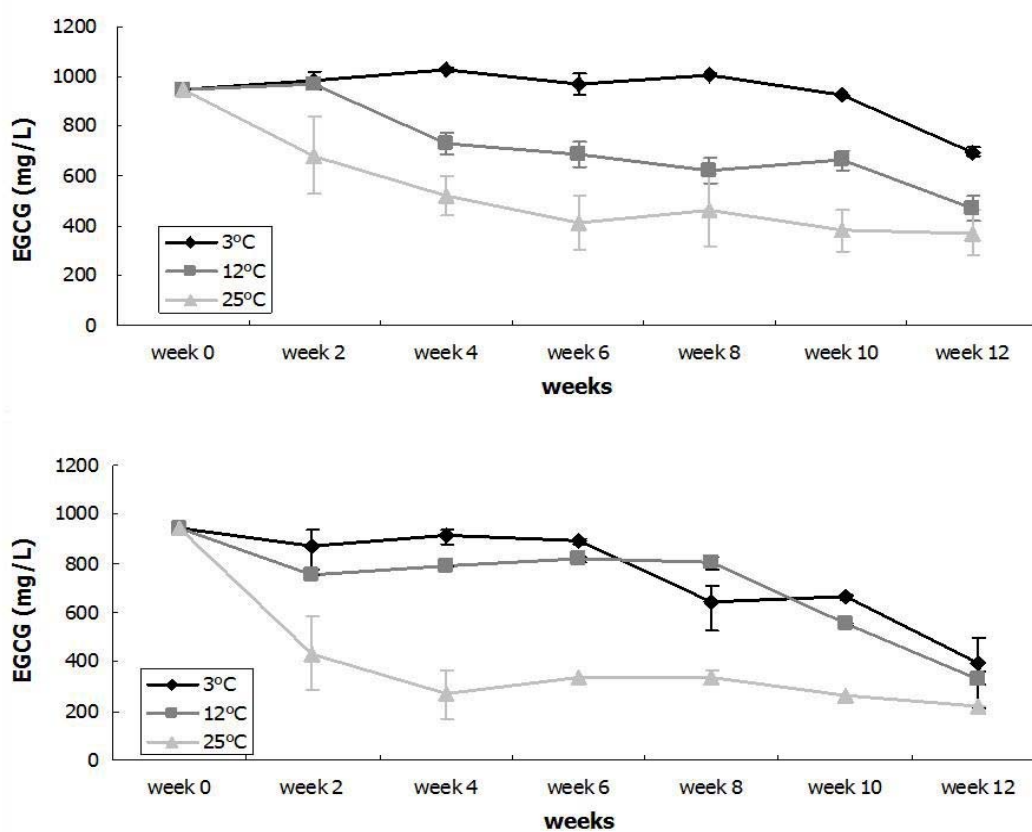


**Figure 6-1.** Changes in EGC (epigallocatechin, mg/L) content in green tea infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean (n=3).

observed only at 3 °C compared to higher temperatures (12 and 25 °C) in the light. Even though EGC concentration at 3 °C was higher between Week 8 and 10 in the light than dark, the final concentration was higher in the dark due to the quick degradation between Week 10 and 12 in the light. Light lowered the reduction rate of EGC at 12 and 25 °C

after Week 2. This result indicates that dark storage was effective to delay degradation of EGC during 12 week storage.

EGCG was also significantly reduced during 12 week storage in all storage conditions as observed in EGC changes. EGCG was degraded by 26, 51, and 61 % at 3, 12, and 25 °C, respectively in dark storage and higher degradation was found at all temperatures relating to 58, 65, and 74 % during lighted storage (**Figure 6-2**). When tea infusions were stored in the dark, EGCG was higher retained as storage temperature

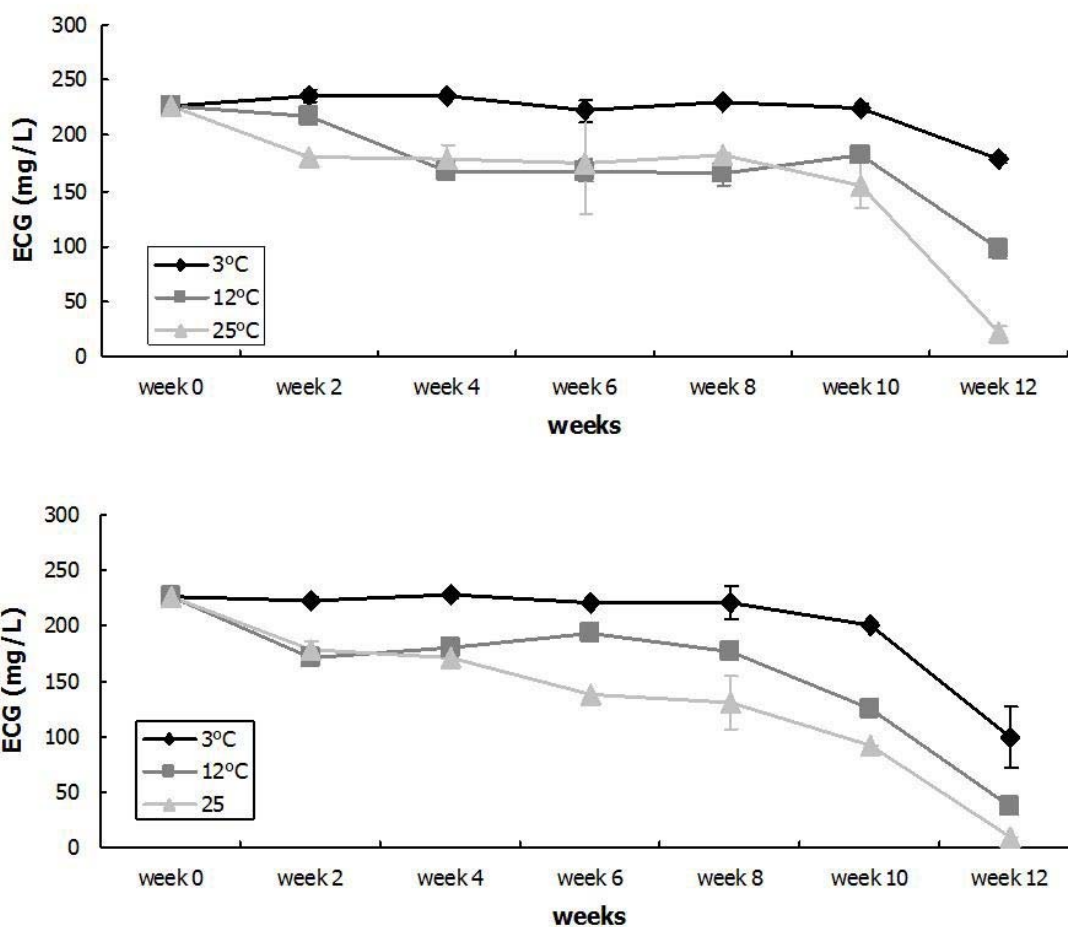


**Figure 6-2.** Changes in EGCG (epigallocatechin gallate, mg/L) content in green tea infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean (n=3).

decreased. However, no difference was observed between 3 and 12 °C in the light and their concentrations were significantly higher at 25 °C throughout the storage. EGCG was highest retained at 3 °C in the dark and lowest at 25 °C under the light, indicating lower temperature was also effective to maintain EGCG concentration and light played a role to reduce the concentration of EGCG. However, the EGCG concentration at 12 °C was higher in the light between 4 and 8 although it was higher at the rest of the storage.

ECG was also degraded during 12 week storage as observed in EGC and EGCG changes. ECG was reduced by 21, 57, and 90 % at 3, 12, and 25 °C, respectively in the dark and the reduction was 56, 83, and 96 % in the light (**Figure 6-3**). The lower temperature retained significantly higher concentration of ECG in both dark and light ( $P < 0.05$ ). ECG was not degraded until Week 10 but most of reduction occurred between Week 10 and 12 in the dark while ECG degradation was observed between Week 8 and 12 in the light indicating dark storage lowered the reduction rate of ECG at cold temperature (3 °C) during 12 week storage compared to the light storage. Even though ECG concentration in the dark was higher between Week 4 and 8 than that with the light at 12 °C, it was significantly reduced after Week 8 and lower in the dark than in the light. In case of the storage at 25 °C, ECG concentration in the dark was higher than in the light after Week 4 until the end of 12 week storage. Overall light effect was to slow the reduction rate of ECG for long term storage (storage longer than 6 week) but there was no or a little effect on delaying reduction for the short term storage (storage shorter than 6 week). The three most predominant phenolic compounds (EGC, EGCG, and ECG) showed higher stability at lower temperature in the dark, indicating the two

storage conditions (temperature and light) were a significant factor to influence phenolic stability during green tea storage.



**Figure 6-3.** Changes in ECG (epicatechin gallate, mg/L) content in green tea infusion during 12 week storage at 3, 12, and 25°C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean (n=3).

It has been regarded that temperature is the most important factor to affect the overall stability of various beverages during storage (79, 211). In the present study, the

tea infusions at 3 °C generally showed higher stability than those at 12 or 25 °C and could be explained by lowered oxidation reaction by temperature effect. Previous studies have described that lower temperature induced higher stability of polyphenolics in various beverages. For example, Chang et al. (77) reported that tea catechins were higher retained at low temperature (4 °C) in fruit juice compared to higher temperature (23 °C) and the degradation pathway was related to oxidative processes. Autooxidation occurs under a variety of conditions from ambient conditions even to supercritical water or even at very high temperature (218), indicating oxidation could occur in brewed tea, which was brewed with heated water (90 °C). The oxidation rate is greater at higher temperature than lower temperature and this reaction rate was confirmed with dissolved oxygen in aqueous solution (218, 219), which explains the dissolved oxygen may cause oxidation in green tea infusion in this study because there was no extra oxygen intake during storage due to the use of no gas permeable glass packaging. Even though these oxidation processes can occur even at low temperature, reaction rates are generally faster than higher temperatures (218, 219).

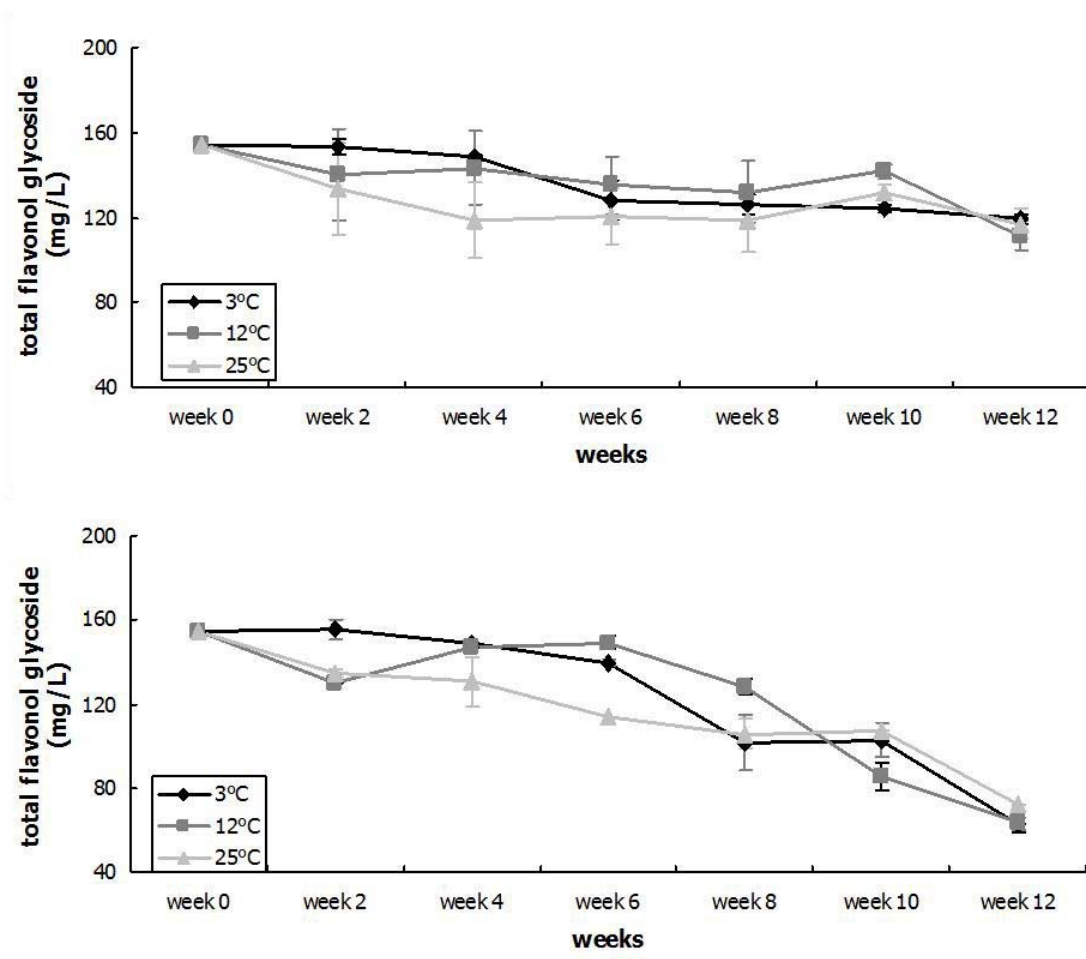
Light is also considered as an important factor that influences overall quality of RTD beverages during storage. It is known that light induces oxidation of carotenoids, proteins, lipids, and vitamins (81, 220, 221) and the light induced oxidation mechanism on these compounds was defined. In the presence of copper, which is present in green tea (222, 223), oxidation of EC and EGCG were accelerated when exposed to light in aqueous solution by a photogenerated superoxide anion (224). In model system, catechin was more stable in aqueous solutions stored in the dark versus lighted storage and its

stability was further enhanced when stored at refrigeration temperature (4 °C) over 80 days storage (225). There are two possible explanations on light induced degradation of tea catechins. First, the ascorbic acid present in green tea was degraded due to light exposure and this is in agreement with Andrews and Driscoll, (82), Pesek and Warthesen, (220), and Wishner (221) who reported ascorbic acid degradation during storage by light exposure. The degradation of ascorbic acid induced higher degree of reduction in flavan 3-ols in green tea infusion, whereas ascorbic acid was previously reported to improve the stability of flavan 3-ols in green tea infusion during storage. The mechanism of this protection was believed to be caused by a suppression of radicals and reduction of *o*-quinones from polyphenolics (33, 60, 226). Secondly, light might accelerate free radical production in the infusion during storage in the term of photooxidation. Photooxidation is defined as oxidation reactions induced by light including the two common processes: the loss of one or more electrons from a chemical species as a result of photo-excitation of that species and the reaction of a substance with oxygen under the influence of light (227). Hydroxyl groups on the A and B ring of flavonoids may donate their hydrogen atom to neutralized free radicals at the higher rate result in oxidative degradation since this hydrogenation reaction was catalyzed when green tea infusion was exposed to light. In conclusion, flavonoids in green tea in the dark were better retained than in the light during storage due to the loss of protective effect from ascorbic acid and the presence of oxidation catalyst, light.

Total flavonol glycoside from myricetin 3-glycoside, quercetin 3-rutinoside, quercetin 3-glycoside, kaempferol 3-glycoside, and kaempferol 3-rutinoside found in

green tea infusion was reduced from the initial concentration of 154.42 mg/L in all storage conditions and significantly higher reduction rate was observed in the light storage compared to the dark storage conditions. There was no temperature effect on total flavonol glycoside under both conditions. The reduction in total flavonol glycoside was 23, 28, and 24 % at 3, 12, and 25 °C, respectively in the dark and the difference was none at the end of the storage ( $P < 0.05$ ) (**Figure 6-4**). Otherwise, lighted storage exhibited 59, 59, and 53 % degradation was observed at 3, 12, and 25 °C and the difference was also not significant at the end of storage ( $P < 0.05$ ). The flavonol glycosides were relatively stable and where less influenced by the conditions of storage due to lowered reactivity from glycosidic moieties attached to flavonols. However, flavonol glycosides were degraded at high rates under exposure to light an observation also reported by Herrmann (228) who reported that light was a deleterious factor that influences flavonol glycosides. The reduction of flavonol glycosides may be due to the accelerated oxidation rate or loss of protective effect from ascorbic acid reduction as discussed in flavan 3-ols changes.



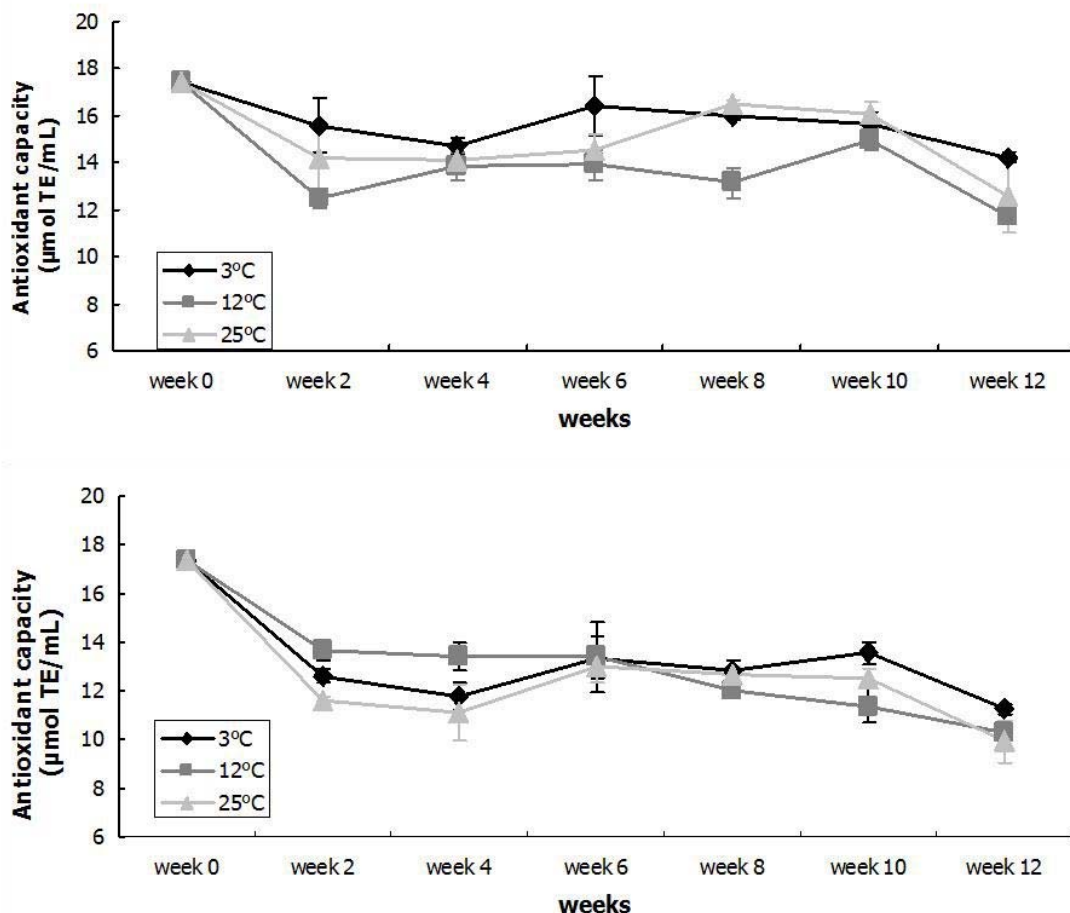


**Figure 6-4.** Changes in total flavonol glycosides (myricetin 3-glycoside, quercetin 3-rutinoside, quercetin 3-glycoside, kaempferol 3-glycoside, and kaempferol 3-rutinoside, mg/L) content in green tea infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Data was expressed as quercetin equivalent. Error bars represent standard error of the mean (n=3).

***Changes in antioxidant capacity and total soluble phenolics in green tea infusion by various storage conditions***

Antioxidant capacity, as determined using ORAC assay, decreased in all storage conditions during 12 weeks storage. As observed for changes in individual

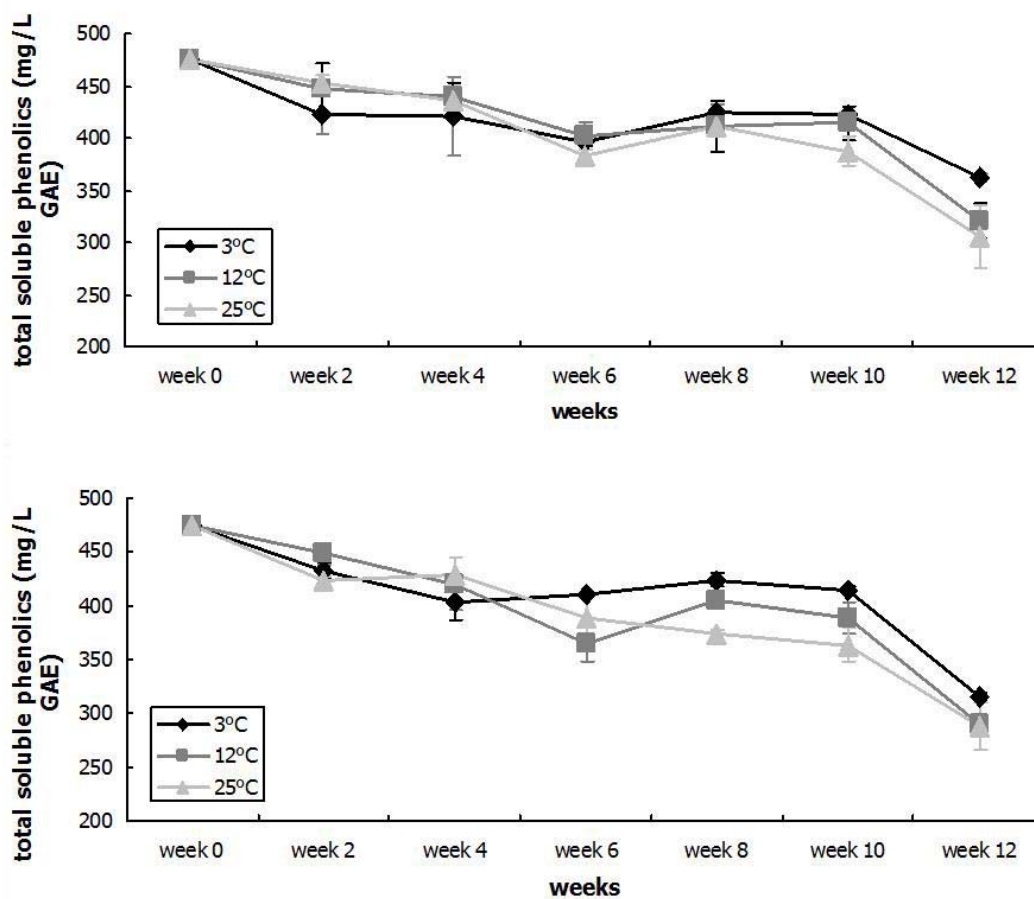
polyphenolics, low temperate storage in the absence of light was instrumental in maximal retention of antioxidant capacity. When tea infusions were stored in the dark, a 19, 32, and 28 % reduction in antioxidant capacity was observed from the initial 17.4  $\mu\text{mol Trolox equivalent/mL}$  at 3, 12, and 25 °C, respectively by the end of 12 weeks storage (**Figure 6-5**). However, no differences were observed for storage temperatures during the first 10 weeks of storage whereas major changes were observed between 10 and 12 weeks at 25 °C in a similar trend observed for ECG. For teas stored in the light, the antioxidant capacity decreased by 35, 41, and 43 % at 3, 12, and 25 °C, respectively at higher reduction rate compared to the antioxidant capacity in the dark with the majority of change observed in the first two weeks. Especially in the light, a quick reduction in antioxidant capacity was found within 2 weeks and this trend was observed in changes of EGC, EGCG, and ECG which have the highest antioxidant properties among all the polyphenolics present in green tea. Overall reduction of antioxidant capacity of green tea infusion was due to decrease of antioxidant polyphenolics and the reduction rate was higher as storage temperature increased under the presence of light.



