EFFECTS OF WATER CHEMISTRY AND PANNING ON FLAVOR VOLATILES AND CATECHINS IN TEAS (*Camellia sinensis*)

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ABSTRACT

In the first experiment, effects of brewing time, chlorine, chloramine, iron, copper, pH and water hardness were investigated for their effects on extraction of epigallocatechine gallate (EGCG) and caffeine in green tea and oolong tea aqueous infusions. The extraction of EGCG and caffeine were lower when green tea was brewed in hard water compared to distilled water. Brewing green tea and Oolong tea in tap water resulted in higher extraction of caffeine but had no effect on EGCG compared to distilled water. The extraction of EGCG and caffeine were significantly increased ($P<0.05$) when green tea and Oolong tea were brewed in the chlorinated water at 4.0 mg free chlorine per liter.

The purpose of the second experiment was to optimize SDE conditions (solvent and time) and to compare SDE with SPME for the isolation of flavor compounds in Jin Xuan oolong tea using Gas Chromatography- Mass Spectrometry (GC-MS) and Gas Chromatography- Olfactrometry (GC-O). The concentration of volatile compounds isolated with diethyl ether was higher ($P<0.05$) than for dichloromethane and concentration was higher at 40 min ($P<0.05$) than 20 or 60 minutes. For SDE, 128 volatiles were identified using GC-MS and 45 aroma active compounds using GC-O. The number of volatiles identified using GC-MS was lower in SPME than SDE. For SPME, 59 volatiles and 41 aroma active compounds were identified. The composition of the volatiles isolated by the two methods differed considerably but provided complementary information.

The goal of the third experiment was to determine effects of panning on flavor volatile compositions of oolong using GC-MS and GC-O. Simultaneous Distillation and Extraction (SDE) and Solid Phase Microextraction (SPME) techniques were applied for extraction of volatiles in panned and unpanned teas. A total of 190 volatiles were identified from SDE and SPME extractions using GC-MS and GC-O. *Trans*-nerolidol, 2-hexenal, benzaldehyde, indole, gernaiol, and benzenacetaldehyde contents were significantly decreased ($P<0.05$) by panning; however, panning increased ($P<0.05$) contents of linalool oxide, cis jasmone, methyl salicylate in oolong tea. Overall, panning significantly changes the volatile compositions of the tea and created new aroma active compounds.

DEDICATION

I dedicate this manuscript to my parents for all love and support through my life.

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CHAPTER I

LITERATURE REVIEW

1.1 Tea and the Global Market

Camellia sinensis is the species of plant whose leaves and leaf buds are used to produce tea. Tea belongs to genus of *Camellia* which is a genus of flowering plants in the family Theaceae. Tea is a native to Southeast Asia, but is currently cultivated in more than 30 countries around the world. More than 3 million hectares of the world has been used for planting tea (Ravichandran & Parthiban, 1998). Tea beverage is an infusion of the dried leaves of *C. sinensis*. Traditionally, tea beverage is prepared by steeping the dried young leaves and leaf buds in boiling water. More than three billion cups of tea are consumed daily worldwide (Hicks, 2009).Tea has been one of the most popular drinks in the world for over 4000 years. Tea, next to water, is the most consumed beverage in the world, with per capita consumption of more than 120 mL/d (Katiyar et al., 1996). In recent years, teas are becoming more popular due to the promotion of the health benefits of tea consumption. In addition, their pharmaceutical and industrial applications of tea products are in development.

Tea is an evergreen shrub or tree. It can grow to a height of 30 feet, but is usually clipped to a height of 2.5 feet in cultivation. The tree is heavily branched and the dark-green, hairy, rectangle, ovate leaves cultivated and normally picked as young shoots (Sharma et al., 2007). Low-grown teas are cultivated at 0 to 600 m above sea level, mid-grown from 600 to 1200 m above sea level, while the high-grown teas are cultivated between 1200-2000 m above sea level (Hicks, 2008). High-grown (high mountain) teas have a bright liquor and excellent flavor. The reason for higher quality of high-grown teas is the cooler temperatures at these altitudes which induce slower growth than in the hot, moist and low country. The seasonal rains also greatly affect the quality of tea (Hicks

2001). The fresh tea leaves are harvested by hand plucking or mechanical plucking. Hand plucking compared to mechanical plucking is more labor intensive and time consuming, and less efficient, but with higher uniformity (Ho et al., 2008).