**Figure 6-5.** Antioxidant capacity changes ( $\mu\text{mol TE/mL}$ ) in green tea infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean ( $n=3$ ). Abbreviation used: TE. Trolox equivalent.

Total soluble phenolics including contribution from ascorbic acid, reducing sugar, and likely small amount of soluble protein also decreased from the initial 475.3 mg/L in all storage conditions and total soluble phenolic at 3 and 12 °C was found higher in the dark than in the light while no light effect was observed at 25 °C (**Figure 6-6**). In the dark storage, no difference was found between 12 and 25 °C but total soluble

phenolic was higher at 3 °C when determined at the end of storage. During 8 week storage in the dark, no difference was observed in total soluble phenolics between the temperatures but the differences occurred after Week 8 at 25 °C and Week 10 at 12 °C. In the light storage, no difference was found for the first 4 weeks but at week 6, total soluble phenolic at 12 and 25 °C was significantly lower.



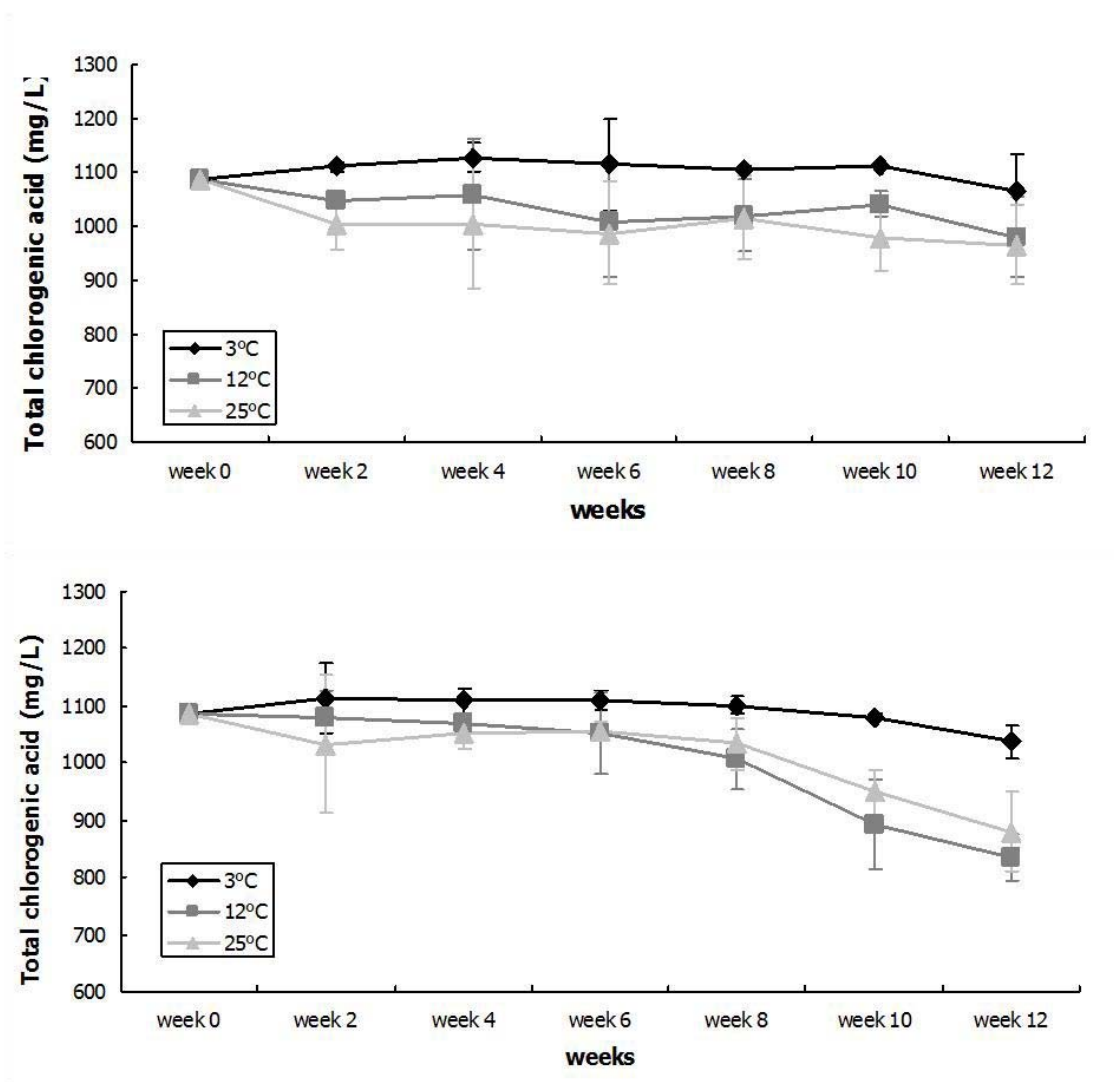
**Figure 6-6.** Total soluble phenolics changes ( $\mu\text{mol TE/mL}$ ) in green tea infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean ( $n=3$ ). Abbreviation used: GAE. Gallic acid equivalent.

***Changes in polyphenolics present in yaupon holly infusion by different storage conditions***

Chlorogenic acid (5-caffeoylquinic acid) and its two isomers (3 and 4-caffeoylquinic acid) were found in yaupon holly infusion as the predominant compounds and their total concentration was 1086.15 mg/L, which is 68 % of total phenolic compound present in yaupon holly infusion. Also, two 3, 4 and 3, 5-dicaffeoylquinic acids and two flavonol glycosides such as quercetin 3-rutinoside and kaempferol 3-rutinoside were detected in the infusion. Both total dicaffeoylquinic acid and total flavonol glycoside were present at 16 % of total phenolic compounds.

During 12 week storage, no changes in total chlorogenic acid were observed at 3 °C in both in the dark and light storages (**Figure 6-7**). However, total chlorogenic acid was reduced by 10 and 12 % at 12 and 25 °C, respectively in the dark and their concentrations were significantly higher at 3 °C throughout 12 week storage ( $P < 0.05$ ). Otherwise, no difference was observed for the first 6 week storage in the light between the temperatures ( $P < 0.05$ ) but total chlorogenic acid at 12 and 25 °C started degrading after week 6 while no degradation was observed at 3 °C. Even though total chlorogenic acid was not different between at dark and light storages at 3 °C during storage, the concentrations at 12 and 25 °C were higher in the dark than the light storage. Total dicaffeoylquinic acid found at the concentration of 261.94 mg/L also showed the similar tendency with total chlorogenic acid changes (data not shown). No changes in total dicaffeoylquinic acid were observed at 3 °C during storage but the concentrations decreased at 12 and 25 °C at both dark and light storages. According to the data trend

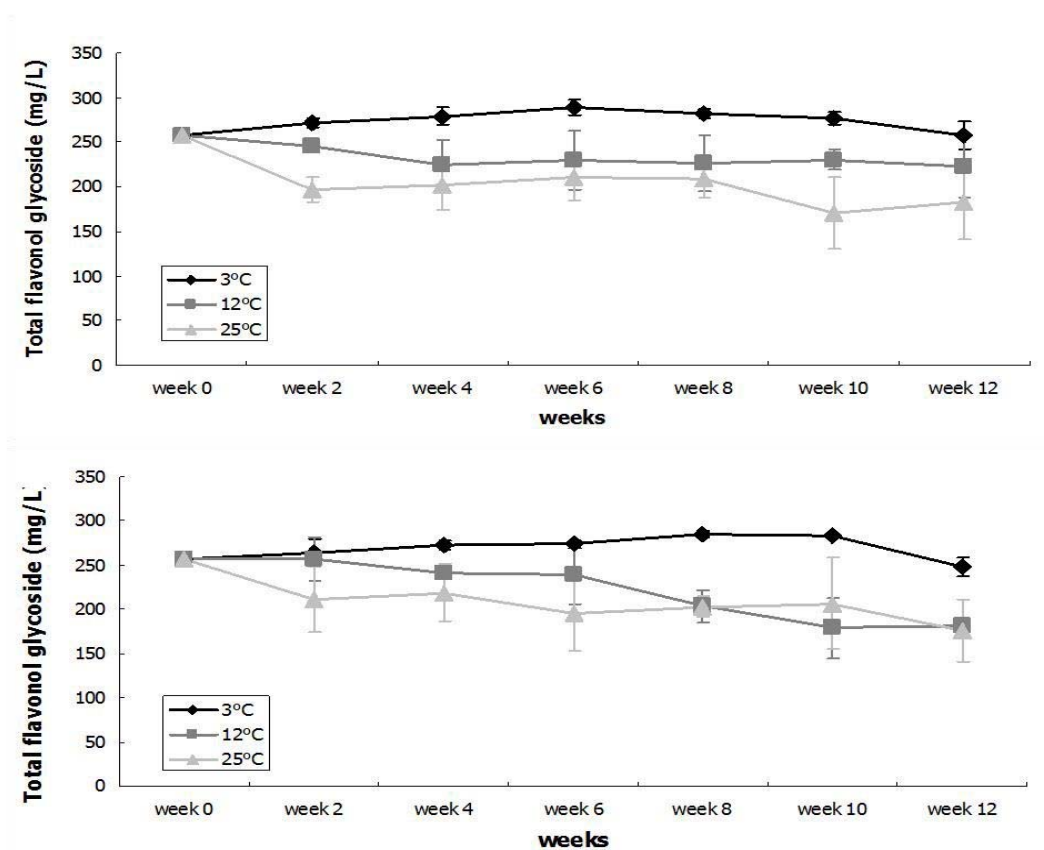
observed here, chlorogenic acid and its mono- and di-isomers did not change only at cold temperature but were degraded at higher temperatures (12 and 25 °C). Light was not a factor to influence the concentrations at 3 °C, but the degradation rate was higher with light when stored at higher temperatures (12 and 25 °C).



**Figure 6-7.** Changes in total chlorogenic acid (chlorogenic acid and its isomers) (mg/L) in yaupon holly infusion during 12 week storage at 3, 12, and 25°C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean (n=3).

High stability of chlorogenic acid and its isomers at cold storage (3 °C) was previously discussed in chapter V. Total chlorogenic acid was significantly reduced at higher temperature (12 and 25 °C) during storage, yet their stability was higher than flavan 3-ols in green tea. Chang et al. (77) reported the similar result, which degradation rate of chlorogenic acid was lower than that of tea catechins at 23 °C in fruit juice for 24 week storage and the authors suggested the degradation of chlorogenic acid at higher temperature was due to faster oxidative degradation during long term storage. Even though the light effect on stability of chlorogenic acid and isomers was not as high as observed in flavan 3-ols changes, photodegradation of total chlorogenic acid was still observed especially at 12 and 25 °C showing synergistic effect of light and temperature. Even though many studies have illustrated changes in chlorogenic acid by light exposure, the studies have conducted only with non-processed crops such as apples (229), carrots (230), and potatoes (231) who reported light affected the biosynthesis of chlorogenic acid in plant tissue. Ascorbic acid content in yaupon holly infusion was determined in the present study and the concentration was as high as that of green tea. Since ascorbic acid is present at significant amount in yaupon holly infusion as observed in chapter IV, the protective effect of ascorbic acid on polyphenolics may be reduced by unfavorable storage conditions such as high temperature and light exposure (82, 173, 220, 221), explaining degradation of chlorogenic acid under high temperature and light exposure. The protective effect of ascorbic acid on chlorogenic acid and its isomers was already observed in chapter IV which illustrated that addition of ascorbic acid into tea infusions reduced the reduction rate of chlorogenic acid during 24 week storage.

Total flavonol glycoside found at the concentration of 257.37 mg/L was stable only at 3 °C while the concentrations decreased at 12 and 25 °C regardless of light exposure. In the dark storage, total flavonol glycoside from quercetin 3-rutinoside and kaempferol 3-rutinoside was reduced by 13 and 29 % at 12 and 15 °C, respectively (Figure 6-8). Otherwise, total flavonol glycoside was degraded with higher reduction rate by 30 and 42 % at 12 and 15 °C in the light storage. This reduction tendency was very similar with total chlorogenic acid changes.



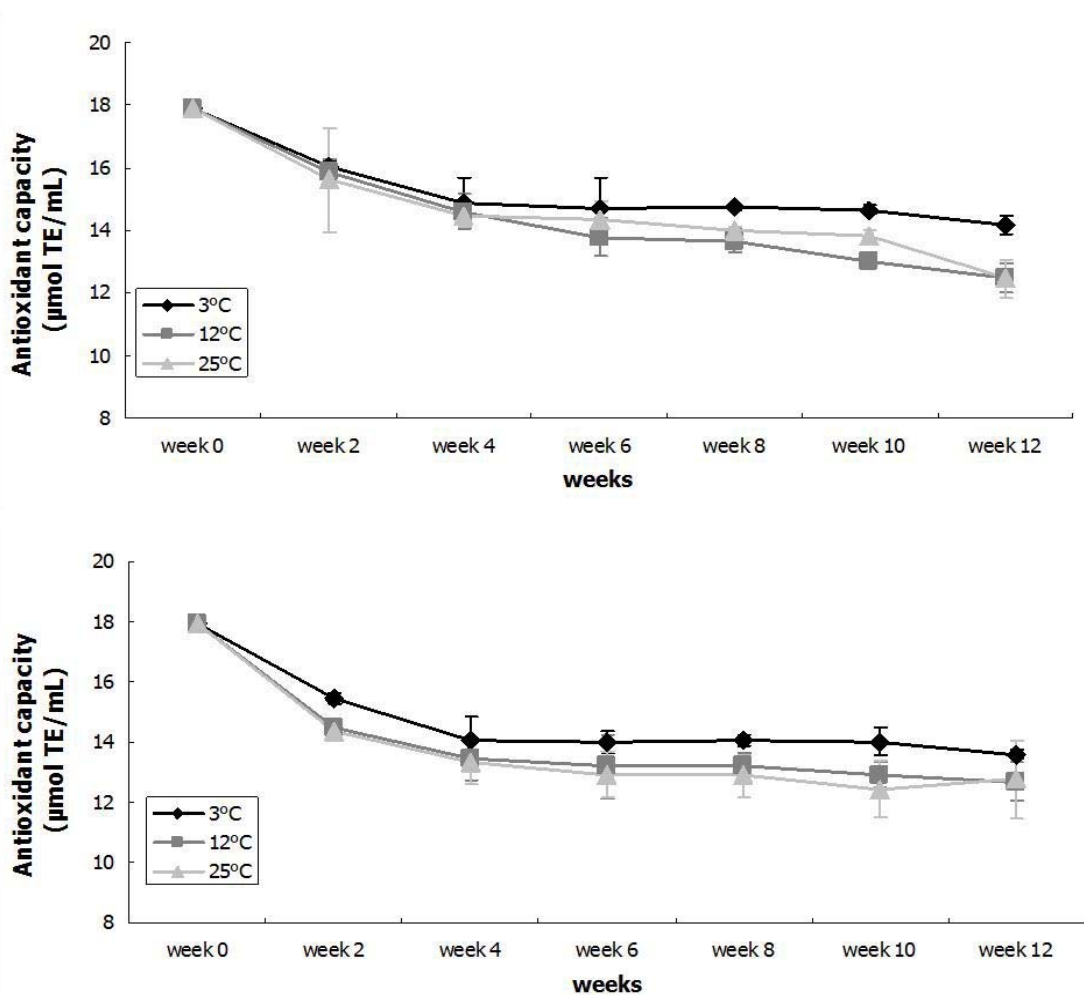
**Figure 6-8.** Changes in total flavonol glycosides (quercetin 3-rutinoside and kaempferol 3-rutinoside) (mg/L) in yaupon holly infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Data was expressed as quercetin equivalent. Error bars represent standard error of the mean (n=3).



Higher storage temperature (higher than refrigeration temperature at 3 °C) was a factor to reduce the concentration of flavonol glycosides and higher degradation in flavonol glycosides was found in the light storage. The degree of reduction in flavonol glycosides was lower than that of green tea during storage. There was one noticeable difference between green tea and yaupon holly infusions, which is the amount of saponin present in each infusion. Since significantly higher amount of saponin is present in yaupon holly infusion as found in chapter VII, the saponin may improve phytochemical stability as briefly explained in chapter V. According to Du et al. (232), saponin was degraded during storage at 5, 20, and 30 °C and the reduction rate significantly increased at higher temperature as observed in the reduction of flavonol glycosides.

***Changes in antioxidant capacity and total soluble phenolics in yaupon holly infusion by various storage conditions***

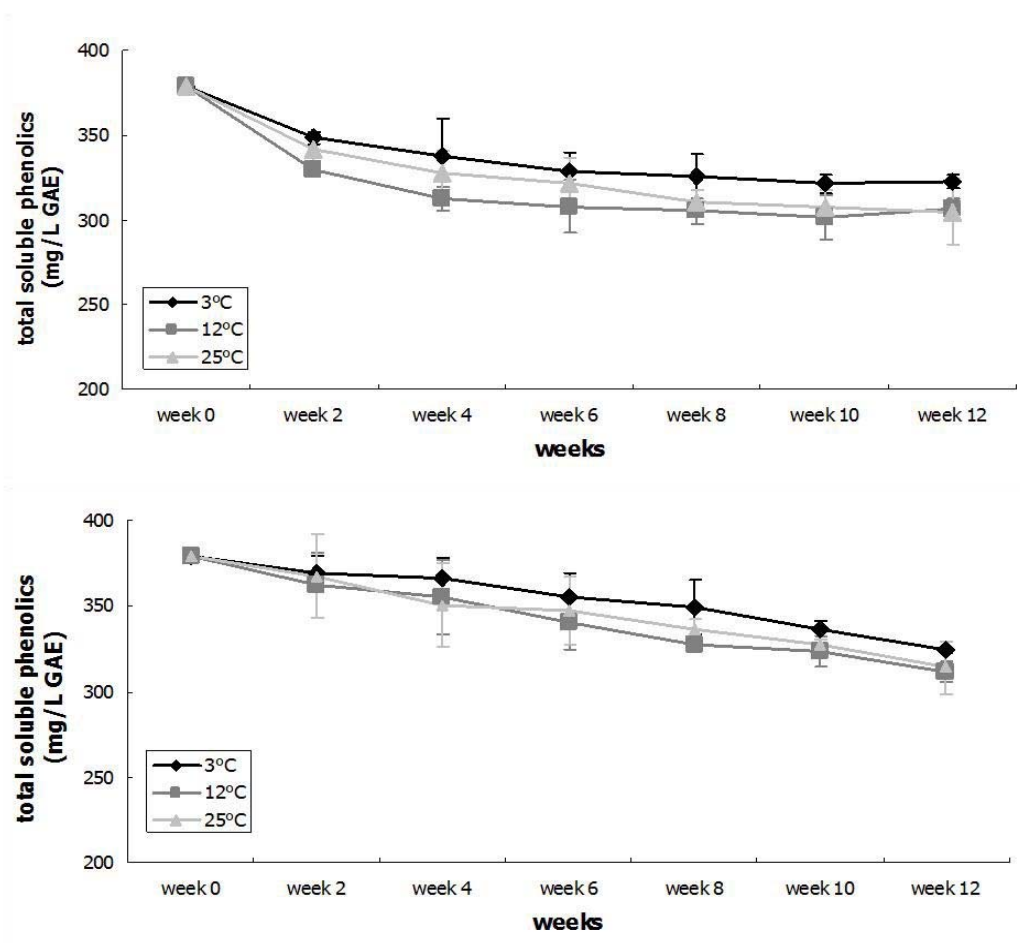
Antioxidant capacity decreased in all the storage conditions and was higher at 3 °C at both dark and light storage than 12 and 25 °C (**Figure 6-9**). In the dark storage, antioxidant capacity was reduced by 21, 31 and 31 % at 3, 12, and 25 °C, respectively from the initial antioxidant capacity of 17.93  $\mu\text{mol Trolox equivalent/mL}$  in yaupon holly infusion. No difference in antioxidant capacity was observed between the dark and light at 12 and 25 °C while it was higher in the dark at 3 °C. In the light storage, 23, 29, and 29 % of reduction was found at 3, 12, and 25 °C, respectively during storage.



**Figure 6-9.** Antioxidant capacity changes ( $\mu\text{mol TE/mL}$ ) in yaupon holly infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean ( $n=3$ ). Abbreviation used: TE. Trolox equivalent.

Antioxidant capacity was lower at higher temperature as observed in all the polyphenolic changes in yaupon holly infusion. The initial total soluble phenolics of yaupon holly infusion was 387.8 mg/L and it was reduced in all storage conditions during 12 week storage. In the dark storage, 15, 19, and 20 % reduction was observed at

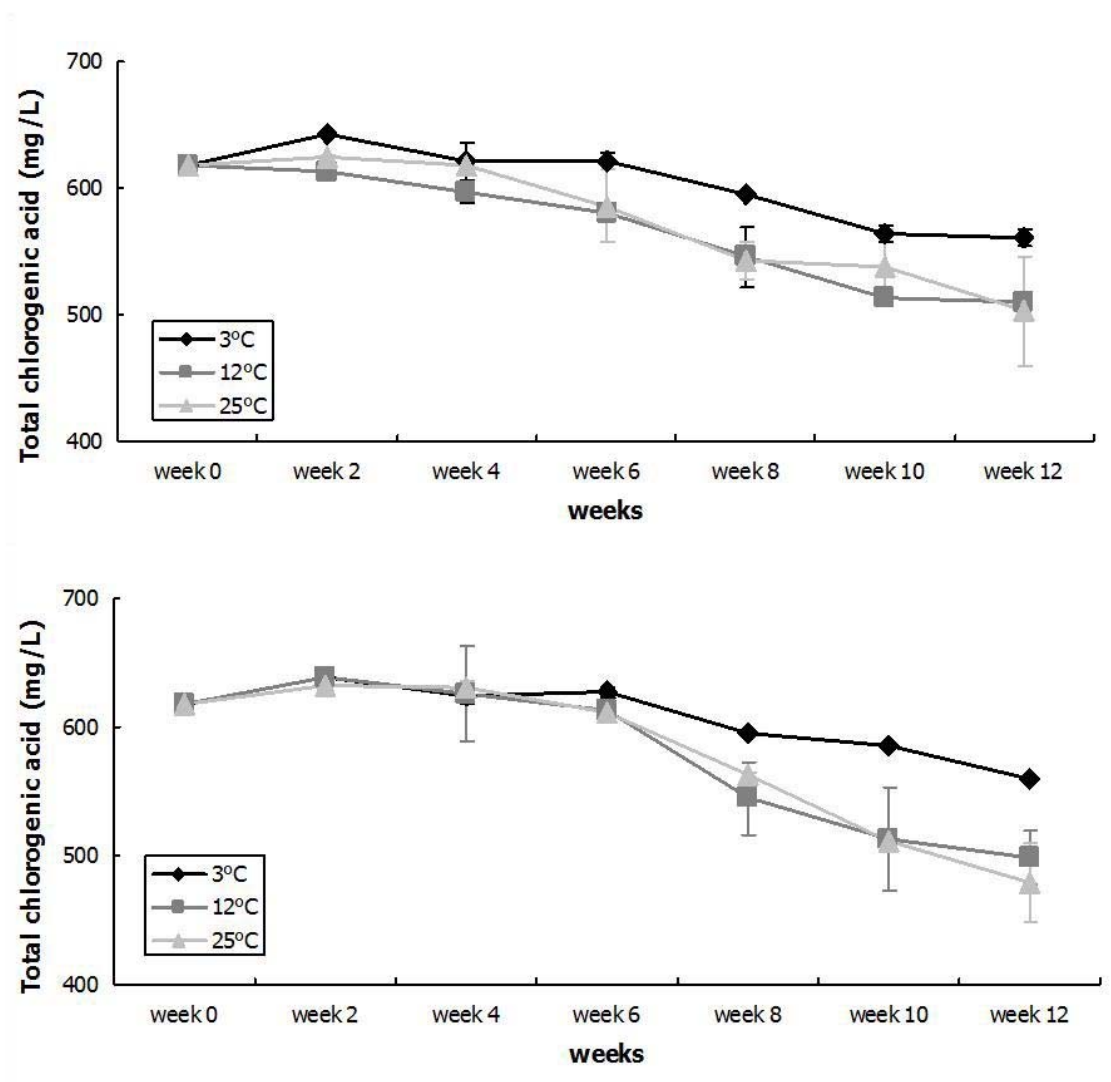
3, 12, and 25 °C, respectively and total soluble phenolics was higher at 3 °C than other temperatures (**Figure 6-10**). A similar degree of reduction was observed in the light and their reduction was 15, 18, and 17 % at 3, 12, and 25 °C, respectively showing no difference between the storage temperatures.



**Figure 6-10.** Total soluble phenolics changes ( $\mu\text{mol TE/mL}$ ) in yaupon holly infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean (n=3). Abbreviation used: GAE. Gallic acid equivalent.

***Changes in polyphenolics present in mamaki infusion by different storage conditions***

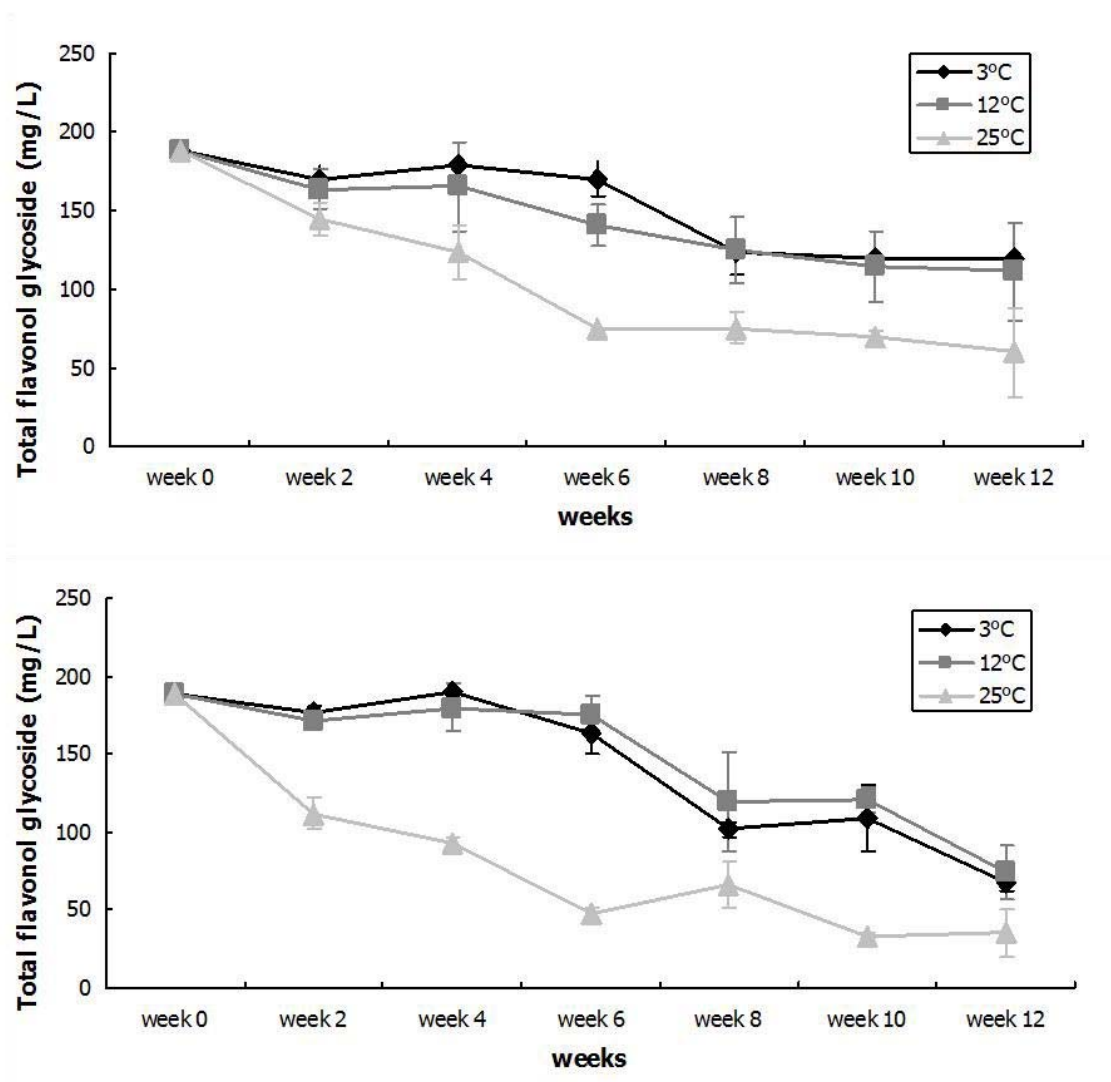
In mamaki infusion, the total concentration of total chlorogenic acid (chlorogenic acid and its isomers) was 617.78 mg/L, which is about 56 % of that of yaupon holly. During 12 week storage, 10 % reduction in total chlorogenic acid was found at 3 °C at both dark and light storages (**Figure 6-11**). Higher storage temperatures induced more degradation of total chlorogenic acid that decreased by 17 and 19 % at 12 and 25 °C in the dark and by 19 and 23 % in the light. While no difference was found at 3 and 12 °C in the dark and light, lower concentration of total chlorogenic acid was observed at 25 °C. Even though it was known that chlorogenic acid shows high storage stability, total chlorogenic acid was reduced in mamaki infusion during storage. This tendency was observed in chapter V which illustrated higher stability of chlorogenic acid in yaupon holly infusion compared to mamaki infusion at 3 °C. Likewise, a similar result was observed at 3 °C and higher reduction of chlorogenic acid was also found at 12 and 25 °C.



**Figure 6-11.** Changes in total chlorogenic acid (chlorogenic acid and its isomers) (mg/L) in mamaki infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean (n=3).

Total flavonol glycoside from two apigenin 3-glucosides, quercetin 3-rutinoside, and kaempferol 3-rutinoside was found in mamaki infusion at the concentration of 188.67 mg/L with continuous reduction in all the storage conditions. In the dark storage,

there was no difference in total flavonol glycoside between at 3 and 12 °C except Week 6 and 8 (**Figure 6-12**). A similar trend was also found in the dark storage, which showed no difference in total flavonol glycoside between at 3 and 12 °C. This indicates that temperature did not influence or showed only minor impact on flavonol glycosides stability when the storage temperature is lower than 12 °C. However, at 25 °C, about 68 % reduction in total flavonol glycoside was found in the dark while it was degraded by 37 and 41 % at 3 and 12 °C, respectively. In the light storage, it was reduced by 64, 61, and 82 % at 3, 12, and 25 °C, respectively and all the concentrations at each storage temperature were significantly lower than those in the dark ( $P < 0.05$ ). Even though temperature did not affect the stability of total flavonol glycoside, light was a significant factor to degrade it. Flavonol glycosides were not stable in mamaki infusion and also showed higher reduction rate compared to yaupon holly infusion. This trend was due to fewer amounts of ascorbic acid and saponin (determined in chapter VII) in mamaki infusion or naturally high pH (7.5) which causes faster reduction in polyphenolics including flavonol glycosides and this trend was in agreement with previous studies (33, 180, 211–213). However, when teas are utilized for RTD teas, pH of tea infusion is lowered using organic acids such as citric acid and malic acid. Thus, even though a significant reduction in polyphenolics was observed in brewed tea, it may not be a real problem in tea industry if mamaki teas are commercialized as RTD teas.



**Figure 6-12.** Changes in total flavonol glycosides (two apigenin 3-glucosides, quercetin 3-rutinoside and kaempferol 3-rutinoside) (mg/L) in mamaki infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Data was expressed as quercetin equivalent. Error bars represent standard error of the mean (n=3).

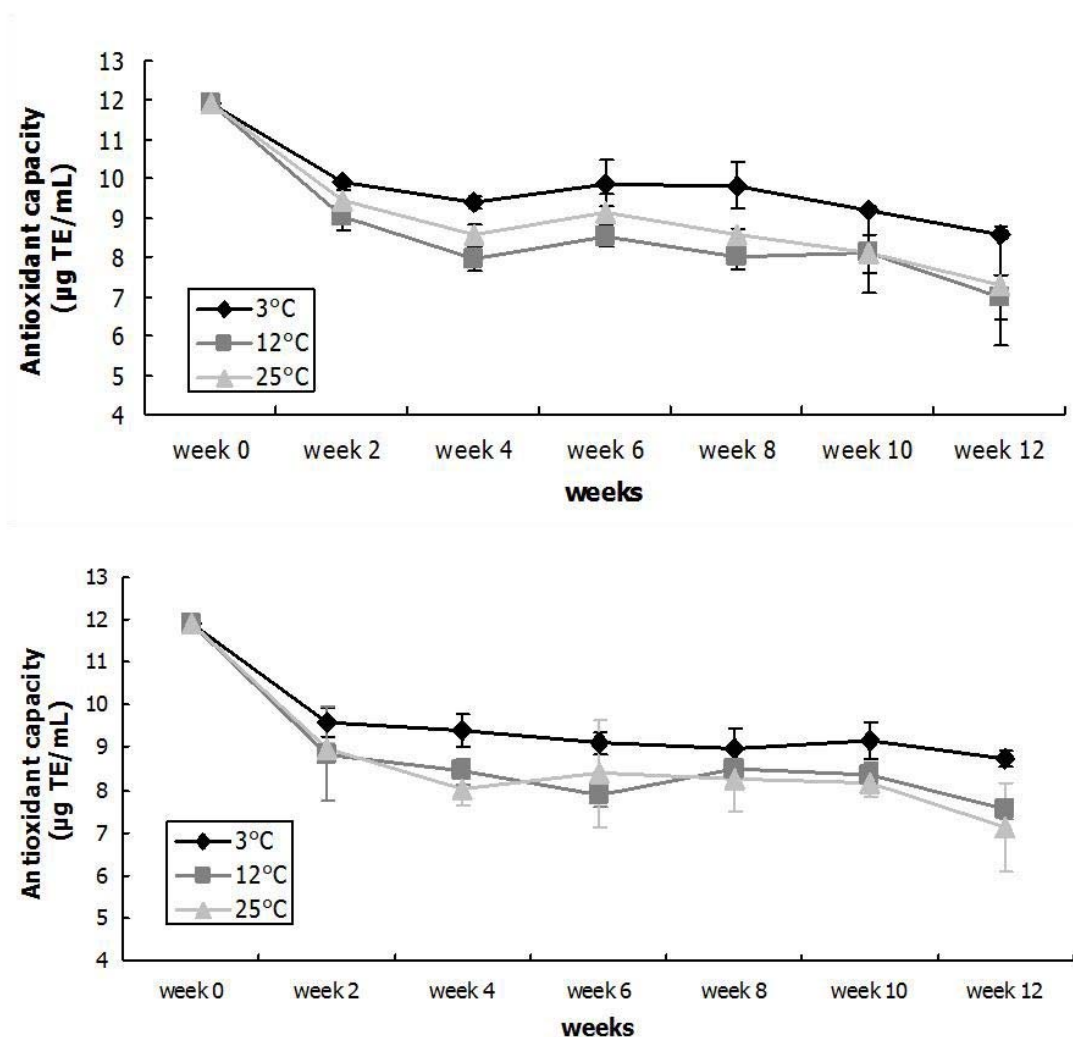
***Changes in antioxidant capacity and total soluble phenolics in mamaki infusion by various storage conditions***

Antioxidant capacity of mamaki infusion decreased at all the temperature and the degree of reduction was lower at 3 °C than at 12 and 25 °C at both dark and light storages as observed in chlorogenic acid changes (**Figure 6-13**). Overall antioxidant capacity from mamaki (11.9 µmol Trolox equivalent/mL) infusion was lower than those of green tea (17.4 µmol Trolox equivalent/mL) and yaupon holly (17.93 µmol Trolox equivalent/mL) due to lower amount of phenolic compounds (806.46 mg/L), which is 47 and 51 % of total phenolic compound in green tea and yaupon holly, respectively and lower concentration of other antioxidants such as ascorbic acid and saponin. The changes in antioxidant capacity in mamaki infusion was more similar with total chlorogenic acid change since chlorogenic acid shows higher antioxidant capacity than rutin (65 % of total flavonol glycoside in mamaki infusion) (151) and total chlorogenic acid concentration was significantly higher by 70 % than total flavonol glycoside.

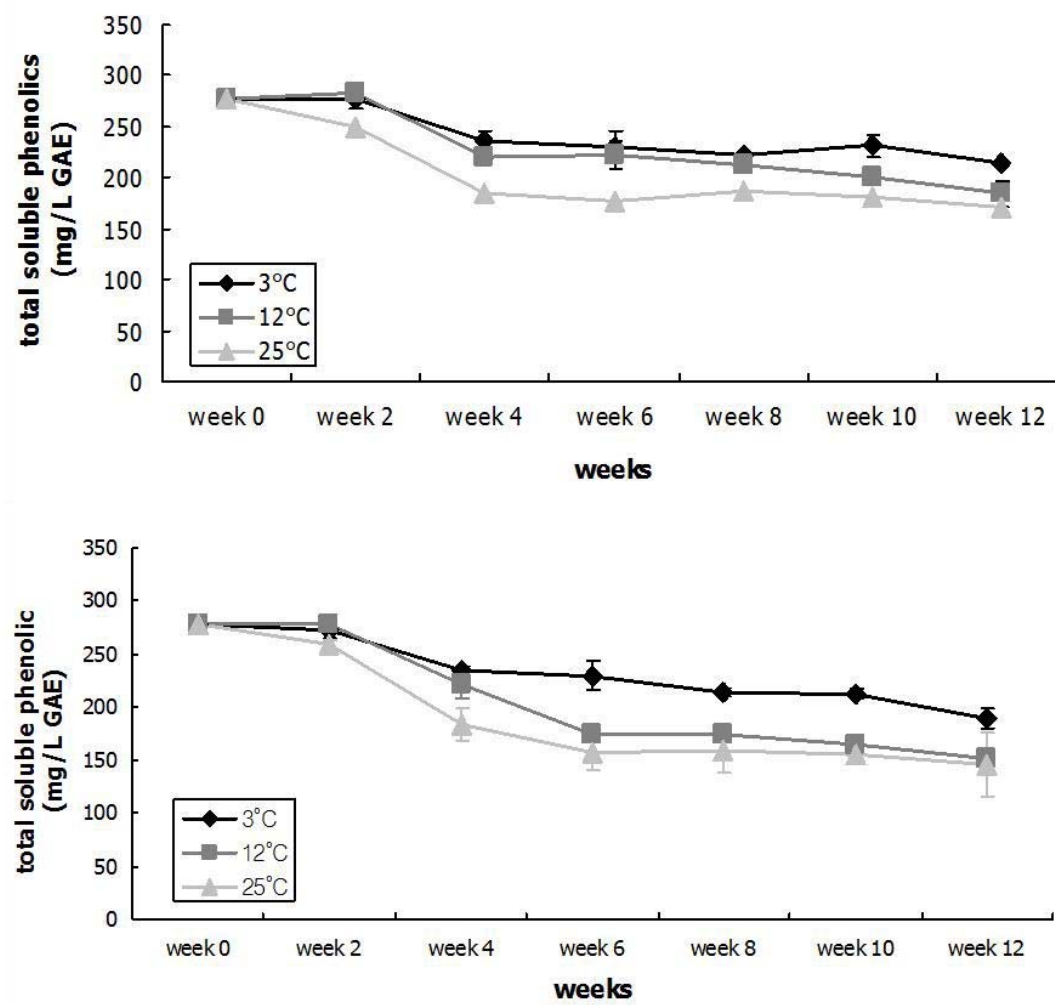
Total soluble phenolics in mamaki infusion determined by Folin-Ciocalteu assay was also reduced from 277.3 mg/L GAE (Gallic acid equivalent) throughout 12 week storage as observed in total chlorogenic acid and flavonol glycoside. During the first 8 week storage in the dark, there was no difference between at 12 and 25 °C but started degrading at 25 °C after Week 8 in the dark storage while total soluble phenolics at 25 °C was consistently lower than that at 3 °C (**Figure 6-14**). A similar trend was also observed for bottles stored in the light. No difference was observed in the light for the first 4 weeks between at 3 and 12 °C but there was significantly degradation at 12 °C



after Week 4. Total soluble phenolics at 25 °C were lower throughout the storage. As observed in antioxidant capacity changes in mamaki infusion, general reduction of total soluble phenolics trend was similar with phenolic compounds changes.



**Figure 6-13.** Antioxidant capacity changes ( $\mu\text{mol TE/mL}$ ) in mamaki infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean ( $n=3$ ). Abbreviation used: TE. Trolox equivalent.



**Figure 6-14.** Total soluble phenolics changes ( $\mu\text{mol TE/mL}$ ) in mamaki infusion during 12 week storage at 3, 12, and 25 °C without (top, in the dark) or with (bottom, in the light) exposure to light. Error bars represent standard error of the mean (n=3). Abbreviation used: GAE. Gallic acid equivalent.

**CHAPTER VII**

**IDENTIFICATION OF THE MECHANISM OF THE FORMATION OF TEA  
CREAM AND DEVELOPMENT OF NOVEL CHEMICAL OR PHYSICAL  
MEANS OF INHIBITING TEA CREAMING**

**Introduction**

Tea cream, the complex formed by interaction between polyphenolics, caffeine, protein, and metal ions can form when brewed tea infusions are stored at cool temperatures. Tea cream may appear as a “muddy” or “hazy” appearance that results in loss of clarity and is considered to be highly undesirable in the beverage industry. As a consequence of tea cream formation, teas may lose certain physical attributes or biological activities due to interactions with compounds responsible for sensory attributes (i.e. astringency, aroma, color, and taste) as health-promoting characteristics (64, 88). Turbidity of final Ready-To-Drink (RTD) tea products is reduced in tea beverage industry, however pursuing high degree of clarity by advanced filtering process causes loss of bioactive polyphenolics in tea and results in loss of health related benefits to consumers (64). Previous studies have used both chemical and physical means in attempt to remove tea cream from black tea without decreasing concentrations of bioactive compounds. Wright (233) used tannase treatments to reduce tea cream in black tea but polyphenolic compounds were degraded that resulted in a loss of astringency and overall quality. The use of strong metal chelators such as EDTA (ethylenediaminetetraacetic acid) also inhibited tea cream by up to 50 % but reformation

of the cream was subsequently observed during storage (234). Other efforts to reduce tea cream have included calcium removal using a two-stage extraction at different temperature stages but resulted in only a partial success followed by reformation of the tea cream (64, 235). So far, no studies have shown complete removal of tea cream without losing sensory properties, organoleptic profile, or bioactive polyphenolic compounds.