The main tea producing countries, in Asia are Bangladesh, China, India, Indonesia, Taiwan, Sri Lanka, and Vietnam; in Africa, they are Burundi, Kenya, Malawi, Rwanda, Tanzania, Uganda, and Zimbabwe; in South America, they are Argentina and Brazil (Hicks 2001) and in Near East, they are Iran and Turkey. In addition, Russia and a number of Commonwealth of Independent States (CIS) countries also produce quantities of tea (Hicks, 2009). More than 75% of global production is in China, India, Kenya and Sri Lanka (Bordoloi, 2012). The majority of the international trade consists of black tea. People from different region have different preferences for tea especially black tea. For example, people from the Persian Gulf region prefer strong teas with dark liquoring. So, these teas are dried longer and collected from the lower elevations while tea drinkers in UK tend to prefer the milder, light-liquored teas which come from higher elevations (Hicks, 2009). Tea bags are very popular with Australians, closely followed by Saudi Arabia and Egypt. Culture also affects the method of preparation of tea around the world. According to Venditti et al. (2010), in the UK, Ireland and in Canada, black tea is mostly consumed and it prepared using boiling water and milk and often sugar are added to the tea. Americans mostly consume iced tea which is made from hot tea and then cooled with ice. In China, tea leaves are steeped in hot water (70–80 °C for green tea, 80–90 °C for oolong and 100 °C for black) for 20–40 s, and the same tea leaves are usually repeatedly steeped up to seven times (Venditti et al., 2010). Recently in Taiwan, cold water (4 or 25 °C) steeping is become a new popular way of preparing tea (Venditti et al., 2010).

Apparently, tea prepared using cold water contains lower amounts of caffeine, reduced bitterness and higher aroma (Yang et al., 2007).

More than 4,000 million kilograms of tea was produced globally in the year 2010. And 1700 million kilograms of total production (43%) was placed in the international export market (Bordoloi, 2012). Since 2004, the global tea production growth rate has been about 3%, an estimated 4,820 million kilograms (Hicks, 2009; Statista, 2014). This significant increase was mainly due to production expansion in China, Vietnam and India. China become the number one producer by production increased 9.5% in 2005, to 1050 million kilograms in 2006, through government policies to increase rural household incomes (Hicks, 2009). In China, green tea is around 50%, black tea around 30% and other teas 20% of the total export market (Hicks 2001). Growth of 28 percent in Vietnam results in an output of 133,000, 000 kilograms as tea bushes reached optimum yields. India had a 3% increase in harvest output of 945,000,000 kilograms for the year (Hicks, 2009). In 2006, world net imports of tea declined by 1.7%, reflecting reduced tea imports by Pakistan, the Russian Federation, and the Netherlands; however, increase in imports by traditional markets such as the United Kingdom, United States, Egypt and Germany did not offset these declines

It is expected in the next decade the world black tea production will grow but at a slower rate due to the slowing of production growth in Africa (Hicks, 2009). Black tea production is anticipated to grown at 1.9% annually to reach 3100 million kilograms by 2017. India continues to be the largest producer of black tea with a projected growth rate of 2% annually and an output of 1200 million kilograms, followed by Kenya and Sri Lanka each with growth rates of 1%,

projected production of 344,000,000 kilograms, 341,000,000 kilograms, China with 312,000,000 kilograms by 2017 (Hicks, 2009). On the other hand, world green tea production anticipated to grow faster than black tea, 4.5% annually compared to 1.9% for black tea. This growth is due to the growth in China where production expansion through rehabilitation, replanting and conversion, and it is expected to reach 1350 million kilograms by 2017 (Hicks 2009). And also black tea consumption in importing countries is anticipated to increase by 0.5% annually. The largest growth is expected to be in the Russian Federation market, where imports are projected to increase by 2.6% annually followed by Pakistan and the US and Canada at a marginal rate of 0.3% (Hicks, 2009).

1.2 Tea Market in the United States

The first processed tea was imported to the U.S. by the Dutch in 1650; however, *Camellia sinensis* was the first in the genus *Camellia* to enter the country in 1744. According to U.S. Patent Office Report in 1805, seeds sent to the Trust Gardens in Savannah Georgia did not survive due to insufficient capital and a devastating malaria outbreak in the Savannah region (Odom, 2007). Now, tea is consumed in nearly 80.0% of US households (Carter, 2014). It ranks sixth in overall beverage consumption (not including tap water), after coffee, bottled water, soft drinks, milk and juice (Carter, 2014). In recent years, increasing scientific evidence has associated consumption of tea with positive health and has raised consumer perceptions of tea, increasing consumption and boosting industry revenue.

Americans drank over 79 billion servings of tea in 2012 (Carter, 2014). The tea production industry's revenue is expected to rise at an annualized rate of 6.3% over the five years. In 2014, tea consumption is expected to rise and boost revenue by 3.2% (Carter, 2014).Tea manufacturers' marketing campaigns are focusing more on the various health benefits of tea consumption, such as its effect on lowering cholesterol. As Americans continue to become more health conscious, they are seeking flavorful alternatives to sugary carbonated beverages.

Most region of the U.S. do not have an ideal climate for growing tea and almost all of tea leaves consumed is imported. However, the warmer regions of the U.S. have excellent conditions for growing tea (Odom, 2007). In U.S., tea leaves are grown in South Carolina, Alabama, Washington, Hawaii, Oregon, Michigan, Mississippi, Louisiana and California (World of Tea, 2014). Between 2009 and 2014, imports increased at an average annual rate of 20.9%. Total imports for 2014 are projected to reach $698.5 million (Carter, 2014). Canada because of its closeness and ease of access facilitated by the North American Free Trade Agreement (NAFTA) has continued to dominate at 21.8% of tea imports. Mexico is the second-largest source, followed by Germany and Brazil. Nevertheless, companies in this industry blend and package some varieties domestically and many of these packaged teas are then exported. The expected 21.5% annualized increase in export revenue over the past five years has consequently supported industry growth (Carter, 2014) (Table 1.1). In 2014, exports are on track to grow 13.6% to $456.8 million (28.6% of industry revenue). Canada has been the main destination for US tea exports, accounting for 57.9% of total exports, followed by Russia, Japan and Mexico (Carter, 2014).