Numerous studies have reported the tea cream formation in either black tea or oolong tea, which may be a preferred medium for this reaction since it is partially or fully oxidized during fermentation (64, 87, 89, 92, 104, 233, 235–240). The mechanism of tea cream formation in black tea with its characteristic polyphenolics compounds, thearubigin and theaflavin, was early identified, while causes of tea cream in green tea infusion that contains flavan-3-ols (tea catechins) are poorly described in the literature (88). Moreover, no study has reported on tea cream formation in other botanical teas, such as yaupon holly and mamaki leaf infusions. These botanicals have a chemical composition that may promote tea cream such as chlorogenic acid, flavonol glycosides, protein, metal ions, and caffeine.

The objectives of these studies were to determination of a degree of contribution to tea creaming by addition or removal of potential tea cream forming compounds and application of metal chelating agents so as to investigate the degree of participation or inhibition in tea creaming. Evaluation of innovative novel chemical and physical methods in tea cream reduction without detrimental effects on tea stability was also determined in this study.

## **Materials and Methods**

### ***Tea preparation and treatments***

The tea varieties which previous used in chapter III were utilized in this study. Briefly, hot air dried tea leaves were powdered with a mortar and pestle and brewed using hot water (95 °C) for 30 min with constant stirring (tea leaf/water = 5 %, w/v) . After cooling to 25 °C and filtering through cheese cloth, the infusions were filtered through Whatman #4 filter paper containing a 1 cm bed of diatomaceous earth under vacuum to ensure particle-free infusions.

Three tea infusions (15 mL each) from each tea variety were placed into pre-weighed aluminum dishes and held at 85 °C for 12 hrs to determine total solids. These initial tea infusions were divided into ten groups containing 70 mL of tea infusion each. The first aliquot was held as an untreated as a control, the next six aliquots were fortified with protein (bovine serum albumin), caffeine, gallic acid, calcium chloride, EDTA (ethylenediaminetetraacetic acid) and citric acid (70 mg/70 mL), and the eighth was decaffeinated by repeated extraction with chloroform. For decaffeination, each infusion was placed into a separatory funnel with an equal volume of chloroform mixed vigorously for 1 min. The mixture was allowed to settle and the lower chloroform layer containing the caffeine was discarded and the process repeated two more times. The last ninth and tenth aliquots were remained untreated for incubation at different temperatures with the first eight aliquots. After fortification and decaffeination, each aliquot (70 mL) was transferred into four 15 mL eppendorf tubes (60 mL total) and held in cold storage

(4 °C) to induce tea cream formation in the absence of light while the ninth and tenth groups were held at 25 and 40 °C, respectively. The remaining 10 mL of tea infusions (70 mL of prepared tea infusion minus 60 mL of tea infusion transferred to 4 Eppendorf tubes) were immediately moved to screw top test tubes for chemical analysis. After 12 hour incubation at refrigerator, the first three out of four infusions in eppendorf tubes were immediately centrifuged at 4000 r.p.m. at 7 °C for 30 min (eppendorf centrifuge 5810R, Westbury, NY) to remove tea cream. The supernatant was removed for phytochemical analysis whereas the tea cream sediment was carefully moved to a pre-weighed aluminum dish by washing with two 5 mL aliquots of distilled water and dried for 12 hrs at 85 °C. The remaining infusion in the 4th eppendorf tube was used for measuring turbidity and then centrifuged to obtain tea cream for chemical analysis. The obtained tea cream was dissolved using 15 mL of 50 % ethanol aided by sonication for 2 hours and then moved to screw top test tubes for chemical analysis. The dried tea cream in the aluminum weighting dishes from the oven was carefully weighed and the amount of tea cream formed was determined by calculating the difference between total solids in the initial infusion and the dried tea cream described by Nagalashmi et al. (93).

For aeration and deaeration, prepared tea infusion using previously described method was divided into three groups and the first group was remained unchanged while pH of the other two groups was changed to 3 and 7 by adding either 0.3 M hydrochloric acid or 0.3 M sodium hydroxide, respectively and each adjusted to a constant volume with deionized water. The pH of the original infusions was 5.50 for green tea, 5.21 for yaupon holly, and 7.74 for mamaki. Each infusion was then divided into three groups

and the first group was remained untreated while other two groups were aerated and deaerated by following treatments. Macroaeration was used for aeration and the air containing about 21 % oxygen was directly introduced into triplicated tea infusions (100 mL each) for two minutes. For deaeration, nitrogen gas generated by nitrogen generator (Domnick Hunter, Charlotte, NC) was also sparged into the infusions for two minutes. The aerated and deaerated infusions were compared to an untreated infusions and each equally transferred to four eppendorf tubes (15 mL) and placed into refrigeration at 4 °C for 12 hrs while an aliquot of each treatment was retain for immediate chemical analyses. Remaining procedures for tea cream formation were followed as previously described.

#### ***Determination of polyphenolics and antioxidant capacity***

Individual polyphenolics and antioxidant capacity were determined as previously described in chapter III. The polyphenolic and antioxidant constituents in tea cream were determined by calculating the difference between original tea infusion and clarified infusions following centrifugation.

#### ***Protein analysis***

The amount of soluble protein present in tea was determined using Biuret assay as early described by Gornall et al. (240). Filtered and diluted tea infusions were mixed with an equal volume of Biuret reagent against a standard curve and allow to react for 20 min for absorbance reading at 540 nm. Properly diluted tea samples (1 mL/4 mL) in test tubes in duplicated was moved to another test tubes and 4 mL of the Biuret reagent

(Biuret reagent TS (USP) test solution. Ricca Chemical Company, Arlington, TX) was added to each test tube. After thorough mix by vortex, the samples were placed on the bench top for 20 min at room temperature. The absorbance values were measured using spectrometer (Helios gamma UV-Vis spectrometer. Thermo scientific, Waltham, MA) at 540 nm. The amount of protein was obtained by computation of the absorbance of the tea samples against standard curve (concentration vs. absorbance). The standard curve was prepared by serial dilutions of 10, 8, 6, 4, 2, and 1 mg/mL (Bovine serum albumin/water, w/v) and blank (0 mg/mL) was applied first for the spectrometer before measuring the absorbance of samples.

#### ***Total metal determination (calcium and magnesium)***

EDTA titration was performed to determine the amount of calcium and magnesium in each tea as described by Ntailianas and Whitney, (241). The trace metal ions such as manganese, iron, aluminum, copper, nickel, and cobalt which interfere with the test were removed by the incorporation of sodium sulfide in borate buffer. A combination of 1mL of borate buffer (pH 9.5) and 3 drops of Eriochromeblack T indicator solution was added to properly diluted tea infusion in beaker (0.5/9.5 mL, v/v) and the mixture titrated with a standardized solution of EDTA to a light blue end point. EDTA standard solution was added by a unit of 10  $\mu$ L.

The EDTA standard solution (0.05 N) was prepared by mixing 10 g of disodium dihydrogen ethylenediamine tetraacetate dehydrate and 2 g of sodium hydroxide pellets in 500 mL of distilled water and diluted up to 1 L. For borate buffer preparation, 3.332 g



of sodium tetraborate decahydrate was first dissolved in 80 mL of distilled water. The mixture of 0.54 g of sodium hydroxide and 0.4165 g of sodium sulfide dissolved in 10 mL of distilled water was added to previously prepared sodium tetraborate decahydrate solution and the final volume was diluted up to 100 mL. Commercial Eriochromeblack T solution was purchased from Sigma (Sigma Chemical Co., St. Louis, MO). The metal concentration was calculated as follows:

$$\text{meq (Ca + Mg) / liter} = \text{mL of EDTA solution} \times \text{normality of EDTA solution} \times 100 / \text{mL of tea}$$

where the volume is measured in mL; meq, milliequivalent.

#### ***Determination of total saponin***

Saponin analysis was conducted as described by Gnoatto et al. (242) with a modification. Each tea infusion was prepared by brewing 15g of powdered tea leaves with 200 mL of distilled water at 90 °C for 30 min followed by filtering through cheesecloth and then allowed to cool to 25 °C for a final filtrations first through Whatman #4 filter paper followed by a 1 cm bed of diatomaceous earth under a slight vacuum to remove suspended particles. The obtained tea extract was divided into 3 groups containing 20 mL of infusion and treated with 3 mL of chloridric acid in view to yield an acid concentration of 4 mol/L prior to hydrolysis for 2 hours. The saponin fraction was extracted with an equal volume of chloroform using separatory funnel. This

extraction was performed three times. The saponin aglycone was obtained with 10 mL of acetonitrile after evaporated to dryness.

The saponin extract from each tea infusion was diluted 3-fold using deionized water and filtered through a 0.45  $\mu\text{m}$  PTFE filter (Whatman, Clifton, NJ) prior to injection. Polyphenolic separations was conducted on a Waters 2695 alliance HPLC system using a Water 996 photodiode array (PDA) detector with a Dionex 250 x 4.6 mm Acclaim 120- $\text{C}_{18}$  column run at 0.8 mL/min. The pH of 100 %  $\text{H}_2\text{O}$  was adjusted with pH 2.4 using *o*-phosphoric acid for mobile phase and it was run for 60 min at 0.8 mL/min. Total saponin (saponin) in each infusions was detected and quantified at 280 nm against external standards of ursolic acid procured from Sigma Adrich (Sigma Chemical Co., St. Louis, MO).

### ***Turbidity***

Turbidity of each tea infusion was measured using Micro TPW portable field turbidimeter (HF scientific, Inc. Ft. Myers, FL). Properly diluted 5mL of tea infusion (1 mL/4 mL) was transferred to a glass sample cuvette and measured 3 times using previously calibrated turbidimeter with the unit of NTU (Nephelometric Turbidity Unit).

### ***Statistical analysis***

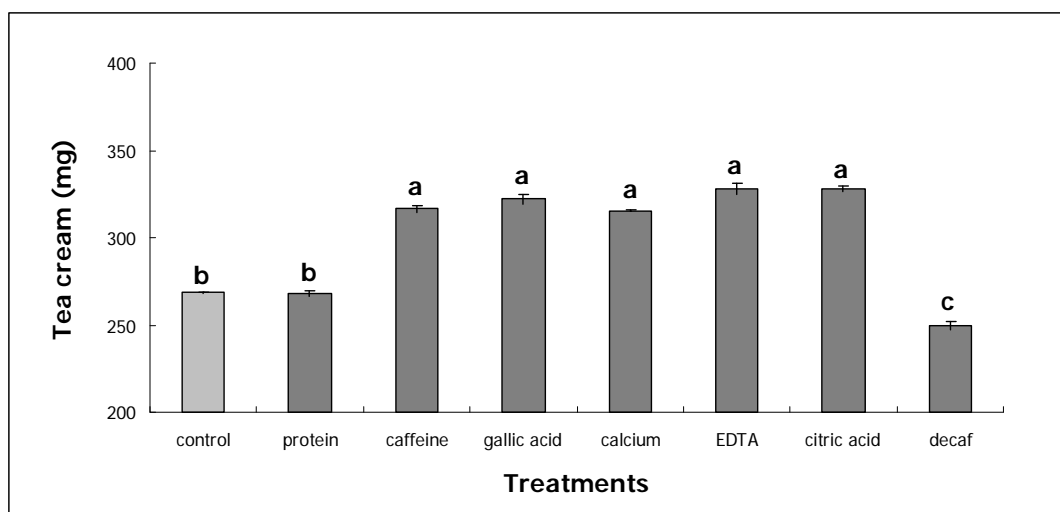
Data obtained from triplicated tea infusions was analyzed by ANOVA using JMP software version 5 (110) and the mean separation was determined by the LSD test ( $P < 0.05$ ).

## Results and Discussion

### *Tea creaming and creaming compounds changes in green tea creaming*

The tea drinking culture in the US is markedly different from other countries whereas teas are mostly consumed as a hot beverage in Europe and Asia about 80 % of tea is served cold in the US. When tea is consumed as a cold drink, black tea has been preferred because the characteristic flavor and taste of black tea are preferred than those of green tea. However, quality deterioration is a frequent occurrence when black tea is stored under refrigerated conditions. Formation of tea cream has been indicated as the causative factor and has led to numerous hypotheses as to the chemical mechanism of the defect. However the recent surge in popularity of green tea in the US, based primarily on its purported health benefits, has created a growing market for RTD refrigerated green tea products. Limited information is available on the causes of green tea creaming and potential preventative measures. Therefore, these studies investigated the mechanism of green tea creaming as well as the potential for tea cream in two non-fermented botanical teas and evaluated proposed treatments to reduce creaming without loss of chemical or physical attributes impacting consumer quality.

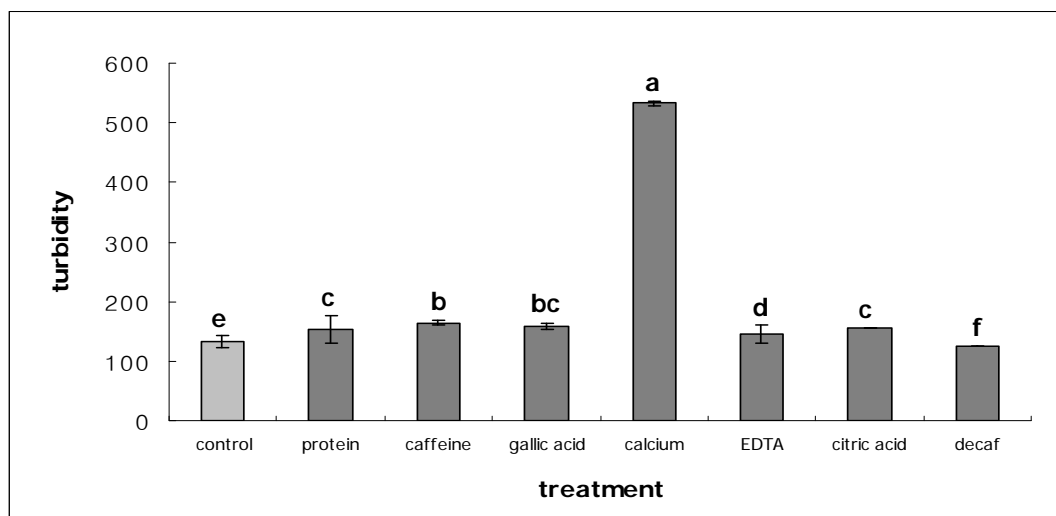
The amount of cream formed in the clarified green tea infusion evaluated herein was 268.4 mg/L after 12 hrs of storage at 4 °C (**Figure 7-1**). The addition of caffeine, gallic acid, calcium, EDTA, and citric acid significantly increased the amount of tea cream by 15.2, 16.7, 14.9, 18.2, and 18.3 %, respectively while 18.4 % less tea cream was found in decaffeinated tea infusion compared to control.



**Figure 7-1.** The amount of tea cream (mg/L) in green tea infusion formed as affected by addition of protein (bovine serum albumin), caffeine, gallic acid, calcium, ethylenediaminetetraacetic acid (EDTA), and citric acid (mg/mL) or removal of caffeine at 4 °C for 12 hours. Bars with different letters for each treatment represent significant difference between triplicated samples (LSD test,  $P < 0.05$ ).

Turbidity was measured due to its direct relationship with tea creaming.

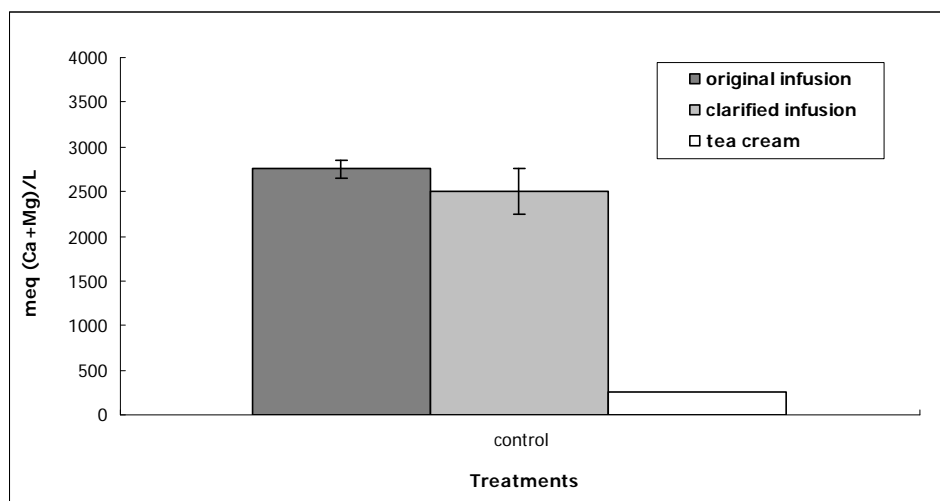
Turbidity is a measurement of not only suspended sediment concentrations but also particle size, shape and color (243). Turbidity increases by higher amount of particles but decreases by smaller particle size due to stronger light scattering ability (235, 244). Previous study proved that turbidity was significantly correlated to the tea cream weights (linear regression coefficient  $r = 0.99$ ,  $P < 0.01$ ) and volume concentration ( $r = 0.986$ ,  $P < 0.01$ ) (235). The turbidity of infusions which directly related to haziness of tea beverage was calcium > caffeine  $\geq$  gallic acid > citric acid = protein > EDTA > control > decaf in tea infusion when measured after 12 hr storage at 4 °C ( $P < 0.05$ ) (**Figure 7-2**). Turbidity increased by addition of protein, caffeine, gallic acid, calcium, EDTA, and



**Figure 7-2.** Turbidity (NTU) of green tea infusion influenced by addition of protein (Bovine serum albumin), caffeine, gallic acid, calcium, EDTA, and citric acid (mg/mL) or removal of caffeine after 12 hour storage at 4 °C. Bars with different letters for each treatment represent significant difference between triplicated samples (LSD test,  $P < 0.05$ ). Abbreviations used: EDTA, ethylenediaminetetraacetic acid.

citric acid but decreased by removal of caffeine as a result of the amount of tea cream changes. Even though addition of protein did not influence on tea cream formation, it increased turbidity by 19 %. Proteins and polyphenolics can form either soluble or insoluble complexes that result in increased turbidity (244, 245). When caffeine and gallic acid were added to green tea infusion, turbidity of the infusions increased by 19 and 16 %, respectively as a result of increased the amount of tea cream by 15 and 17 %. The turbidity that resulted from added calcium had a marked effect on turbidity and increased by 339 % because not only tea cream increased but also added calcium chloride which is highly water soluble formed soluble complex with polyphenolics resulting in significant increase of turbidity (244, 246). Even when calcium was not

added to tea infusion, the concentrations of the second and third prevalent metal contents (Ca and Mg) present in green tea next to potassium (K) were found in green tea cream, indicating green tea contains enough metal content contributing to tea creaming (**Figure 7-3**). The metal ions contribute to tea creaming by binding to already formed tea cream (222), which is called secondary reaction. Addition of chelating agents (EDTA and citric acid) was effective to reduce total metal content but significantly increased tea cream and turbidity. This result is disagreement with Smith (234) which reported adding EDTA decreased the extent of black tea creaming by up to 50 % but caused reformation of tea cream during storage. Both chelating agents, EDTA and citric acid, sequestered total metals by 71 and 47 %, respectively inducing 18 % more tea cream formation in both EDTA and citric acid added infusions. From this result, it was hypothesized that metal-EDTA (citric acid) complex may bind to the other tea creaming compounds such as polyphenolics via their carbonyl groups by hydrogen bond resulting in higher volume of tea cream by increasing radius of tea cream particles and/or the complexes present as an insoluble form in tea infusion may contribute to the total volume of tea cream. To confirm this hypothesis, a further study needs to be conducted to recover metal compounds from EDTA-metal complex in tea cream by dissociating complex using electrolysis method as described by Juang and Wang, (247).



**Figure 7-3.** Changes in total metal content (meq/L) of original infusion, clarified infusion, and tea cream in green tea infusion at 4 °C for 12 hours. Bars represent standard error of the mean (n=3).

According to Liang et al. (88), the tea cream was dissolved using 50 % ethanol (v/v) for chemical analysis, but in the present study the tea cream was not fully dissolved even after 2 hours of ultrasonification. It was early reported that formed tea cream was not redissolved in water at room temperature after ultrasonic bath due to its high insolubility (89). Also, in this study, the tea cream was not fully redissolved by 50 % ethanol and it was supported by HPLC analysis that showed fewer peaks of polyphenolics compared to original and clarified infusions (**Figure 7-4**). If tea cream was fully dissolved in the solution, the phenolic compounds and caffeine should have been detected by HPLC. The amount of polyphenolics, caffeine, protein, and metal in tea cream were determined by subtracting the value of clarified infusion from that of the original infusion as described by Chao and Chiang, (92) and Smith (248).