Table 1.1 Revenue growth for Tea Manufactures in the U.S (Carter, 2014).

Year	Revenue $ million	Growth %
2002	348.4	0.0
2003	469.6	34.8
2004	625.8	33.3
2005	836.3	33.6
2006	1,017.3	21.6
2007	1,139.8	12.0
2008	1,126.5	-1.2
2009	1,174.4	4.3
2010	1,239.3	5.5
2011	1,444.8	16.6
2012	1,548.6	7.2
2013	1,546.2	-0.2
2014	1,595.8	3.2

According to Mintel, U.S. retail sales of tea and ready to drink (RTD) tea grew 19.8% from 2009 to estimated 2014 to reach $7.3 billion, resulting from its perception as an emerging, healthful beverage (Mintel, 2014). Sales of refrigerated RTDs and bagged/loose leaf/single-cup teas were the main drivers in category growth. The canned and bottled RTD segment lead in category sales; however, the instant tea segment continues to decline in sales. Bagged tea was the most consumed type for the tea category. Consumers, especially young adults, are looking to new flavors and products to keep them interested in the tea segment. In addition, types of tea that have shown strong growth in appearance on restaurant menu are oolong tea (50%), herbal tea (33%), green tea (30%), and tea lattes (30%); this provide great opportunities for restaurants to incorporate unique flavor combinations to set themselves apart (Mintel, 2013). The main competitors in tea markets are Pepsi Lipton tea (23% of market share), Ferolito Vultaggio & Sons (18%) and Unilever (13%).

Mintel (2014) projects tea sales will grow 16.8% from 2014 to 2019. As RTD teas continue to grow in popularity, consumers will show more interest in bagged, loose leaf, and single-cup offerings; however, the instant tea segment is expected to drop in both market share and sales. Millennials will be the most likely to consume RTD tea in the most variety of offerings. Regular-calorie canned/bottled RTD tea will be the most popular tea product due to its convenience and flavor (Mintel, 2014).

1.3 Tea Classification

Based on the combination of processing (usually degree of fermentation) and the characteristic quality of manufactured tea, tea is classified into six types: green tea, yellow tea, dark tea (containing brick tea and pu-erh tea), white tea, oolong tea and black tea. In addition, *Ilex paraguayensis* is a species of tea from South America that is processed to obtain a final commercial product named yerba mate. The mate is a popular caffeine containing tea consumed in Argentina, Brazil, Uruguay, and Paraguay (Graham, 1992; Kawakami M, Kovayashi, 1991; Dall Orto et al., 2005). The Rooibos red tea is another type of tea plant grows in the South Africa. Rooibos is a caffeine-free tea with a distinctive sweet taste. Rooibos naturally contains protective antioxidants and its consumption is associated with some health benefits (Craig, 2012).

The fermentation term that used in tea processing is not the anaerobic breakdown of energy-rich compounds such as seen in fermenting wine or beer but it is the natural browning reactions induced by oxidative enzymes in the tea leaves; a more accurate definition is “the oxidative polymerization and condensation of catechins catalyzed by endogenous polyphenol oxidase and peroxidase” (Chaturvedula & Prakash, 2011). The

three basic types of tea based on fermentation/oxidation levels are: unfermented green teas, semi-fermented oolong teas, and fully-fermented black teas. Each variety has different quality characteristics, including aroma, taste, color, and appearance. Of the total amount of tea produced and consumed in the world, 698 is oolong tea (Wan, 2004). Green tea is prepared from the fresh tea leaf and widely consumed primarily in Japan and China. However, in U.S. and Europe, many stores now carry green tea products. In recent years, numbers of different trade books have been published related to green tea and health. These are all indicators of increased demand for green tea in the U.S. Black tea is consumed all around the world. Most western cultures prefer black tea. Black tea is processed through the oxidation, curing process of maceration and exposure to atmospheric oxygen (Graham, 1992; Langley-Evans, 2000). The consumption and production of oolong tea is mainly limited in China and Taiwan (Katiyar et al., 1996).

1.4 Tea Chemical Components

In processing of green tea, the tea leaves are heated to inactivate the oxidative enzymes and are dried, the constituents of the tea leaves are preserved in the dried tea leaves. However, when tea leaves are brewed, many of the solid materials are extracted into infusion. A tea infusion contains a moderate amount of caffeine, volatile oils, tannin and several B-complex vitamins (Hicks, 2009). Carbohydrates contribute approximately 11 % wt/wt of extract solids (Graham, 1984). A most dominant carbohydrate fraction in tea extract has been found to comprise the disaccharide 2-0-(β-L-Arabinopyranosy l)-myo-inositol (Sakata et al., 1987). The volatile oils are responsible for tea volatiles, while astringency and color come from tannins. A cup of tea can contain as few as four calories which is consider a low-energy beverage (Hicks, 2009).