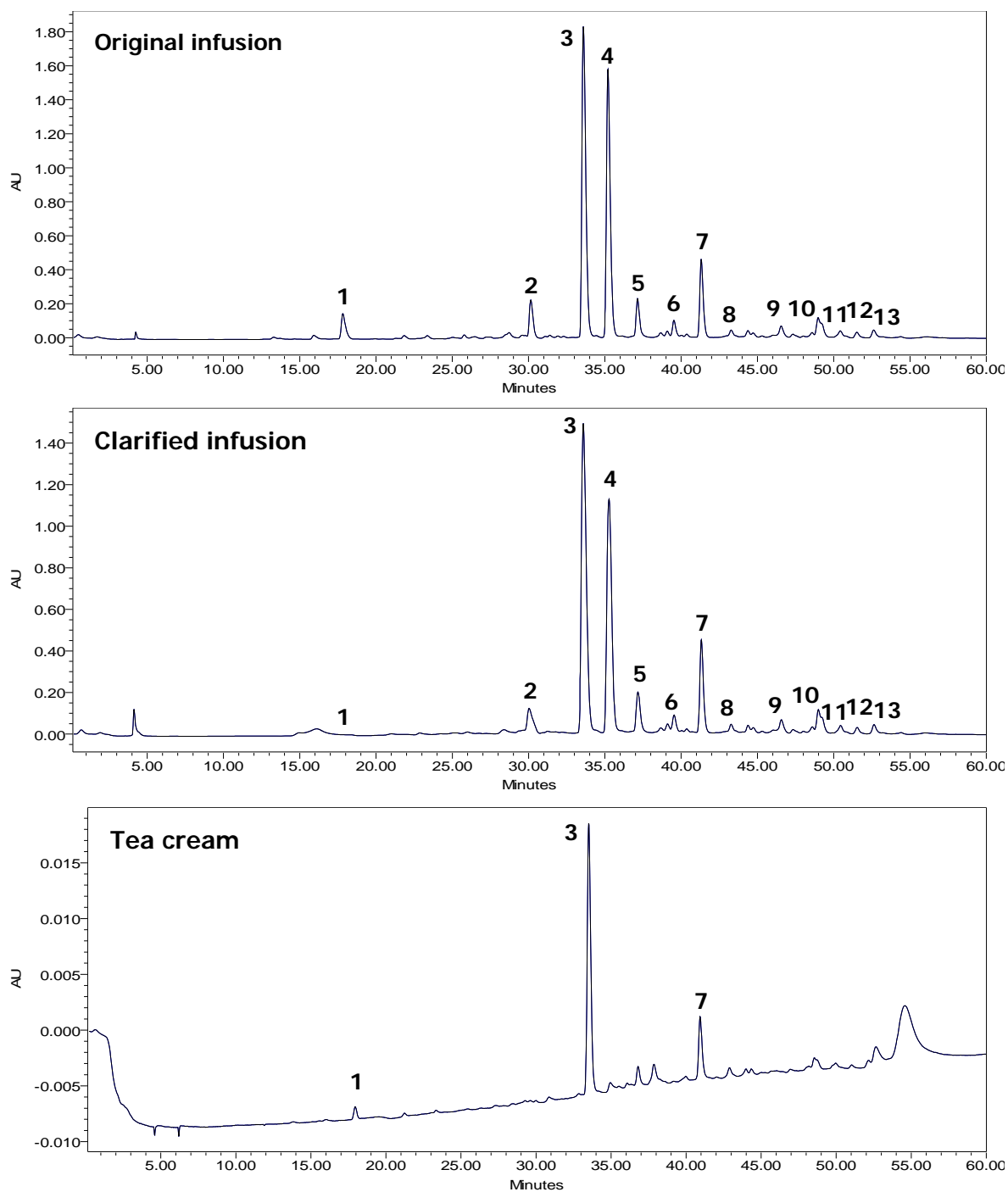


Figure 7-4. HPLC chromatograms of original infusion, clarified infusion, and tea cream. Peak assignment was described in **Table 7-1**.



The highest concentrations of EGCG was present in green tea cream next to caffeine as shown in **Table 7-1**, but 86 % of gallic acid was participating in tea creaming indicating gallic acid most actively contributed to tea creaming. As a result of tea creaming, some phenolic compounds such as gallic acid, EGC, EGCG, myricetin glycosides, and rutin significantly contributed to tea creaming while the participation of the rest was negligible. Protein and caffeine which are known as main compounds participating in primary tea cream formation with polyphenolics was also found in the concentration of 1369 and 54.57 mg/L, respectively in green tea cream (**Figure 7-5**). As a result of tea cream formation, protein and caffeine which are involved in primary tea creaming reaction were also significantly reduced in clarified infusion compared to original infusion as observed polyphenolic changes ( $P < 0.05$ ). It is widely accepted that caffeine has a major role in forming tea cream, additionally supported in the present study, and significantly less amount of tea cream was found in decaffeinated green tea.

**Table 7-1.** The concentration of chemical compounds in different fractions of green tea.

	Original infusion	Clarified infusion	Tea cream	% in tea cream
1. Gallic acid	26.31 ± 2.81 <sup>a</sup>	3.73 ± 0.25	22.58	11.74
2. (-)-epigallocatechin	127.73 ± 2.23	101.70 ± 5.12	26.03	13.54
3. Caffeine	603.14 ± 9.77	548.58 ± 12.46	54.57	28.38
4. (-)-epigallocatechin gallate	435.20 ± 8.08	397.26 ± 15.77	37.94	19.73
5. (-)-epicatechin	84.82 ± 4.04	84.19 ± 1.12	0.63	0.33
6. (-)-gallocatechin gallate	19.52 ± 0.72	17.00 ± 0.89	2.52	1.31
7. (-)-epicatechin gallate	99.22 ± 4.22	97.18 ± 1.81	1.95	1.01
8. Myricetin 3-glycoside-1	29.76 ± 1.97 <sup>b</sup>	22.18 ± 0.88	5.94	3.09
9. Myricetin 3-glycoside-2	39.84 ± 2.59	32.42 ± 2.31	9.07	4.72
10. Quercetin 3-rutinoside	118.85 ± 4.09	89.03 ± 2.75	29.81	15.50
11. Quercetin 3-glucoside	21.34 ± 2.77	21.29 ± 0.12	0.05	0.03
12. Kaempferol 3-glucoside	19.12 ± 0.64	19.02 ± 0.98	0.10	0.05
13. Kaempferol 3-rutinoside	26.33 ± 0.90	25.21 ± 1.02	1.12	0.58

a. Data was expressed as concentration (mg/L) ± standard deviation.

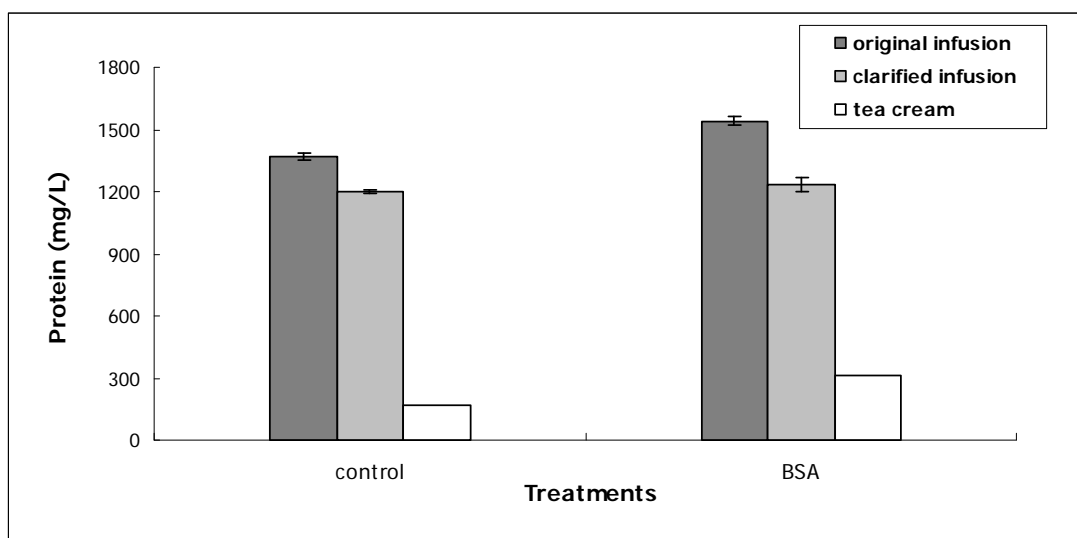
b. Peaks from 8 to 13 were expressed as rutin equivalent.

It was early defined that black tea cream is first formed by binding actions of caffeine or protein to oxidized polyphenolics with galloyl esters (theaflavin and thearubigin) and was believed to be the primary reaction. Other tea constituents such as carbohydrates and metal ions such as calcium were thought to bind to pre-formed tea cream as a secondary reaction that served to increase the mass and radius of the cream (88–92, 104). In green tea, caffeine and tea catechins may form tea cream via hydrogen bonding between and keto-amino group of caffeine and gallyl and/or galloyl group of tea catechins. It has been described that methyl gallate of theaflavin and thearubigin is complexed with caffeine in black tea, however, in green tea the gallyl moiety on tea

catechins may have a role in formation of caffeine-polyphenol or protein-polyphenol complexes. In these trails, gallo-flavanols which three hydroxyl groups attach to the B rings such as EGCG and ECG were found at significant concentrations in tea cream, while catechol-flavanol which contains two hydroxyl groups on their B rings such as ECG and EC was present at relatively lower concentrations (**Table 7-1**). This is supported by that the concentrations of gallo-flavanols were 37.94 (EGCG) and 26.03 mg/L (EGC) in green tea cream but less concentrations of catechol-flavanol were observed in ECG (1.95 mg/L) and EC (0.63 mg/L). Even though ECG has galloyl moiety on its B rings as observed in the structure of EGCG, the degree of participation forming tea cream was negligible. When comparing the concentrations of ECG and EC in the original infusion against clarified infusion, no significant difference was found between two infusions indicating insignificant contribution on green tea creaming. However, with free gallic acid 86 % of the initial amount was present in the tea cream, indicating three hydroxyl groups are playing an important role in tea creaming.

In whole plant tissues, including green tea, flavonols are mostly present as *O*-glycosides (bound to sugars via  $\beta$ -glycosidic bonds) whereas aglycones (flavonoids without attached sugars) are rarely present (249). In the present study, green tea flavonol glycosides from quercetin, and kaempferol, which share a 3-hydroxyflavone backbone, participated in green tea cream formation. But only small portion of quercetin-3-glucoside and two kaempferol glycosides participated in creaming by 0.2, 1, and 4 %, respectively from the concentrations in original infusion compared to significantly higher portion for myricetin glycosides (22 %) and rutin (25 %). No prior studies have

reported rutin in tea creams, which present at the highest concentration among flavonol glycosides in green tea and was not seemingly instrumental in cream complexes with compounds such as caffeine and protein because no increase in rutin in tea cream was observed when caffeine and protein were added to green tea infusion (Data not shown). However, rutin was reported to bind to many different metal ions that are known to be present in green tea such as Cu, Mg, and Fe, indicating rutin may bind to metals ions rather than caffeine and protein (222, 250–253). Polyphenol-protein complex is formed via hydrogen bond between peptide carbonyl group of protein and hydroxyl groups of polyphenol (254). Since polyphenol-protein complex induce haziness and cloudiness in protein rich beverages such as beer, wine and tea, the complex formation has been a problem in beverage industry (90, 98, 255). According to Asano et al. (256), proline ( $\alpha$ -amino acid) containing polypeptides do a major role to form haze and the amount of formed haze was proportional to percentage of proline in the peptide while polypeptides have no proline in their structure formed insignificant amount of haze. This result was supported by Outtrup et al. (257) who reported that proline-containing peptides produced significantly more haze compared to non-proline peptides. In this study, the amount of protein was significantly higher in original infusion than in clarified infusion due to the participation in tea creaming as shown in **Figure 7-5**.

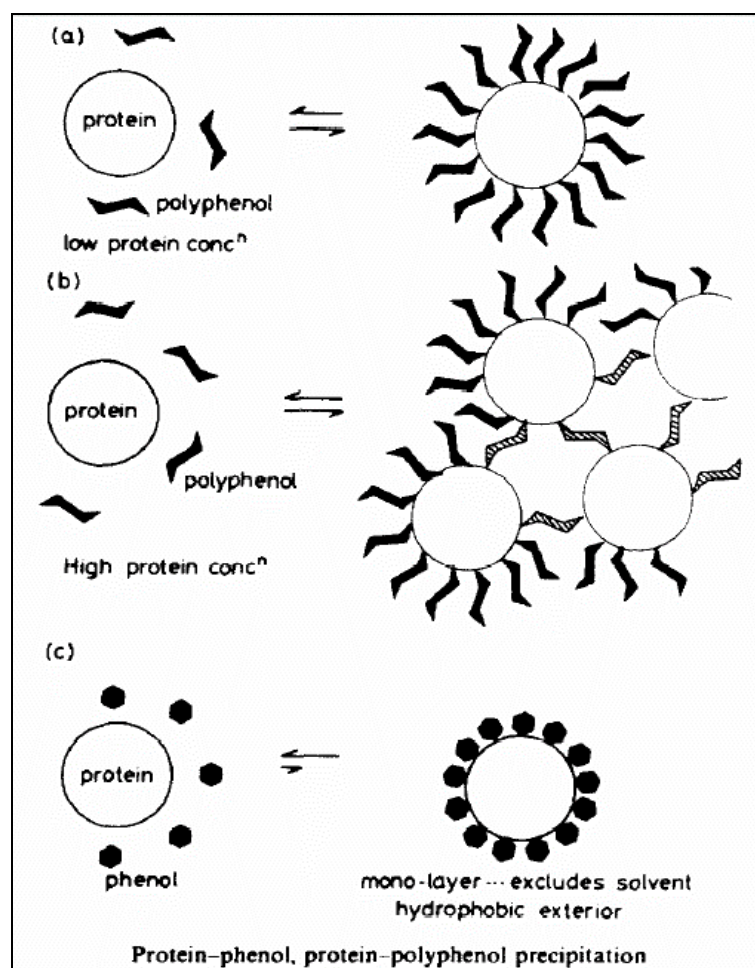


**Figure 7-5.** The protein concentration (mg/L) of different green tea fractions (original infusion, clarified infusion, and tea cream) in control and protein added (mg/mL) infusions. Bars represent standard error of the mean (n=3). Abbreviation used: BSA, Bovine serum albumin.

When bovine serum albumin was added to green tea infusion, the amount of protein in tea cream increased by 45 % indicating protein significantly contributed to green tea creaming. Theanine which makes up approximately 1 -2 % of the dry weight of green tea is proline ( $\alpha$ -amino acid) (154, 258–260) and this explains why protein present in green tea significantly contributed to tea creaming.

The hydrogen bonding between hydroxyl groups of phenolic compounds and peptide bonds of protein is well known interaction in forming strong insoluble polyphenol-protein complex in aqueous solution resulting in increase of haze and cloudiness (90, 98, 245). At relatively low concentration of protein in solution,

polyphenols bind to multiple sites on the protein surface via hydrogen bonding to give a mono-layer which is less hydrophilic than protein inducing development of hydrophobic interaction (**Figure 7-6**). However, when protein present at high concentration in solution, a hydrophobic surface layer is formed by polyphenol-protein complexation and then by cross-linking of different protein molecules by glue-like polyphenol action (260). In case of simple phenols with protein, if those can be present in solutions at high enough concentration to break the equilibrium in favor of protein-simple phenol complex, a hydrophobic monolayer is formed on the protein surface causing hydrophobic interaction (260). As shown in **Figure 7-6**, Tea catechins may bind to protein by either (a) or (b) reaction due to its relatively complex structure while chlorogenic acid and its isomers may form complex via (c) reaction due to its relatively simple structure indicating less or no hydrogen bonding may be involved in tea creaming by protein-polyphenol.



**Figure 7-6.** Polyphenol or simple phenol-protein complexation (260).

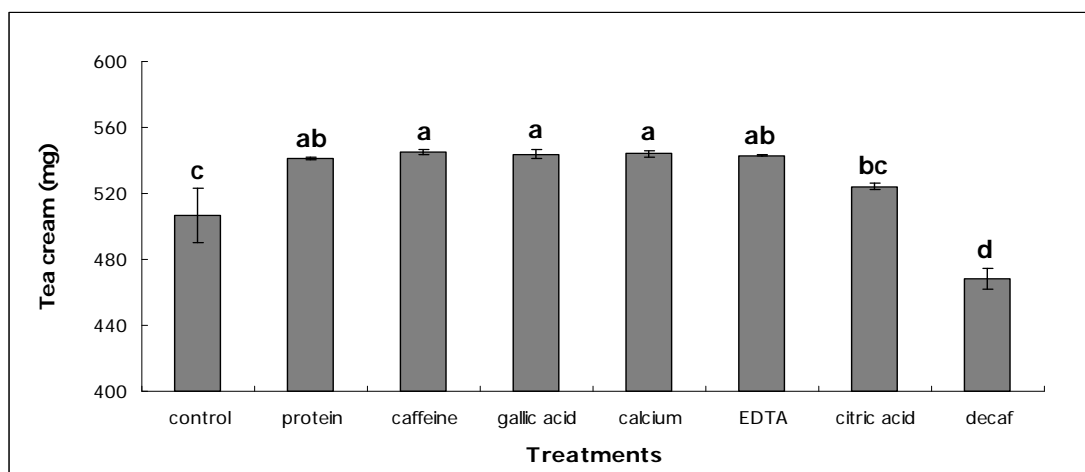
***Tea creaming and creaming compounds changes in yaupon holly***

Yaupon holly contains high concentration of chlorogenic acid and its isomers, which were reported to participate in complexation with caffeine, protein, and metal ions. The studies regarding to tea creaming solely focused on the teas from *camellia sinensis* but no studies reported tea creaming with chlorogenic acid although it is the predominant compound in botanical teas. In the present study with yaupon holly infusion, tea

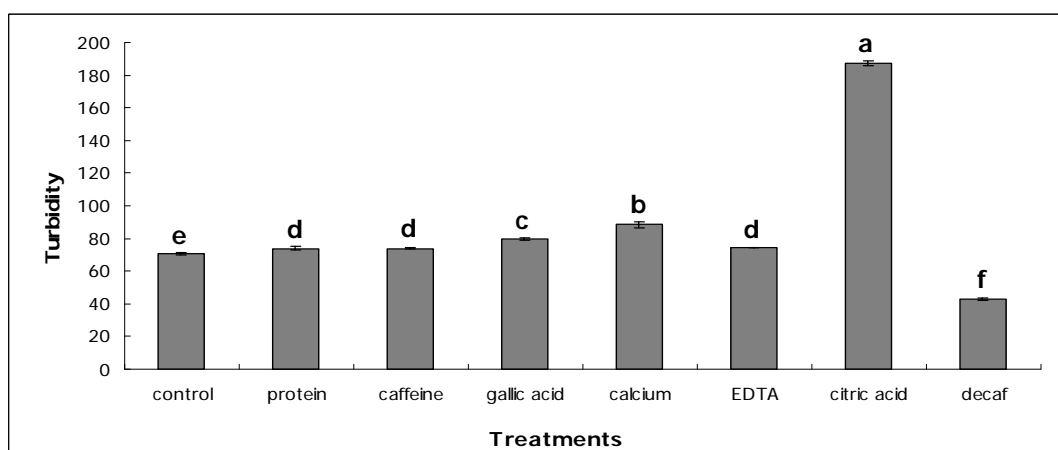
creaming with chlorogenic acid and other tea creaming compounds such as caffeine, protein, and metals ions was investigated.

The amount of tea cream in yaupon holly was higher by 47 % than in green tea and the weight of yaupon holly tea cream was 506.60 mg/L (**Figure 7-7**). The tendency to form tea cream in the presence of additives was very similar to green tea, with exception of added protein and citric acid. In green tea, addition of BSA was not a factor to increase the amount of tea cream, however, BSA fortification to yaupon holly infusion significantly increased the amount of tea cream. On the contrary, citric acid addition, which significantly contributed to green tea cream, did not influence the tea cream formation in yaupon holly infusions. The amount of tea cream formed was highest when caffeine was added (545.23 mg/L) and lowest when caffeine was removed (468.40 mg/L). Even though the amount of tea cream was higher in yaupon holly infusion compared to that of green tea, its overall turbidity was lower (**Figure 7-8**). As observed in green tea creaming, all the treatments significantly increased turbidity of yaupon holly infusion while decaffeination lowered turbidity. Addition of citric acid to yaupon holly infusion as a chelating agent drastically increased turbidity of the infusion by 266 %, which was not observed in turbidity changes in green tea.





**Figure 7-7.** The amount of tea cream (mg) in yaupon holly infusion formed as affected by addition of protein (Bovine serum albumin), caffeine, gallic acid, calcium, EDTA, and citric acid or removal of caffeine at 4 °C for 12 hours. Bars with different letters for each treatments represent significant difference between triplicated samples (LSD test,  $P < 0.05$ ). Abbreviations used: EDTA, ethylenediaminetetraacetic acid.



**Figure 7-8.** Turbidity of yaupon holly infusion influenced by addition of protein (bovine serum albumin), caffeine, gallic acid, calcium, EDTA, and citric acid or removal of caffeine after 12 hour storage at 4 °C. Bars with different letters for each treatments represent significant difference between triplicated samples (LSD test,  $P < 0.05$ ). Abbreviations used: EDTA, ethylenediaminetetraacetic acid.

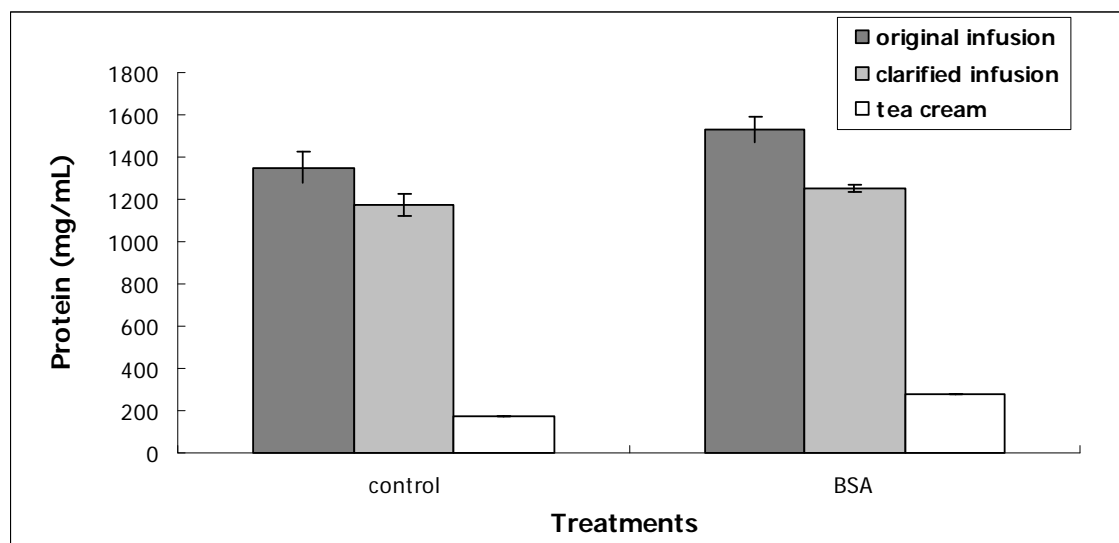
Chlorogenic acid (5-caffeoylquinic acid) is an ester of caffeic acid and quinic acid (118). Chlorogenic acid can form an insoluble complex with caffeine resulting in tea creaming in various types of beverages which contain caffeine and chlorogenic acid via hydrogen bond and hydrophobic interaction (100, 101, 103, 260). The formation of chlorogenic acid-caffeine complex was also reported by Hamidi and Wanner, (261) who illustrated the complex might be formed during the extraction process. Even though the plant genus *Ilex* which belongs to the holly genus such as yerba maté (*Ilex paraguariensis*) and yaupon holly (*Ilex vomitoria*) contains significant amount of chlorogenic acid and its isomers and caffeine (48, 117, 262), no studies have reported the formation of tea creaming in *Ilex* infusions. The total concentration of hydroxycinnamic acids was 1004.2 mg/L and it was 20 % higher than flavan 3-ols concentration in green tea (**Table 7-2**). The participation of dicaffeoylquinic acids in tea creaming was negligible compared to chlorogenic acid and isomers of chlorogenic acid and was in agreement with Sondheimer et al. (103) who described lower complexing ability of dicaffeoylquinic acid with other tea creaming compounds such as caffeine, protein, and metal ions compared to chlorogenic acid. Two flavonol glycosides (kaempferol 3-glycoside and quercetin 3-rutinoside) found in yaupon holly infusion participated in tea creaming and especially rutin (quercetin 3-rutinoside) contribute to tea creaming at the highest rate (25.72 % of total tea cream) followed by chlorogenic acid (25.12 %), 3-caffeoylquinic acid (23.65 %), and 4-caffeoylquinic acid (23.65 %).