The main chemical components of tea leaves are the polyphenol group, accounting for 25 to 35% on a dry weight basis (Hara et al., 1995d; Balentine, 1997). The main polyphenols in tea belonged to six groups of compounds: flavanols, hydroxyl-4-flavanols, anthocyanins, flavones, flavonols and phenolic acids (Mukhtar et al., 2000). The most important tea polyphenols are the catechins (flavan-3-ols).The most dominant catechins are: (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)- epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (+)-catechin (C), and (+)-gallocatechin (GC) (Hara et al., 1995a; Liang et al., 2003). These compounds are responsible for the bitterness, astringency and sweet aftertaste of tea beverages (Hara et al., 1995b). Tea favonols are mainly quercetin, kaempferol, myrecetin, and their glycosides (Chaturvedula & Prakash, 2011). In black tea, the oxidation of polyphenols during fermentation results in the formation of catechins and gallic acid complexes such as theaflavins, theaflavinic acids, thearubigins or theasinensis, and of proanthocyanidin polymers (Balentine et al., 1997; Hara et al., 1995c; Lee et al., 2008). "Methylxanthines are present with 2-4% as caffeine theophylline and of theobromine in a small amount" (Hara et al., 1995a).

Many amino acids exist in tea, but theanine is the most dominant one which accounting for 50% of the total amino acids. Amino acid degradation is involved in the biogenesis of the tea aroma (Balentine et al., 1997). The free amino acids content of tea are increased during step of withering of the fresh tea leaves but decreased during fermentation to black tea. And they likely consumed during aroma biogenesis and through other routes. These reactions have strong impacts on the aroma of the finished product (Harbowy et al., 1997).

Although, other chemical components such as chlorophyll, carotenoids, lipids and volatile compounds are not major constituents in a tea brew, they play a significant role in the development of the aroma (Hara et al., 1995d). In addition, tea contains carbohydrates, vitamins A, E, K, and low levels of vitamins B and Vitamin C that can only be found in green tea. Tea also contains good amounts of potassium, manganese and fluoride (Hara et al., 1995d). Caffeine accounts for 3% to 6% of dry weight. Fermentation does not have any significant effects on the caffeine content of tea leaves (Sharma et al., 2007). The quantity of caffeine in the infusion depends on brewing time and by leaf style. Longer brewing times result into greater quantities of caffeine in a tea beverage. Larger tea leaves and uncut leaves lead to weaker infusions with respect to caffeine content but smaller sized tea leaves give a more rapid and stronger infusion (Harbowy et al., 1997). There is no significant difference between green and black tea infusions caffeine contents (Table 1.2).

During fermentation in black tea, chemical oxidation of the flavanols and flavanol gallates, the flavanol glycosides (especially myricetin) and the non-flavanoid theagallin occurred which is driven by polyphenol oxidase (Sharma et al., 2007). These transformations generate a series of pigments including the brownish thearubigins and the red-orange theaflavins, theaflavic acids and theaflavins (Harbowy & Balentine, 1997). The thearubigins are the major polyphenols of black tea leaf and tea beverages (3-6%) and theaflavins contribute to the taste and account for 2% to 6% of the dry weight of black tea extracts. The major theaflavins are theaflavin-3-digallate, theaflavin-30-gallate and theaflavin-3, 30- digalalte (Sharma et al., 2007). In general, the composition varies

with the cultivation conditions, climate and methods of processing of the tea (Odom, 2007).

Table 1.2 Comparison of the chemical composition of green and black tea infusions %wt/wt solids (Harbowy et al., 1997).

Chemical	Green Tea	Black Tea
Catechins	30%	9%
Theaflavins	-	4%
Simple polyphenols	2%	3%
Flavonols	2%	1%
Other polyphenols	6%	23%
Theanine	3%	3%
Amino acids	3%	3%
Peptides/Protein	6%	6%
Organic acids	2%	2%
Sugars	7%	7%
Other carbohydrates	4%	4%
Lipids	3%	3%
Caffiene	3%	3%
Other methylxanthines	<1%	<1%
Potassium	5%	5%
Other minerals/ash	5%	5%
Aroma	Trace	Trace

1.5 Catechins in Tea

Catechins are members of the flavan-3-ols (also referred to as flavanols) which is a class of flavonoid. Afzelechin, catechins and gallocatechin are three subgroupings of the flavanols which representing varying degrees of B-ring hydroxylation (Harbowy & Balentine, 1997). The epi-isomers of the catechins and gallocatechins are the dominant forms in tea. The “tea catechins” is a term commonly used to refer to both catechins and gallocatechins make up as much as 30% wt/wt of dissolved solids. A large percentage of

the catechins present in tea exist as gallic acid esters. Gallation is found to be occured mainly at the 3-position (Harbowy & Balentine, 1997). Other than tea, catechins have also been found in chocolate, cocoa, apples, beer, black, red and white currants, blueberries, cacao liquor , gooseberries, grape seeds (*Vitis vinifera*), kiwi fruit, strawberry, red wine, etc. (Sutherland et al., 2006). Green tea is prepared by drying and steaming whereas black tea is fermented, converting its catechin content into the theaflavins. Catechin behavior during green tea brewing divided in two groups, the time-dependent compounds (EGC and EC) and the time/temperature dependent compounds (EGCG, GCG, ECG) (Labbé et al., 2006). The addition of milk to tea does not affect the bioavailability of catechins but may alter the antioxidant potential depending on the fat content (Johnson et al., 2012). Green and oolong teas typically contain 30-130 mg of EGCG per cup (237 mL), whereas black tea may contain up to 70 mg of per cup (Balentine & Paetau-Robinson, 2000). The average catechin content in China cultivars is 157±4 mg/g (Sabhapondit et al., 2012).