**Table 7-2.** The concentration of chemical compounds in different fractions of yaupon holly.

	original infusion (mg/L) (C)	clarified infusion (mg/L) (C)	tea cream (mg/L) (C)	original infusion (mg/L) (D)	clarified infusion (mg/L) (D)	tea cream (mg/L) (D)
1. 3- <i>O</i> -caffeoylquinic acid	423.02 ± 2.59 <sup>a</sup>	399.37 ± 3.80	23.65	462.11 ± 2.83	461.02 ± 6.17	1.09
2. 5- <i>O</i> -caffeoylquinic acid	317.63 ± 0.35	292.51 ± 1.94	25.12	345.37 ± 2.14	340.49 ± 4.04	4.88
3. 4- <i>O</i> -caffeoylquinic acid	124.82 ± 0.34	114.57 ± 0.97	10.25	133.25 ± 0.08	128.45 ± 1.04	4.80
4. Caffeine	139.55 ± 0.47	122.66 ± 0.36	16.89	ND	ND	ND
5. 3,4-dicaffeoylquinic acid	67.73 ± 0.24	66.09 ± 1.08	1.64	77.73 ± 0.56	75.72 ± 0.49	2.01
6. Kaempferol 3-glycoside	102.11 ± 9.90	98.18 ± 3.92	3.93	136.74 ± 1.92	128.87 ± 1.13	7.87
7. 3,5-dicaffeoylquinic acid	18.94 ± 0.22	16.80 ± 0.57	2.14	22.24 ± 0.05	20.45 ± 0.46	1.79
8. Quercetin 3-rutinoside	392.44 ± 0.07	366.72 ± 0.39	25.72	455.75 ± 0.10	412.16 ± 2.24	43.59
9. 4,5-dicaffeoylquinic acid	52.06 ± 0.41	44.58 ± 0.55	7.48	60.43 ± 1.26	56.09 ± 0.13	4.34

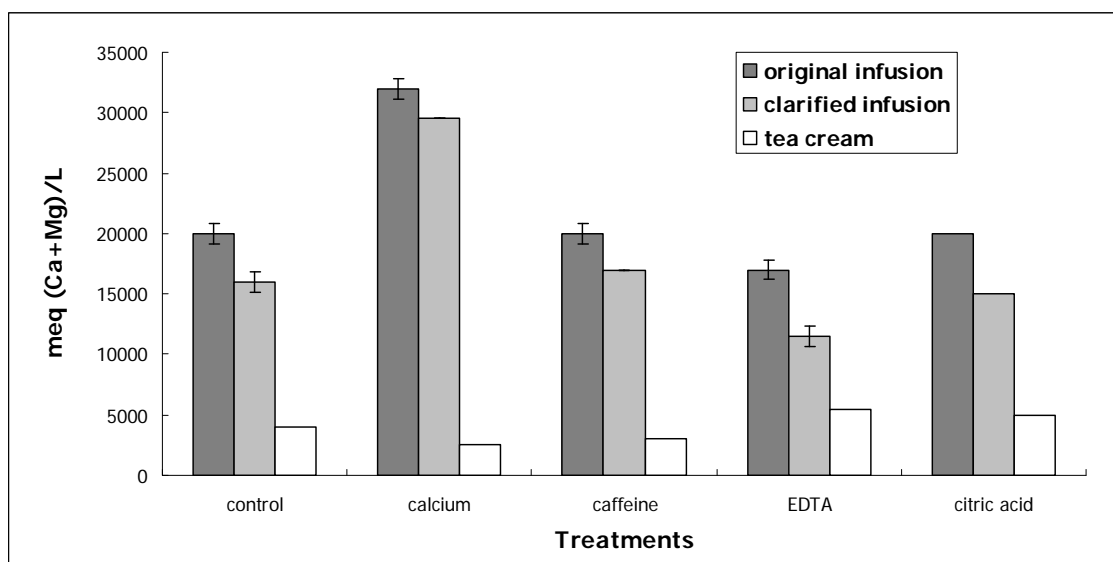
- a. (C) represents fractions from control infusion.
- b. (D) represents fractions from decaffeinated infusion.
- c. Data was expressed as concentration (mg/L) ± standard deviation.
- d. Peaks 6 and 8 were expressed as rutin equivalent.
- e. ND. Not detected.

As shown in **Figure 7-9**, protein in yaupon holly infusion participated in tea creaming. When BSA was added to yaupon holly infusion, 36 % more protein and 41 % more chlorogenic acid and its isomers (data not shown) were found in tea cream of yaupon holly. However, when caffeine was removed from yaupon holly infusion, the participation of chlorogenic acid and its isomers and dicaffeoylquinic acid in tea creaming significantly decreased as shown in **Table 7-2**. This indicates that chlorogenic acid and its isomers may bind to caffeine with higher priority than protein in yaupon holly infusion. Chlorogenic acid-protein complex has been also reviewed in other foods (263–265).



**Figure 7-9.** The protein concentration (mg/L) of different yaupon holly fractions (original infusion, clarified infusion, and tea cream) in control and protein added (mg/mL) infusions. Bars represent standard error of the mean (n=3). Abbreviation used: BSA, Bovine serum albumin.

Garcia et al. (266) reported that significant amount of metal ions such as Mg, K, I, and Ca present in both leaf and infusion in yerba maté (*Ilex paraguariensis*), the same genus as yaupon holly, whereas significant amounts of metals were also found in yaupon holly infusions (**Figure 7-10**). Metals in yaupon holly infusion contributed to tea creaming but addition of calcium did not increase participation of metals on tea creaming, indicating only certain amount of metal contributes tea creaming. According to Kolaylı et al. (267), complex formation capacity of caffeine with metals such as Ca and Mg was significantly lower than EDTA. Likewise, higher amount of metals were found in tea cream when EDTA and another chelating agent, citric acid, were added to yaupon holly infusion.



**Figure 7-10.** Changes in total calcium and magnesium content (meq/L) in yaupon holly infusion influenced by addition of calcium, EDTA, and citric acid at 4°C for 12 hours. Bars represent standard error of the mean (n=3). Abbreviations used: EDTA, ethylenediaminetetraacetic acid.

In yaupon holly infusion, noticeable amount of saponin is present and the concentration was significantly higher than green tea (**Table 7-3**). According to West et al. (268), saponin bound to metal ions and formed precipitation. Moreover, it was observed that saponin also formed a complex with soluble vitamins *in vitro* studies (269). Thus, it is hypothesized that saponin may bind to metal ions and/or soluble vitamins in yaupon holly infusion and induced more tea creaming compared to green tea infusion which contains very small amount of saponin.

**Table 7-3.** Total saponin concentrations in green tea, yaupon holly, and mamaki.

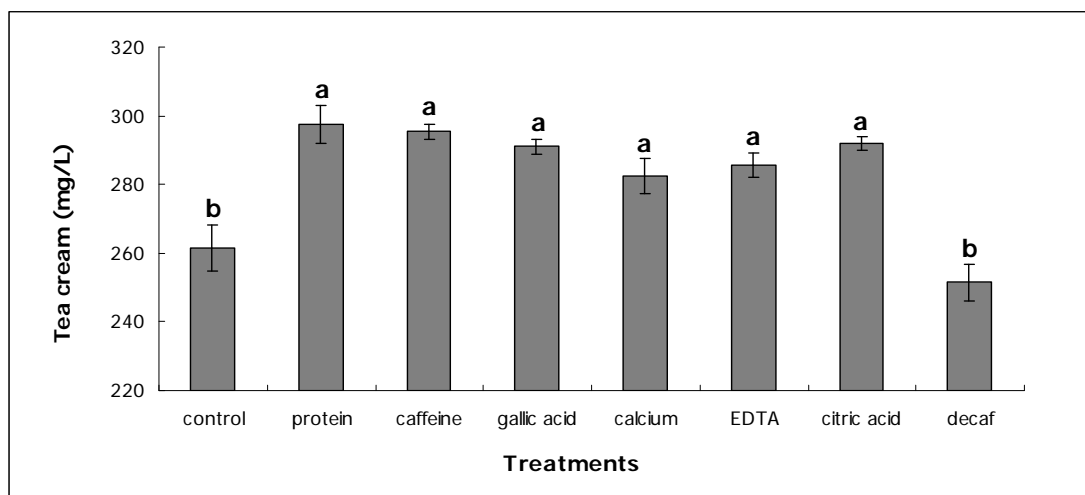
Tea	Green tea	Yaupon holly	Mamaki
Concentration (mg/L)	49.98 ± 1.31 <sup>a</sup>	1031.64 ± 18.50	470.83 ± 10.56

<sup>a</sup>. Data was expressed as mean concentration (mg/L) ± standard deviation of n=3.

#### ***Tea creaming in mamaki and changes in creaming compounds***

The amount of tea cream formed in mamaki infusion was 261.33 mg/L, which is almost equal amount of cream with green tea (268.4 mg/L) but it was significantly lower than that of yaupon holly by 48 % (**Figure 7-11**). The trends in tea creaming in mamaki infusion was similar with that in green tea and yaupon holly because all the treatments significantly increased the tea cream formation. Even though no caffeine presence in mamaki infusion was found by preliminary study but the same decaffeination treatment was applied to keep the consistency and as predicted, the treatment did not affect on tea creaming in mamaki infusion (**Table 7-4**). In mamaki

infusions, quercetin 3-rutinoside was the predominant compound and its concentration was higher than sum of chlorogenic acid and its isomers in both the original mamaki infusions and tea cream. Even though chlorogenic acid and its isomers significantly contributed to tea creaming in yaupon holly infusion, it was found that those compounds less significantly participated in creaming in mamaki infusion due to less concentration. Turbidity of mamaki infusion was elevated by addition of protein, caffeine, gallic acid, calcium, EDTA, and citric acid as observed in previous two teas (**Figure 7-12**). When calcium was added to mamaki infusion, the turbidity increased by 215 % as observed in turbidity changes in green tea.



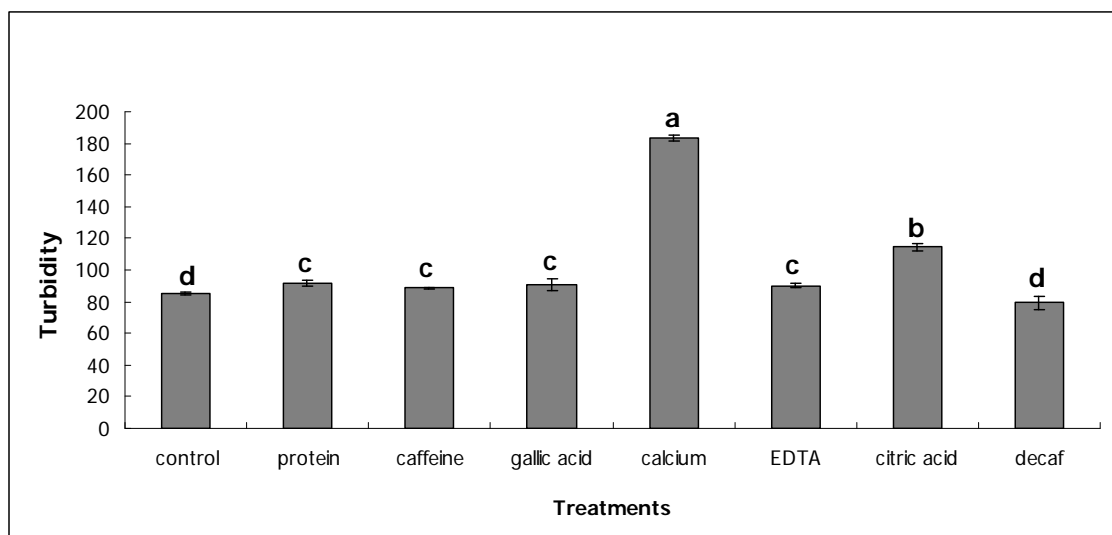
**Figure 7-11.** The amount of tea cream (mg) in mamaki infusion formed as affected by addition of protein (Bovine serum albumin), caffeine, gallic acid, calcium, EDTA, and citric acid and chloroform treatment at 4 °C for 12 hours. Bars with different letters for each treatments represent significant difference between triplicated samples (LSD test,  $P < 0.05$ ). Abbreviations used: EDTA, ethylenediaminetetraacetic acid.

**Table 7-4.** The concentration of chemical compounds in different fractions of mamaki.

	original infusion (mg/L)	Clarified infusion (mg/L)	tea cream (mg/L)
1. 3- <i>O</i> -caffeoylquinic acid	119.23 ± 2.16 <sup>a</sup>	113.61 ± 4.50	5.62
2. 5- <i>O</i> -caffeoylquinic acid	146.40 ± 1.25	134.93 ± 19.91	11.47
3. 4- <i>O</i> -caffeoylquinic acid	97.51 ± 0.96	92.56 ± 3.29	4.94
4. Ester of caffeic acid	74.27 ± 2.36	73.89 ± 2.58	0.28
5. Apigenin 7-glucoside_1	52.80 ± 0.35 <sup>b</sup>	49.27 ± 0.27	3.54
6. Apigenin 7-glucoside_2	75.23 ± 1.01	71.72 ± 0.18	3.50
7. Quercetin 3-rutinoside	699.50 ± 3.38	635.99 ± 22.06	63.51
8. Kaempferol 3-rutinoside	119.44 ± 1.70	108.14 ± 8.90	11.29

a. Data was expressed as concentration (mg/L) ± standard deviation.

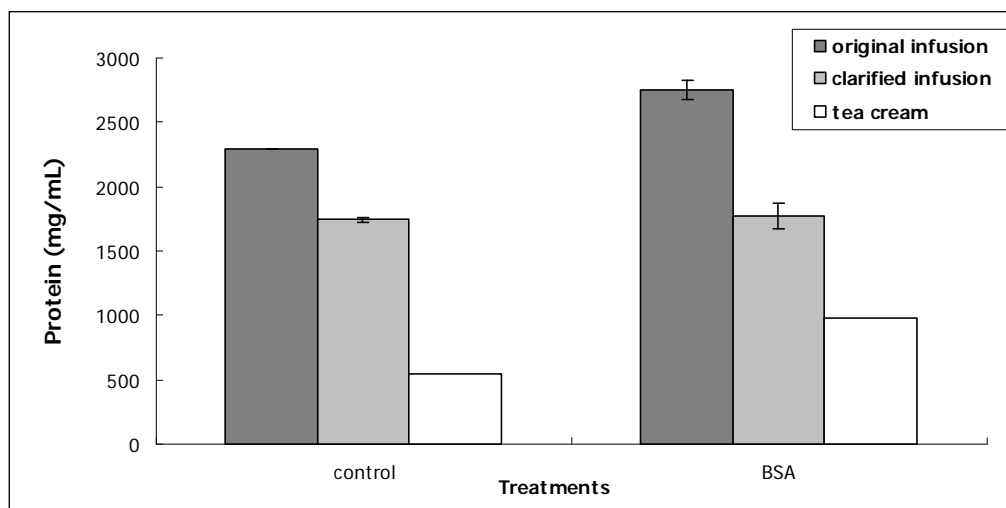
b. Peaks from 5 to 8 were expressed as rutin equivalent.



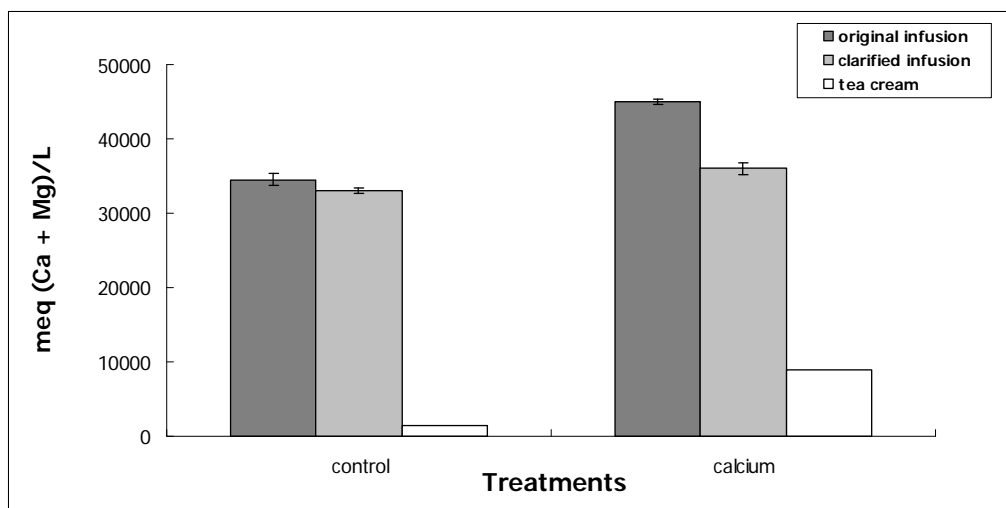
**Figure 7-12.** Turbidity of mamaki infusion influenced by addition of protein (bovine serum albumin), caffeine, gallic acid, calcium, EDTA, and citric acid or removal of caffeine after 12 hour storage at 4 °C. Bars with different letters for each treatments represent significant difference between triplicated samples (LSD test,  $P < 0.05$ ). Abbreviations used: EDTA, ethylenediaminetetraacetic acid.



The naturally present protein and metals were significantly higher than those of green tea and yaupon holly (**Figure 7-13, 14**). Owing to lack of caffeine in mamaki infusion, polyphenol-protein-metal complex is considered as major contributing compounds to tea creaming of mamaki infusion. The most predominant phenolic compound found in mamaki tea cream was rutin present by 64.4 % since it was present in mamaki infusion at highest concentration among all phenolic compounds. The amount of protein and metal found in tea cream was also drastically higher than those of green tea by 322 and 600 % respectively, indicating protein-metal complex was more prevalent in mamaki tea cream than other tea creams. When protein and calcium were added to mamaki infusion, the concentration of each compound in tea cream increased by 44 and 600 %, respectively. This result explains that protein-metal complex in mamaki infusion was active reaction. Protein-metal complex has been reviewed by many literatures. Proteins make use of metal ions which exist primarily as a free ion species in foods to bind substrates, to maintain structure and to effect analysis (270). Metal ions bind to protein atoms and suitable binding sites are sometimes engineered for best suitable binding affinity and protein purification (270).



**Figure 7-13.** The protein concentration (mg/L) of different mamaki fractions (original infusion, clarified infusion, and tea cream) in control and protein added (mg/mL) infusions. Bars represent standard error of the mean (n=3). Abbreviation used: BSA, Bovine serum albumin.

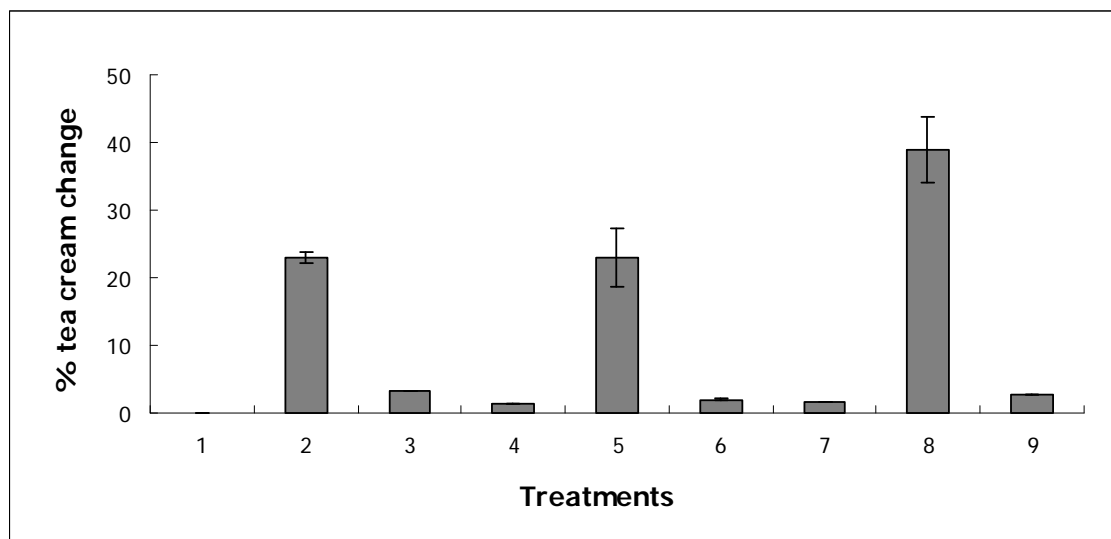


**Figure 7-14.** Changes in total calcium and magnesium content (meq/L) in mamaki infusion influenced by addition of calcium at 4 °C for 12 hours. Bars represent standard error of the mean (n=3). Abbreviations used: EDTA, ethylenediaminetetraacetic acid.