The major catechins in tea are (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epicatechin (EC) and (+)-catechin (C) (Liang et al., 2006). Among them, EGCG usually is the most abundant component, while the contents of EC and C are relatively low. Wang et al., (2008) reported the EGC content can be much higher than EGCG in some teas. Fermentation reduces the concentration of four major tea catechins including EGCG, EGC, EC, and ECG by 74%, 91%, 51%, and 62%, respectively while gallic acid concentration increases by 1.64-fold (Kim et al., 2011). It was also found that increasing exposure time to sunlight results in total increase of catechin of tea leaves. This suggests that catechin

biosynthesis is also environmentally dependent (Mariya et al., 2003). The catechins' role in plants is to provide protection from the damage from UV rays in sunlight, and catechin production is strongly affected by photosynthesis (Mariya et al., 2003; Premkumar et al., 2008). Wei et al. (2011) suggests chlorophyll contents might be associated with catechin biosynthesis. Climate has effects on catechin content of tea. In Wei et al., (2011) study the rise of chlorophyll a content during young leaf development was observed alongside the increase of (-)-epicatechin, (-)-epigallocatechin and the decline of (+)-catechin. They suggested that chlorophyll plays a vital role in the regulation of individual catechins.

The proportions of catechins in tea are important not only for quality assessment but also for the efficiency of cancer prevention (Owuor & McDowell, 1994; Ravindranath et al., 2006). Some studies reported the inhibitory effect of tumour cell growth varied with the type of catechins. The order of radical-scavenging activity was: ECG>EGCG>EGC>EC (Ravindranath et al., 2006). Catechins are known to be synthesized through phenylpropanoid and flavonoid biosynthetic pathways. The creation of dihydroquercetin and dihydromyricetin which are the precursors of dihydroxylated catechins (EC and ECG) and trihydroxylated catechins (EGC and EGCG) is genetically controlled (Gerats & Martin, 1992).Therefore, total catechin and dihydroxylated: trihydroxylated catechins ratio [(EC + ECG):(EGC + EGCG)] could be used as indicators for superior quality in tea breeding programs (Wei et al., 2011).

The conversion of the tea catechins to their corresponding isomers is called epimerization. It can occur during the production of tea and tea beverages (Komatsu et al., 1992). It has shown that catechins can go through epimerisation at the C-2 position in hot aqueous solution (Kiatgrajai et al., 1982). Epimerisation at the C-3 position only

occurs when oxidative degallation is occurred (Coggon, Moss, Graham & Sanderson, 1973). In tea fermentation, monomeric flavanols may undergo oxidative polymerization, converting predominantly into bisflavanols, theaflavins, and thearubigins. Kim at al. (2011) showed fermentation reduce catechins due to the transformation to theaflavins and thearubigins. This phenomenon results in loss of total soluble phenolic content and antioxidant capacity of teas. The same authors also explained reduction in antioxidant capacity was result of oxidative or thermal degradation of antioxidants such as caffeine, ascorbic acid, saponin, and non-catechin polyphenolics (flavonol glycosides) rather than the conversion of tea catechins to theaflavins during fermentation process (Kim et al., 2011). However, Wang et al. (2008) found neither the total catechin contents nor the individual catechin compositions can accurately separate green teas from oolong teas.

In terms of stability, EGCG and EGC were the most unstable, while EC and ECG were relatively stable among catechins. The reason is the three vicinal hydroxyl groups at positions 3', 4' and 5' in EGCG and EGC being more vulnerable to destruction by producing semiquinone free radicals than the two vicinal hydroxyl groups at positions 3' and 4' in ECG and EC (Yoshioka et al., 1991). Catechins stability in the commercial tea is low (Labbe et al., 2008) and mostly are converted to their corresponding epimers during manufacturing (Chen et al., 2001). In manufacturing of tea drinks, other ingredients in the formulation such as citric acid or ascorbic acid, might interact with green tea catechins and affect their stability (Labbé et al., 2008).

Catechins have antioxidant activity by chelating redox active transition-metal ions, scavenging free radicals, inhibiting pro-oxidant enzymes, inhibiting redox active transcription factors, and inducing antioxidant enzymes (Zanwar et al., 2013). The (+)-

catechin oxidation is pH-dependent and leads to absorption on the electrode surface and the result is non-electroactive, and blocks the electrode surface. The (+)-catechin electron/proton donating capacity and its radical scavenging antioxidant activity impacts the deprotonation of the catechol group (Zanwar et al., 2013).The scavenging activity of catechins is significantly increased when coupled with amine-terminated polyhedral oligomeric silsesquioxane by using horseradish peroxidase as a catalyst (Ihara et al., 2005). Lotito & Fraga (2000) found that the catechin's antioxidant activity results in delays in lipid oxidation and depletes endogenous lipid-soluble antioxidants such as α-tocopherol and β-carotene in human blood plasma. Other than the antioxidant properties of catechin, it also affects the extracellular matrix degradation, molecular mechanisms involved in angiogenesis, regulation of cell death and multidrug resistance in cancer and related disorders (Demeule et al., 2002).

A daily consumption of 3–5 cups per day (approximately 720–1200 ml) of green tea will provide at least 250 mg/day of catechins (Johnson et al., 2012). Green tea diminish the bioavailability of folic acid, so, is not recommended for pregnant women or people with megaloblastic anaemia (Poulter et al., 2004). There is some evidence that green tea reduces iron absorption (Ullmann et al., 2004). Antioxidant properties of green tea have been studied in many research studies. Many of the health benefits of green tea are attributed to the antioxidant capacity of these compounds. Extensive evidence on the chemopreventive efficacy of green tea has shown reduction of the risk of various types of cancer, including esophagus, breast, pancreas, prostate and colon cancer (Gupta et al., 2001; Crespy & Williamson, 2004; Huh et al., 2004; Nihal et al., 2005; Baliga et al., 2005). Other studies suggest that green tea may reduce low-density lipoproteins and total

cholesterol. There are not enough studies to clearly show a reduction in coronary artery disease risk in green tea drinkers (Johnson et al., 2012). Evidence suggests that green tea does reduce body weight in the short term, (Hursel et al., 2009; Phung et al., 2010).

1.6 Solid Phase Microextraction (SPME)

Solid Phase Microextraction (SPME) is a newer sample preparation technique developed by Pawliszyn and co-workers in 1990 using a fused silica fiber that coated on the outside with an appropriate stationary phase (Kataoka et al., 2000). In this method, analyte in the sample is directly extracted and concentrating the fiber coating. The method is fast, simple, affordable and without any solvent purchase and disposal costs (Pawliszyn, 1997). The SPME technique can be used in combination with GC, GC–MS, HPLC and LC–MS (Kataoka et al., 2000). SPME can be applied to a wide variety of compounds, especially for the extraction of volatile and semi- volatile compounds from food, biological and environmental samples (Pawliszyn, 1999).

The SPME device is a syringe like device with fiber holder and fiber assembly with built-in fiber inside the needle. "The fiber holder consists of a spring-loaded plunger, a stainless-steel barrel and an adjustable depth gauge with needle" (Kataoka et al., 2000). The design allows the device to be reusable and fiber assemblies to be replaceable. The fused-silica fiber is coated with a relatively thin film of several polymeric stationary phases. This film concentrates the organic analytes on its surface during absorption (from headspace) or adsorption from the sample matrix. The fiber is exposed to the sample headspace to pre-concentrate and fractionate volatiles by adsorption, partition or mixed mechanisms. Desorption of the volatiles from SPME fiber into the injection port of a GC allows the chromatographic analysis of this volatile

fraction without matrix interferences (D'Agostino et al., 2014). Several types of coating are commercially available for the extraction of analytes. Fibers with different coating and thickness have different properties and based on the affinity of the fiber with an analyte are being selected. There are many studies available on capability of each coating on extraction of various compounds. For example, Polydimethylsiloxane-Divinylbenzene (PDMS–DVB), Carboxen- Divinylbenzene (CAR–DVB), Carbowax (CW: polyethylene glycol)–DVB and Carbowax-Templated Resin (CW–TPR) can be used for the extraction low-molecular mass and polar volatiles (Kataoka et al., 2000).

In this technique, stationary phases are immobilized by bonding, non-bonding, partial crosslinking or high crosslinking. According to Kataoka et al. (2000), bonded phases are stable with all organic solvents except for some non-polar solvents while non-bonded phases are stable with some water-miscible organic solvents. Partially crosslinked and highly crosslinked phases are stable in most water-miscible organic solvents and some non-polar solvents; however, for highly crosslinked some bonding to the core has occurred (Kataoka et al., 2000). In the newer developed SPME technique, an open tubular fused-silica capillary column used as an SPME device instead of SPME fiber. This method is known as in-tube SPME and has been developed for combination with GC-MS or LC–MS (Kataoka et al., 2000). In-tube SPME is useful for automation, and automated sample handling procedures which shorten the analysis time and provide better accuracy and precision relative to manual techniques. However, the precision of SPME technique has frequently questioned the quantitative performance of SPME especially when multicomponent mixtures are to be analyzed (Jeleń et al., 2012). This technique might not provide precise results especially for studies on characterization of