*Changes in tea creaming in green tea infusion and effects on polyphenolics and antioxidant capacity by macroaeration and deoxygenation*

Nine treatments were conducted to investigate changes in tea creaming by aeration and deaeration followed by pH adjustment. The aeration was applied in this study to oxidize phenolic compounds in teas resulting in lowering hydrogen bond and tea infusions were deaerated for comparison. The macroaeration and deoxygenation were performed to add and remove oxygen. The treatments were assigned as Treatment 1. control (no treatment and no pH conversion), Treatment 2. no treatment at pH 3, Treatment 3. no treatment at pH 7, Treatment 4. deoxygenation and no pH conversion, Treatment 5. deoxygenation at pH 3, Treatment 6. deoxygenation at pH 7, Treatment 7. macroaeration and no pH conversion, Treatment 8. macroaeration at pH 3, Treatment 9, macroaeration at pH 7.

Green tea creaming significantly increased by lowering pH from 5.45 (pH of original green tea infusion) to pH 3 regardless of treatments and when macrooxygenation was applied on pH 3 infusion (treatment 8), the amount of green tea cream was highest among all treated infusions (**Figure 7-15**). The weight of tea cream without any treatment and pH conversion was 199.4 mg/L and it increased by 23, 23, and 39 % by treatment 2, 5, and 8, respectively when pH was reduced to 3. Without pH conversion, no effect was observed by oxidation using macroaeration method and deoxygenation using direct nitrogen sparging.



**Figure 7-15.** % tea cream change in green tea by different treatments. Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7. Bars represent standard error of the mean (n=3).

When pH was adjusted to neutral, polyphenolics in green tea showed a quick degradation in the original green tea infusions at pH 7 (treatment 3, 6, and 9) while no significant change in polyphenolics was observed in pH 3 infusions (treatment 2, 5, and 8) compared to original infusions (treatment 1, 4, and 7) (**Table 7-5**). Moreover, no changes of tea polyphenolics were observed when pH of infusion was lowered to 3 without respect to air treatment. Caffeine which is a well-known stable xanthine alkaloid was also not affected by any treatments. However, significantly higher amount of EGC and EGCG were found in tea cream from treatment 2, 5, and 8 (pH 3) and consequently, higher amount of caffeine was also observed in tea cream from treatment 2 and 5.

**Table 7-5.** Polyphenolics and caffeine concentrations in green tea cream affected by macroaeration and deoxygenation followed by pH conversion.

Treatments Compounds	1 <sup>a</sup>	2	3	4	5	6	7	8	9
1.(-)-epigallocatechin	9.33 <sup>b</sup> (10.6%) <sup>c</sup>	17.02 (10.7%)	6.87 (4.8%)	41.74 (29.8%)	41.69 (56.7%)	5.68 (3.9%)	34.29 (59.6%)	88.57 (60.8%)	8.12 (5.8%)
2.Caffeine	28.44 (4.7%)	36.53 (5.7%)	13.95 (2.4%)	19.04 (3.2%)	31.03 (4.9%)	8.48 (1.4%)	21.40 (3.7%)	26.18 (4.1%)	29.01 (4.9%)
3.(-)-epigallocatechin gallate	28.50 (8.5%)	30.51 (6.6%)	14.60 (3.3%)	66.80 (15.5%)	123.56 (42.7%)	14.19 (3.3%)	119.72 (54.6%)	162.58 (36.9%)	31.08 (7.1%)
4.(-)-epicatechin	7.08 (8.0%)	4.58 (4.6%)	6.14 (6.5%)	0.41 (0.4%)	2.25 (2.7%)	2.25 (2.5%)	11.59 (14.4%)	7.54 (7.7%)	4.43 (5.0%)
5.(-)-gallocatechin gallate	1.09 (6.8%)	0.34 (1.6%)	0.49 (2.4%)	3.29 (15.8%)	6.10 (42.2%)	0.46 (2.3%)	3.12 (30.3%)	7.66 (37.7%)	0.72 (3.7%)
6.(-)-epicatechin gallate	5.61 (6.2%)	2.69 (2.7%)	6.37 (6.6%)	2.67 (2.7%)	1.82 (2.2%)	4.22 (4.4%)	9.49 (12.9%)	5.86 (5.9%)	7.85 (8.1%)
7.Myricetin 3-glycoside_1	0.72 (2.8%)	7.82 (23.0%)	4.34 (11.5%)	6.56 (20.4%)	1.12 (4.3%)	5.31 (15.3%)	5.22 (20.5%)	0.63 (2.3%)	3.16 (8.8%)
8.Myricetin 3-glycoside_2	3.11 (10.4%)	2.35 (6.9%)	2.84 (7.8%)	0.18 (0.6%)	4.15 (15.0%)	3.15 (8.6%)	4.09 (16.0%)	4.90 (14.9%)	0.35 (1.0%)
9.Quercetin 3-rutinoside	4.00 (3.2%)	6.43 (4.9%)	0.33 (0.3%)	3.59 (2.8%)	0.41 (0.3%)	14.03 (10.2%)	9.40 (8.1%)	0.92 (0.7%)	2.03 (1.6%)
10.Quercetin 3-glycoside	3.18 (8.3%)	1.11 (2.4%)	0.04 (0.1%)	1.38 (3.2%)	1.06 (2.9%)	5.58 (11.7%)	6.89 (20.8%)	2.83 (5.9%)	1.66 (3.8%)
11.Kaempferol 3-glycoside	2.73 (8.3%)	3.04 (7.3%)	2.28 (6.2%)	1.58 (4.4%)	2.76 (8.5%)	4.90 (13.5%)	5.31 (18.6%)	3.48 (9.2%)	1.98 (5.5%)
12.Kaempferol 3-rutinoside	0.85 (3.9%)	0.75 (3.1%)	0.79 (3.3%)	2.04 (8.8%)	1.01 (4.5%)	1.90 (8.1%)	3.66 (17.4%)	1.72 (7.3%)	1.41 (5.8%)

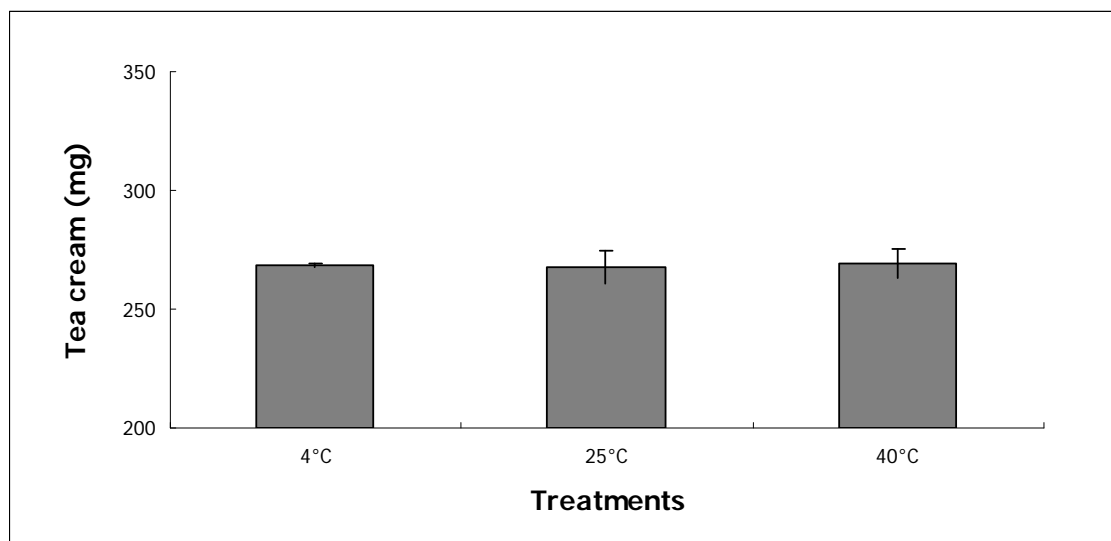
a. Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7.

b. Data was expressed as mg/L.

c. % in parenthesis represents percentage of polyphenolics and caffeine in tea cream from original infusion.

When aeration and deaeration were combined with pH conversion, two general trends were observed: lowering pH increased tea creaming and macroaeration contributed to tea cream formation when combining with pH reduction. It has been reported that lowering pH to around 3 reduced tea cream formation in black and oolong tea (92, 104, 237, 248). Even though the same amount of tea cream contributing compounds were observed in between pH 3 and original infusions (pH 5.50), the concentration in tea cream from pH 3 was higher in green tea cream due to its higher tea creaming power. This indicates that the hydrogen bond was lowered by reduced pH and oxidation, it increased tea creaming in green tea. The other driving force of tea creaming, hydrophobic interaction, was inversely proportional to pH change (271). When pH was elevated from 3.2 to 7.2, the hydrophobic interaction significantly decreased in different aqueous media. This explains that tea creaming power was higher in pH 3 tea infusions by increased hydrophobic interaction in tea infusion. When macroaeration was applied followed by lowering pH, the amount of tea cream was highest. By oxidation, a hydrogen atom is released from the breaking of O-H bond present in a phenolic compound and resulting in reducing hydrogen bond strength (272). This indicates that hydrophobic interaction plays an important role in forming tea creaming in green tea even when hydrogen bond became weaker. This was additionally supported by the result from the study tested tea creaming at different temperatures. Green tea infusions were stored at 4, 25, and 40 °C to determine tea creaming behavior when hydrogen bonding was weakened by elevated temperature (273). The amount of tea cream formed at different temperatures was 268.4, 267.6, and 269.4 mg at 4, 25, and 40 °C, respectively

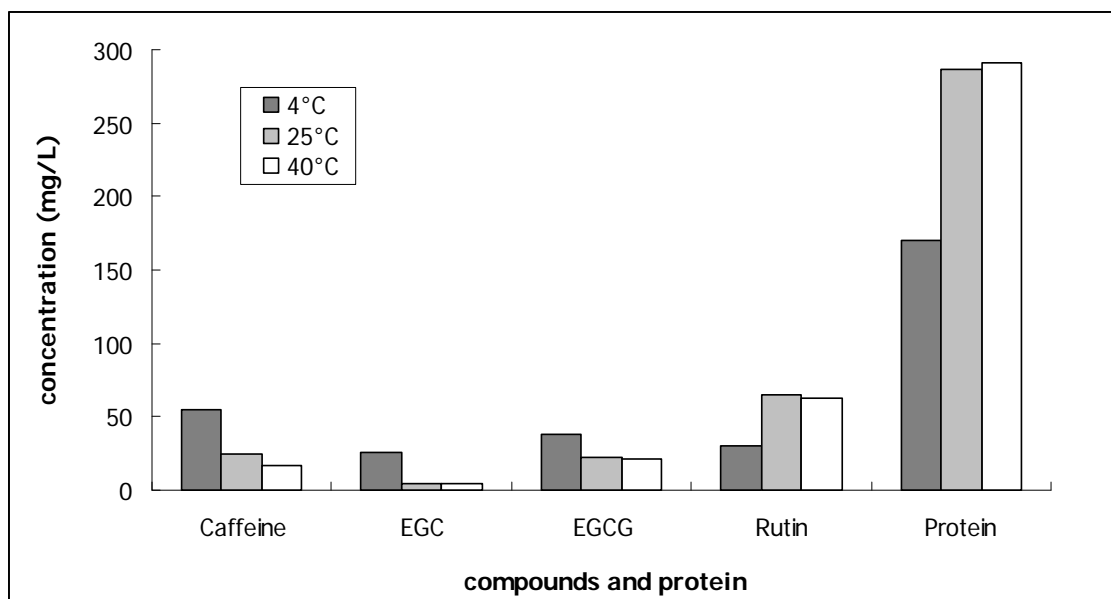
(Figure 7-16). Owing to lowered hydrogen bond, less tea creaming was expected, however no difference of tea cream was observed between three different temperatures due to main driving force in tea creaming, hydrophobic interaction.



**Figure 7-16.** The amount of tea cream (mg) in green tea infusion formed as influenced by different storage temperatures at 4, 25, and 25 °C for 12 hours. Bars represent standard error of the mean (n=3).

As shown in **Figure 7-17**, caffeine and two major tea cream contributing compounds, EGC and EGCG, were significantly higher in the infusion at 4 °C while other tea creaming compounds such as rutin and protein were higher in the infusions at 25 and 40 °C. This result suggested that hydrogen bond was more involved in caffeine-tea catechin complex in green tea, but other complex such as flavonol-protein complex is mostly formed by hydrophobic interaction since hydrophobic interaction is stabilized at

high temperature and destabilized at low temperature (254). Additionally, theanine was known to be more hydrophobic than many other amino acids (260).

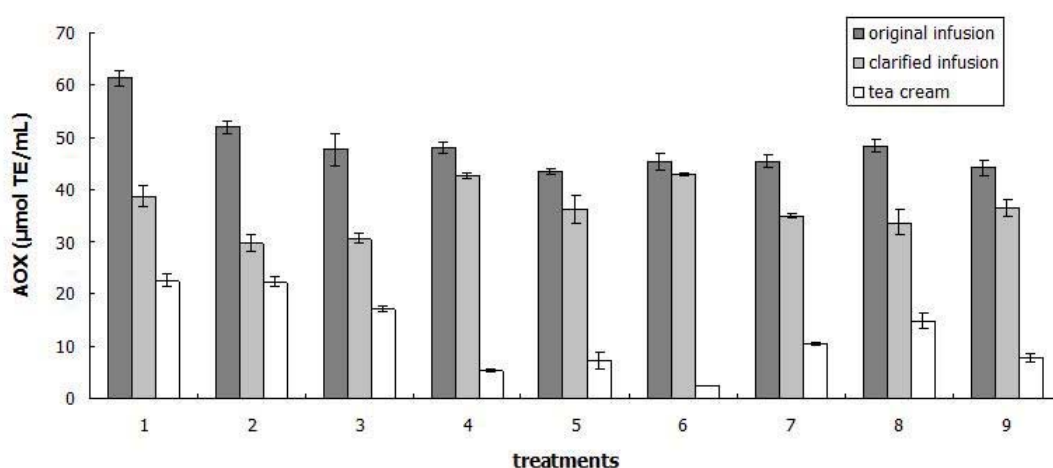


**Figure 7-17.** The concentrations of caffeine, EGC, EGCG, rutin, and protein in tea cream from green tea infusion as influenced by different storage temperature at 4, 25, and 25 °C for 12 hours. Abbreviation used: EGC. Epigallocatechin, EGCG. Epigallocatechin gallate, rutin. Quercetin 3-glycoside.

Antioxidant capacity in original infusion, clarified infusion, and tea cream determined by ORAC assay was shown in **Figure 7-18**. Antioxidant capacity was measured to determine whether loss of compounds in tea infusion by tea creaming affects antioxidant capacity of tea infusion. Significant loss of antioxidant capacity from original infusion by formed tea cream was observed in most infusions and the degree of reduction was highest in no oxygen treated infusions (treatment 1, 2, and 3).



Deoxygenation effectively removed dissolved oxygen in tea infusion resulting in minor losses of antioxidant capacity. Even though macroaeration reduced polyphenolic concentration from original infusion, the antioxidant capacity did not decrease due to the presence of unaffected antioxidant compounds. When higher amount of tea cream formed by treatment 2, 5, and 8, more reduction of antioxidant capacity was observed.



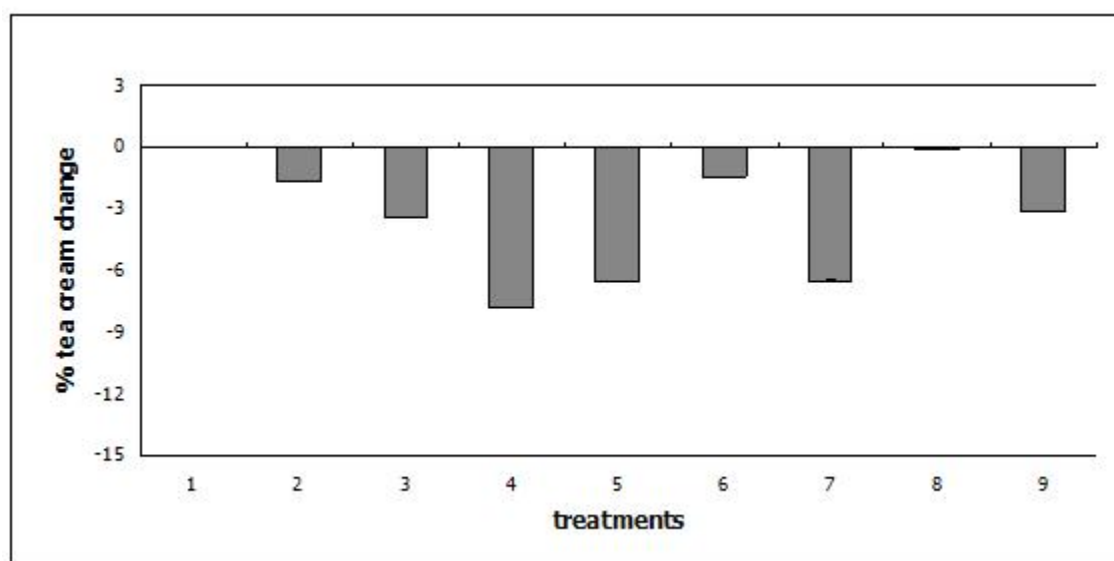
**Figure 7-18.** Antioxidant capacity in original infusion, clarified infusion, and tea cream of green tea as affected by different treatments. Bars represent standard error of the mean (n=3). Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7. Abbreviations used: AOX. Antioxidant capacity, TE. Trolox equivalent.

***Changes in tea creaming in yaupon holly infusion and effects on polyphenolics and antioxidant capacity by oxygenation and deoxygenation***

Tea cream in yaupon holly infusion did not change by any treatments as shown in **Figure 7-19** and only minor reductions in tea creaming were observed. The biggest difference in the amount of tea cream was found between control and treatment 4 by 7.8% with no significant difference ( $P < 0.05$ ). Chlorogenic acid was reported that it is stable in acidic aqueous media while pH neutral or higher makes chlorogenic acid degrade quickly (180) as observed in treatment 3 and 6 except treatment 9 (**Table 7-6**). The chlorogenic acid and its isomers concentrations in pH 3 infusions (treatment 2, 5, and 8) were significantly higher or equal to the original infusions. According to Oszmianski and Lee, (214), reaction rate of oxidation of chlorogenic acid was higher at pH 6.5 than pH 3.5, so likewise the lowest chlorogenic acid concentration was observed in yaupon holly original infusion at treatment 6. As observed in green tea infusions, caffeine in original infusions was not affected by pH conversion. In tea cream portion, lower concentration of chlorogenic acids as well as caffeine was found in pH 7 infusions regardless of treatments.

No difference between treatments although different concentrations of chlorogenic acid and caffeine was due to more amount of protein involved in creaming due to higher binding capacity between chlorogenic acid and protein at neutral pH than pH 3. Chlorogenic acid is an amphiphile compound which possesses both hydrophobic and hydrophilic properties, so contains a hydrophobic phenyl ring at the end of the molecule and hydrophilic nature exists at the other end with a carboxylic acid group and

an OH-substituted cyclohexane ring (265). This suggests that hydrophobic interaction is the most important driving force to form chlorogenic acid-protein complex due to its amphiphile nature. The carboxylic acid group on chlorogenic acid which is ionized at neutral pH and still positively charged side chain of protein forms a complex in a higher rate by lowered ionic strength (264, 265).



**Figure 7-19.** % tea cream change in yaupon holly by different treatments. Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7. Bars represent standard error of the mean (n=3).

**Table 7-6.** Polyphenolics and caffeine concentrations in yaupon holly cream affected by macroaeration and deoxygenation followed by pH conversion.

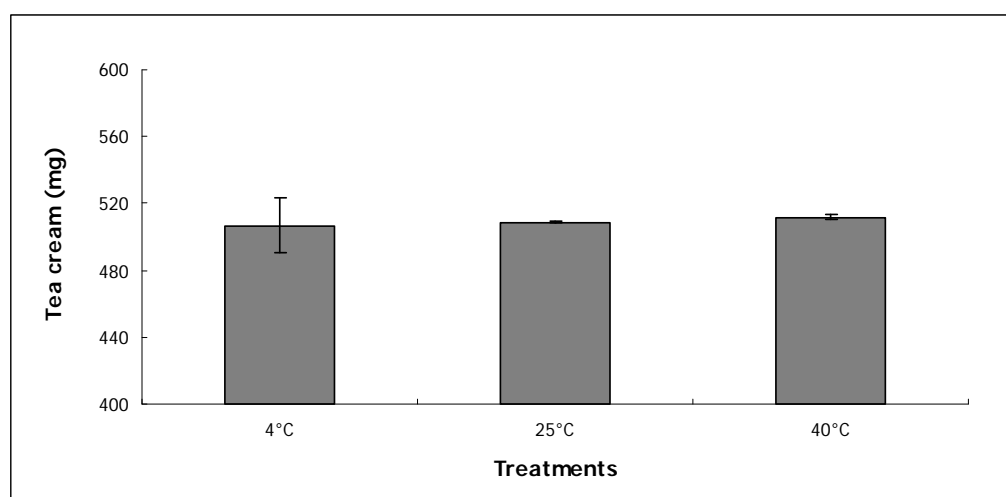
Treatments Compounds	1 <sup>a</sup>	2	3	4	5	6	7	8	9
1. 3- <i>O</i> -caffeoylquinic acid	30.83 <sup>b</sup> (6.60%) <sup>c</sup>	52.03 (10.35%)	17.42 (4.01%)	22.72 (5.10%)	44.54 (11.44%)	4.02 (0.93%)	30.56 (6.67%)	27.93 (6.02%)	24.92 (6.50%)
2. 5- <i>O</i> -caffeoylquinic acid	35.99 (8.93%)	46.47 (11.17%)	4.01 (1.12%)	31.68 (8.37%)	34.08 (10.15%)	3.92 (1.05%)	26.10 (6.63%)	24.18 (7.26%)	16.16 (4.12%)
3. 4- <i>O</i> -caffeoylquinic acid	26.35 (11.97%)	15.67 (7.46%)	2.84 (1.32%)	4.57 (2.24%)	5.28 (2.59%)	0.27 (0.14%)	9.70 (4.38%)	9.99 (4.82%)	8.42 (3.96%)
4. Caffeine	15.57 (16.50%)	7.62 (8.71%)	3.23 (3.60%)	2.56 (2.89%)	1.00 (1.11%)	0.97 (1.14%)	2.53 (2.80%)	10.03 (10.92%)	11.55 (12.96%)
5. 3,4-dicaffeoylquinic acid	4.42 (7.56%)	16.79 (29.33%)	9.16 (25.07%)	24.95 (43.34%)	30.77 (89.08%)	14.51 (28.29%)	17.64 (31.37%)	25.86 (45.18%)	18.65 (66.78%)
6. Kaempferol 3- <i>O</i> -glycoside	37.16 (18.01%)	33.73 (17.14%)	21.30 (13.84%)	39.46 (22.50%)	73.00 (48.87%)	15.59 (8.74%)	39.30 (21.30%)	61.08 (42.39%)	14.13 (7.95%)
7. 3,5-dicaffeoylquinic acid	7.07 (19.47%)	5.58 (17.06%)	2.92 (7.55%)	0.61 (1.85%)	13.48 (37.33%)	2.21 (7.25%)	4.58 (11.90%)	3.83 (11.98%)	11.08 (28.81%)
8. Quercetin 3-rutinoside	33.49 (8.29%)	88.78 (22.24%)	47.13 (15.00%)	75.77 (22.10%)	109.66 (42.34%)	31.93 (8.71%)	90.56 (23.15%)	121.17 (43.98%)	70.91 (18.37%)
9. 4,5-dicaffeoylquinic acid	11.81 (12.12%)	25.97 (27.21%)	28.23 (30.36%)	24.59 (26.92%)	52.99 (63.01%)	12.41 (14.01%)	21.66 (22.79%)	46.93 (56.79%)	17.24 (18.92%)

a. Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7.