samples where precision is the most important analytical parameter (D'Agostinoetal et al., 2014). Some studies compared SPME with other extraction techniques. Majcher & Jelen (2009) found that SPME was not suitable for the isolation of high molecular compounds or for those with a strong affinity to the matrix. In addition, compared to other techniques, the same authors and Cai et al. (2001) found that SPME was more precise than SDE. Nonetheless, the high precision of SPME method has been previously reported where authors controlled the extraction parameters carefully (Iba´n˜ez et al., 1998). Some studies also showed the limits of detection for important flavor compounds of in foods such wheat grains and bread crumbs (Jelen´ et al., 2004; Ruiz et al., 2003). SPME extraction allows identification of low boiling compounds which co-eluting with other methods that use solvents for extraction (Majcher & Jelen, 2009). It was found that SPME was more efficient in extracting low molecular weight compounds of high volatility compared to SDE (Garcia-Esteban et al., 2004; Madruga et al., 2009). This might result from the use of Carboxen®/PDMS SPME fibre that are more suited for the analysis of low molecular weight volatile compounds. Application of SPME methods combined with GC–flame ionization detection (FID) and GC–MS have been reported for the analysis of flavor compounds in teas, vegetables, fruits, beverages and other food products (Kataoka et al., 2000).

1.7 Simultaneous Distillation and Extraction (SDE)

Simultaneous distillation-extraction (SDE) is an extraction technique that is widely used for flavor analysis of foods with a complex matrix because of its simplicity and versatility. SDE method has used routinely for isolation of volatiles from spices (Zawirska-Wojtasiak & Wa˛sowicz, 2002), fruits (Pino et al., 2002) or food subjected to

boiling for many years. In this method, Likens–Nickerson apparatus is used which combines common solvent extraction and vapor distillation extraction. Unlike solvent extraction of a lipid-containing food, the extract from this technique contain nearly all the flavor volatiles present in the raw sample (Reineccius, 1993). Since headspace sampling methods such as Purge and Trap and SPME select only the most volatile molecules, SDE remains a very common method, despite several drawbacks (Pollien & Chaintreau, 1997). One of the main advantages of this extraction method is to simplify the extraction procedures, save organic solvents and decrease loss of samples during the transfer process (Zhang & Li, 2010). In volatile analysis of tea, Zhang et al. (2013) showed the semi-volatile compounds have higher recovery than other compounds compared to SPME. They also discussed the compounds with high volatility may be lost in the processing steps, and the compounds with low volatility may be difficult to be extracted by the SDE technique. The same authors indicated that the recovery results were not equally satisfactory to all compounds, but the good repeatability shows that SDE technique is suitable for extraction of volatile components of teas (Zhang et al., 2013).

The biggest drawback in this technique is high temperature applied during extraction using this technique can potentially lead to breakdown of some flavor compounds and create artificial compounds which do not normally exist. This has been observed especially when dealing with food samples rich in free amino acids and sugars. These components can interact during the Maillard reaction and Strecker degradation to form artifact compounds (Schieberle, 1995). The formation of esters or acetals is also possible in this technique (Weurman et al., 1970). In Siegmund et al. (1997) found that 5,6-dihydro-2,4,6-trimethyl-4H-1,3,5- dithiazine is formed, and another study by

Tarantilis and Polissiou (1997) showed that safranal, the main aroma compound of saffron, breaks down into 2,6,6-trimethyl-1,3-cyclohexadiene-1-carbon acid. Jelen´ (2003) showed in his work on fungal volatile metabolites, high amounts of oxygenated terpene compounds found in SDE extracts were results of long-term temperature influence of highly unsaturated sesquiterpene hydrocarbons. There are some compounds that cannot be detected in distillation techniques (SAFE or SDE) extracts because of the presence of solvent. Majcher & Jeleń (2009) study showed that SPME and SAFE methods were more precise than SDE. The same authors suggested that SDE should not be used for food products rich in carbohydrates, amines or unsaturated fatty acids that can serve as flavor precursors during long-term heat treatment of SDE extraction (Majcher & Jeleń, 2009). Some studies have been conducted for improvements of this technique to overcome its main limitations. Extraction at reduced pressures is one of the solutions that proposed to avoid generation of artifacts (Pollien & Chaintreau, 1997).

1.8 Gas Chromatography-Olfactometry (GC-O)

GC-O analysis is based on sensory evaluation of the eluate from the GC column with a human used as detector. The main goal of GC-O is to discover the aroma active compounds in the sample. This allows identifying compounds that are sensory active at concentration higher than the threshold of sensory detection, and the determination of the odor descriptions and the intensity (Plutowska & Wardencki, 2008). Application of GC-O in flavor analysis is especially important since some of the detected compounds by the human nose are not detectable with conventional detectors due to much higher selectivity and sensitivity of the human nose (Benn & Peppard, 1996; Ferreira et al., 1998). Several comparative studies have indicated that the use of different extraction techniques (even

using different solvents) might affect the compositions of the isolated compounds. Therefore, the olfactograms are significantly influenced by the isolation procedure (Lopez & Gomez, 2000; Nonato et al., 2001; Bonino et al., 2003).The GC-O is designed in a way that the elute from GC column is split, so, the analytes reach both detectors simultaneously. The CharmAnalysis, Time- Intensity, Aroma Extract Dilution Analysis (AEDA), OSME (means smell in Greek), Surface of Nasal Impact Frequency (SNIF) are among the most common methods of odor detection in GC-O analysis.