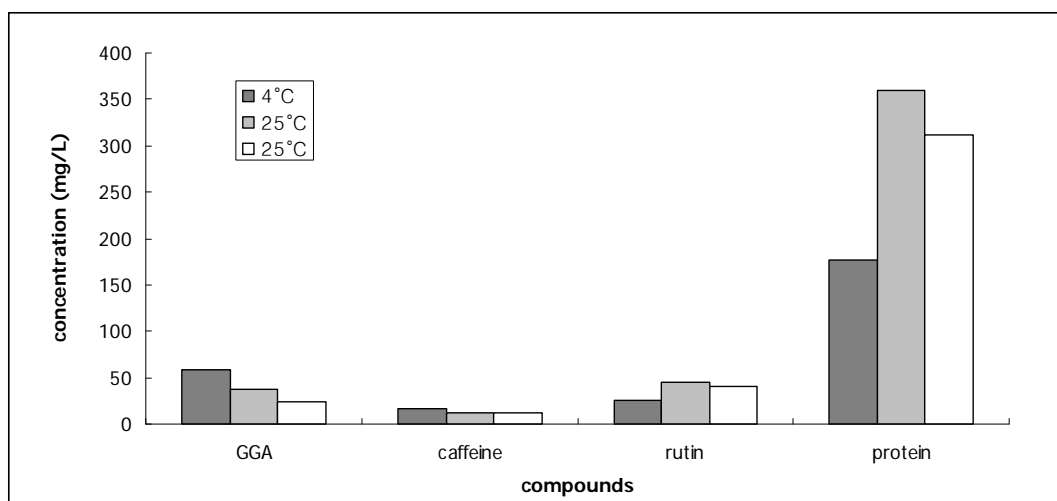
b. Data was expressed as mg/L.

c. % in parenthesis represents percentage of polyphenolics and caffeine in tea cream from original infusion.

As observed in green tea cream changes by lowering hydrogen bonding using storage at different temperatures, no tea cream changes was observed in yaupon holly tea cream (**Figure 7-20**). Additionally, higher amount of caffeine was found in tea cream at lower temperature (4 °C) and more protein was involved in tea creaming when storage temperature was higher (25 and 40 °C) (**Figure 7-21**). It indicates that caffeine-polyphenol complex was mainly formed via hydrogen bond while protein-polyphenolic complex was formed by hydrophobic interaction. According to **Table 7-6**, flavonol glycosides such as kaempferol 3-glycoside and quercetin 3-rutinoside participated in tea creaming of yaupon holly. Due to significant amount of flavonols in tea cream and formation of polyphenol-protein complex by hydrophobic interaction in yaupon holly infusion, no difference in tea creaming was observed although chlorogenic acid concentration is different in tea infusions.

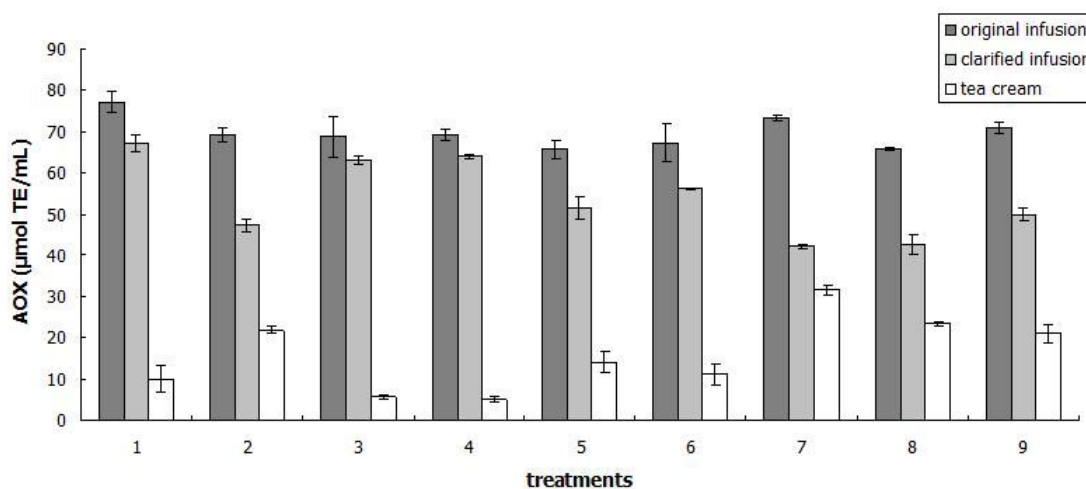


**Figure 7-20.** The amount of tea cream (mg) in yaupon holly infusion formed as influenced by different storage temperatures at 4, 25, and 25 °C for 12 hours. Bars represent standard error of the mean (n=3).



**Figure 7-21.** The concentrations of caffeine, EGC, EGCG, rutin, and protein in tea cream from yaupon holly infusion as influenced by different storage temperature at 4, 25, and 25 °C for 12 hours. Abbreviation used: CGA, total concentration of chlorogenic acid and its isomers, rutin. Quercetin 3-rutinoside.

Antioxidant capacity of yaupon holly original infusion was higher than that of green tea by 21 % due to higher concentration of polyphenolics and protein (**Figure 7-22**). The antioxidant capacity was highest in the cream from the infusions of treatment 7, 8, and 9 where deoxygenation was applied because rutin concentration which shows higher antioxidant capacity than chlorogenic acid in tea cream was higher in 7, 8, and 9 than in other treatments (167). The amount of tea cream formed was not proportional to the antioxidant capacity since the creaming compounds participated in creaming have different antioxidant capacity.

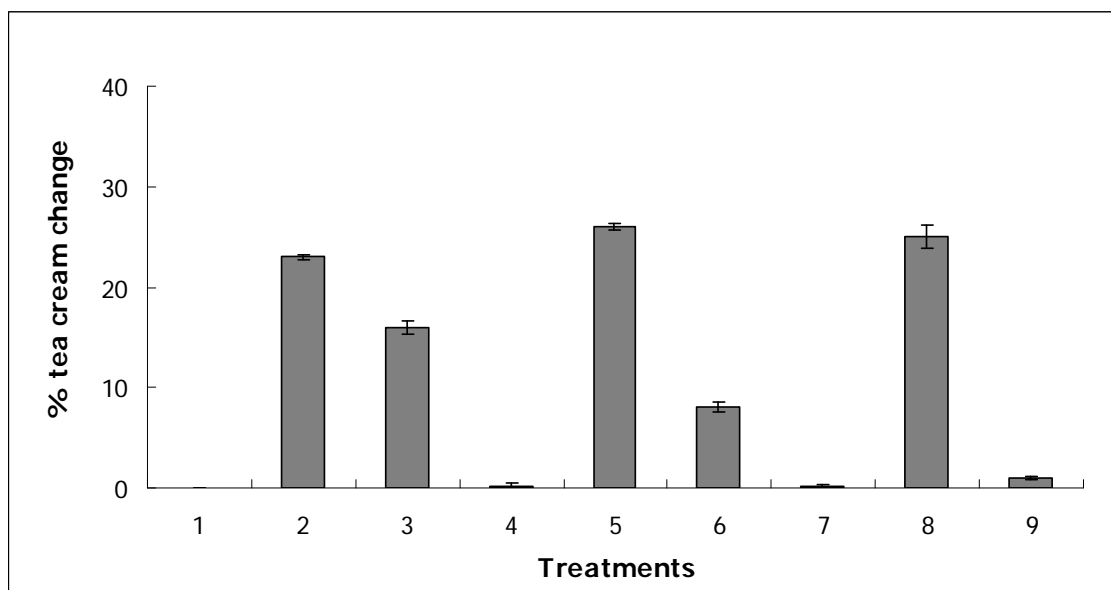


**Figure 7-22.** Antioxidant capacity in original infusion, clarified infusion, and tea cream of yaupon holly as affected by different treatments. Bars represent standard error of the mean (n=3). Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7. Abbreviations used: AOX. Antioxidant capacity, TE. Trolox equivalent.

***Changes in tea creaming in mamaki infusion and effects on polyphenolics and antioxidant capacity by oxygenation and deoxygenation***

As observed in green tea creaming change, the amount of tea cream was higher in all pH 3 mamaki infusions (**Figure 7-23**). Tea cream increased by 23, 26, and 25 % in treatment 2, 5, and 8, respectively. Even though mamaki infusion contains similar polyphenolic profile with yaupon holly such as 3,4,5-caffeoylquinic acid and rutin (quercetin 3-rutinoside), different tea creaming tendency was observed in the two tea infusions. When pH was elevated to 7 without oxygen treatments, 16 % increase in tea

creaming was observed while other pH 7 samples (treatment 6 and 9) showed only minor changes by 8 and 1 %, respectively.



**Figure 7-23.** % tea cream change in yaupon holly by different treatments. Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7. Bars represent standard error of the mean (n=3).

The pH of mamaki infusion is naturally high as 7.75 and it is much higher than those of green tea (5.5) and yaupon holly (5.21) infusions. Thus, chlorogenic acid which is very unstable in neutral pH was quickly degraded and the concentration in the original mamaki infusion was 79 % lower than that in pH 3 infusion (**Table 7-7**). Even though significantly less concentrations of chlorogenic acid and its isomers were found in the



original infusions than pH 3, the concentration present in tea cream was not significantly different due to its higher binding power as observed in yaupon holly tea creaming.

Rutin which is present in mamaki infusion as highest concentration was the most prevalent compound in mamaki tea cream regardless of treatment. Significantly more tea cream was formed in mamaki infusion stored at 25 and 40 °C compared to 4 °C (**Figure 7-24**). Chlorogenic acid present in mamaki infusion may be from chlorogenic acid-protein complex via hydrophobic interaction because more chlorogenic acid was found in the infusions at 25 °C and even higher at 40 °C (**Figure 7-25**). It was early reported that chlorogenic acid binds to protein at higher rate when temperature goes up from 10 to 40 °C since conformational change in polypeptides, which alter binding affinity for small ligand molecules like chlorogenic acid and more accessible binding sites of protein to chlorogenic acid (265). More tea creaming in pH 3 infusions can be explained by that flavonols in mamaki such as rutin and apigenin glycoside aggressively formed a complex with protein which present at very high concentration in mamaki infusion. The high rate of flavonol (rutin) ability in forming complex via hydrophobic interaction is supported by that 8.2 and 11.4 fold more rutin was found in mamaki tea cream at 25 and 40 °C, respectively where hydrogen bonding is weak.

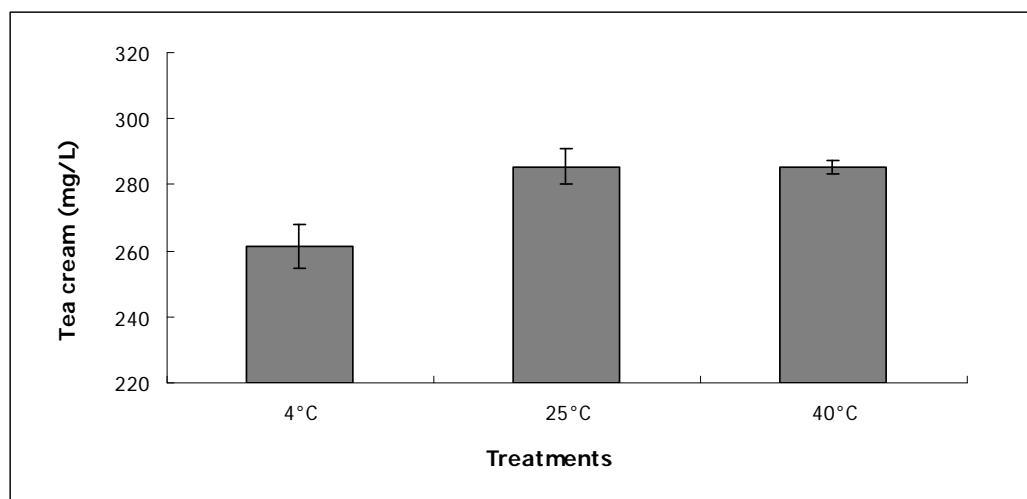
**Table 7-7.** Polyphenolic concentrations in tea cream of mamaki affected by macroaeration and deoxygenation followed by pH conversion.

Treatments Compounds	1 <sup>a</sup>	2	3	4	5	6	7	8	9
1. 3- <i>O</i> -caffeoylquinic acid	6.20 <sup>b</sup> (25.56%) <sup>c</sup>	9.49 (8.35%)	4.96 (6.39%)	10.90 (51.35%)	11.44 (10.33%)	4.02 (8.42%)	12.25 (74.64%)	4.84 (4.48%)	6.55 (9.14%)
2. 5- <i>O</i> -caffeoylquinic acid	5.44 (19.13%)	9.05 (6.71%)	4.55 (5.10%)	3.27 (15.31%)	7.86 (6.06%)	2.62 (5.46%)	4.10 (23.51%)	3.46 (2.72%)	5.93 (7.20%)
3. 4- <i>O</i> -caffeoylquinic acid	1.13 (2.85%)	2.91 (2.88%)	0.87 (1.23%)	11.27 (35.46%)	4.64 (4.77%)	1.20 (2.89%)	5.29 (23.79%)	2.94 (3.05%)	3.64 (5.63%)
4. Ester of caffeic acid	14.71 (51.93%)	4.68 (10.65%)	0.02 (0.07%)	14.04 (51.24%)	4.29 (10.97%)	0.47 (1.73%)	9.41 (43.53%)	0.37 (0.95%)	0.27 (0.89%)
5. Apigenin-7-glucoside_1	10.08 (5.17%)	24.51 (18.64%)	11.42 (4.91%)	8.05 (4.56%)	16.45 (12.88%)	8.51 (4.44%)	7.18 (4.41%)	16.86 (13.18%)	8.19 (4.28%)
6. Apigenin-7-glucoside_2	5.12 (3.35%)	23.37 (11.45%)	1.41 (0.74%)	3.22 (2.74%)	14.33 (7.20%)	0.57 (0.43%)	3.68 (3.64%)	12.52 (6.56%)	4.73 (3.02%)
7. Quercetin 3-rutinoside	51.51 (29.54%)	36.38 (6.50%)	25.54 (5.84%)	30.54 (25.43%)	25.52 (4.74%)	16.70 (6.33%)	22.71 (27.61%)	24.15 (4.52%)	30.15 (7.89%)
8. Kaempferol-3-rutinoside	17.94 (37.14%)	5.69 (6.76%)	0.33 (0.46%)	15.77 (39.67%)	4.17 (5.03%)	1.53 (3.22%)	7.50 (28.87%)	5.17 (6.21%)	2.63 (4.42%)

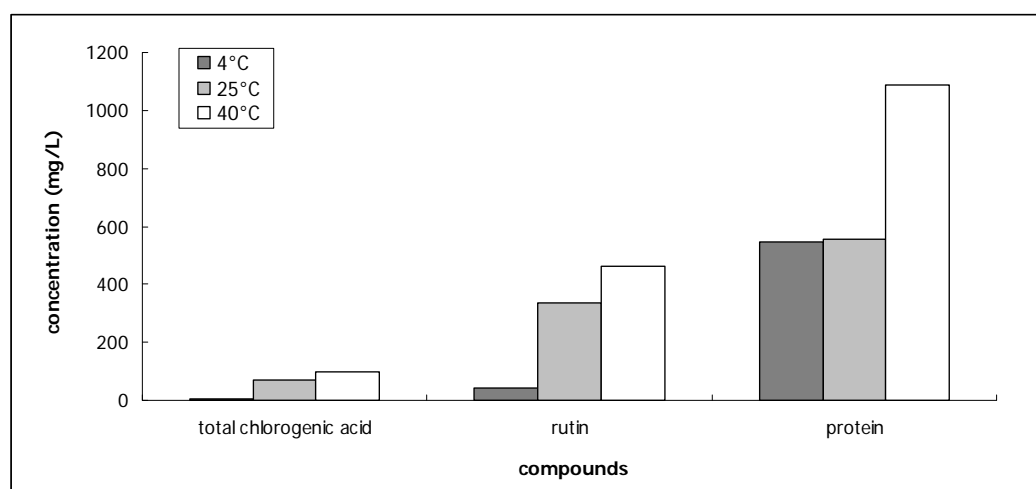
a. Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9, macrooxygenation at pH 7.

b. Data was expressed as mg/L.

c. % in parenthesis represents percentage of polyphenolics in tea cream from original infusion.

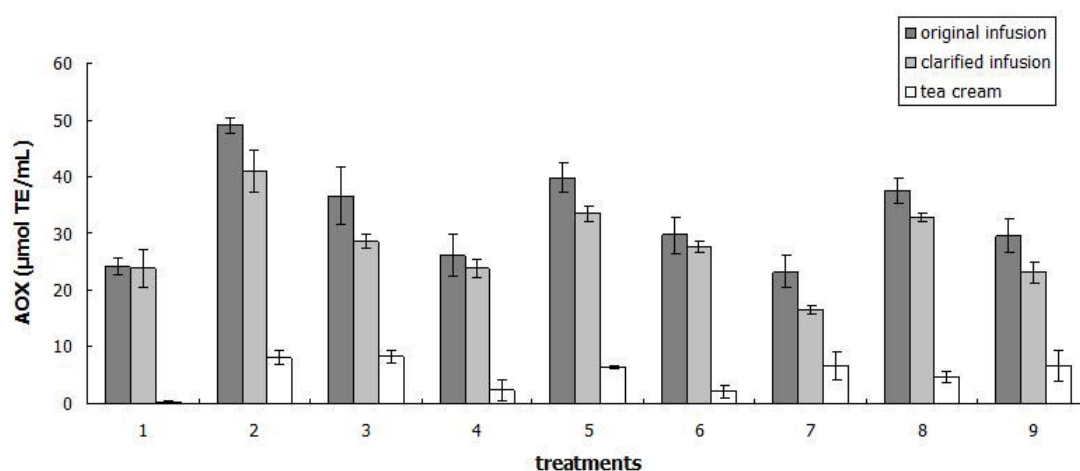


**Figure 7-24.** The amount of tea cream (mg) in mamaki infusion formed as influenced by different storage temperatures at 4, 25, and 25 °C for 12 hours. Bars represent standard error of the mean (n=3).



**Figure 7-25.** % tea cream change in mamaki by different treatments. Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7. Bars represent standard error of the mean (n=3).

As shown in polyphenolic concentration changes in mamaki infusion, antioxidant capacity was higher in all pH 3 tea infusions than original (pH 7.75) and pH 7 infusions (**Figure 7-26**). Even though tea creaming was higher at low pH, antioxidant capacity did not show significant difference at treatment 2 and 8 compared to other pH infusions. As observed above, the amount of tea cream formed was not directly proportional to antioxidant capacity changes but did lose the benefit of tea drinking due to lowered antioxidant capacity of tea infusion by lowered antioxidant capacity.



**Figure 7-26.** Antioxidant capacity in original infusion, clarified infusion, and tea cream of mamaki as affected by different treatments. Bars represent standard error of the mean (n=3). Treatment numbers assignment. 1. control (no treatment and no pH conversion), 2. no treatment at pH 3, 3. no treatment at pH 7, 4. deoxygenation and no pH conversion, 5. deoxygenation at pH 3, 6. deoxygenation at pH 7, 7. macrooxygenation and no pH conversion, 8. macrooxygenation at pH 3, 9. macrooxygenation at pH 7. Abbreviations used: AOX. antioxidant capacity, TE. Trolox equivalent.

## CHAPTER VIII

### SUMMARY AND CONCLUSION

Ready-to-drink tea market has extensively grown due to an increased awareness of the potential health benefits from bioactive polyphenolics present in teas. The tea industry are pursuing new novel methods to improve not only the overall quality and but also phytochemical contents in recent years. The stability of phytochemicals in botanical teas in relation to green tea was evaluated in the present study.

Among the processing treatments applied to green tea, yaupon holly, and mamaki, addition of ascorbic acid and lowered pH were proven to improve phytochemical stability and resultant antioxidant capacity while heating adversely influenced the stability of phenolic compounds in teas. Use of packaging was a significant factor in terms of phytochemical stability and glass was the most appropriate packaging material to store Ready-to-drink tea at cold storage due to delayed oxidation. Other oxygen permeable packagings such as PET and retortable pouch allowed higher degree of oxidation resulted in lowered antioxidant capacity of teas. Storage conditions also significantly influenced phytochemical stability of teas by inducing photooxidation under exposure to light and by accelerating oxidation process as storage temperature increased. The formation of tea cream is a highly undesirable phenomenon due to increased haziness in tea beverages. The protein-polyphenolics complex formed by hydrophobic interaction was the reason to make tea cream insoluble while caffeine-polyphenolics complex via hydrogen bond was dissociated by treatments.

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