1.9 Oolong Tea

Oolong tea is a semi-fermented tea and is manufactured predominantly in China and Taiwan (Lee et al., 2008). Oolong is generally fermented from 20% to 60% to avoid green tea's flavor characteristic (green and grassy) while obtaining some of the black tea's sweet and bold flavor (Kim et al., 2011). Therefore, oolong tea has a taste and color somewhere between green and black teas. Of the total production of tea in the world, 2% is oolong tea (Wan, 2004). Oolong teas usually have a higher unit price than green and black teas in the international tea market, mostly because of the complex processing steps and the limited supply (Wu et al., 2004).

Several studies reported health benefits that are associated with consumption of oolong tea such as: reduced total plasma cholesterol, LDL oxidation, and triglyceride which have benefits in coronary heart disease prevention (Hosoda et al., 2000), the increasing energy expenditure and consequently weight loss, prevention of cardiovascular disease (Yamamoto et al., 2000), anti-oxidant, anti-cancer, and anti-inflammatory activities (Chen et al., 2010), prohibiting the development of hypertension in rats (Tanida et al., 2008) and anti-bacterial activity (Chen et al., 2010). Traditionally, oolong tea has

been reported to have anti-obesity and hypolipidemic effects in humans. These effects might be a result of the unoxidized tea catechins and oxidized theaflavins and thearubigins present in oolong tea (Han et al., 1999).

Oolong tea processing is considered as an art and there are no standard recipes on how to manufacture oolong tea. Different oolong teas are processed in variety of ways. Tea masters or tea gardens decide on processing and the level of fermentation. There are oolong tea processing competitions which tea masters participate to demonstrate their professional skills at this fine art by creating variety of tea flavors (Popec, 2010).

Leaves are harvested mainly as buds and young leaves. Depending on the variety, teas are harvested as “one bud one leaf”, “one bud two leaves” or “one bud three leaves” (Taiwan.gov, 2014). Tea leaves are picked either manually or recently with machines. The leaf should not be broken and this is critical to the quality of the final product. After harvesting, the tea leaves are spread in a thin layer on special bamboo mats under direct sunlight (Popec, 2010) or hot air blowing machines (Taiwan.gov, 2014) that will let most of water evaporate. This step is called withering or wilting. The time of the withering process varies depends on the ambient temperature. The leaves turn progressively darker as they wilt under the sun and soften from moisture loss (Taiwan.gov, 2014).Then, the withered leaves are placed in a large basket and the tea leaves are shacked and gently tumbled in order to bruise the edges of the leaves to start fermentation (Popec, 2014). Fermenting (oxidation) is the process that takes place when the cells of tea leave come into contact with the air and lose part of their moisture content. If the moisture loss is too rapid, the cells die and an incomplete fermentation will occur (dehydration) and the result

will be a tasteless tea. Any scar or excessive forces on leaves may result in breakage of leaves and affect the ideal fermentation condition and, consequently result is an inferior tea quality (Taiwan.gov, 2014). The fermentation should be stopped immediately, once the desired level of fermentation is reached. This is achieved through the heat drying process of raw materials called "panning". In this step, panning or baking destroys the enzymes responsible for the fermentation. The panning requires extensive experience in oolong tea processing. The next step is called kneading. The teas are moved in the roller and they are become slowly curled up and tighten as they roll around. Different tea varieties require different levels of kneading (Taiwan.gov, 2014). This step expedites the steeping when preparing a cup of tea. To completely destroy enzymes and stop fermentation, leaves are machine dried using high temperature. This process will result in 5% moisture loss. Most Oolong are first partially dried and then given a final finish drying (Popec, 2010). Then the teas are refined and classified to ensure consistency of appearance. Refined leaves are then roasted to release their natural aroma. Leaves undergo light roasting (raw tea), medium roasting (raw-ripe tea) and heavy roasting (ripe tea) depending on the variety (Taiwan.gov, 2014). After this stage, tea leaves are placed in the package. The most common packages are can and plastic bag which are sealed with an elastic band, twist tie or vacuum-sealed (Taiwan.gov, 2014).

1.10 Panning

Panning, also known as pan-frying or pan-firing, is a processing step in manufacturing of some varieties of oolong tea that is performed after fermentation. The primary goal of panning is to inactivate the enzymes by heating and inhibiting further fermentation. Nevertheless, some flavor forming enzymes are not deactivated, but

oxidation of polyphenols is not taking place after this step (Changoiwala, 2007). During this process, tea leaves lose significant amounts of moisture. Therefore, leaves are softened, making dehydration easier (Hui et al., 2003). There are no standard recipes on how to pan oolong tea. Usually, selecting the correct time and temperature for panning the oolong tea leaves requires extensive experience in oolong tea processing. Tea masters are responsible for judging the conditions of this procedure (Tea From Taiwan, 2014). The panning period depends on the nature of tea leaves and quantity of the batch (Hui et al., 2003). For this reason, temperatures and period of this process are reported differently in literature. A very wide range of time and temperature (100 ^{o}C- <300 ^{o}C) have been reported in literature (Hui et al., 2003; Changoiwala, 2007; Hojo, 2013). Over-panning leaves causes more prickles on leaves or burnt odor and tea leaves that underpanned will have a greenish odor and red central vein (Hui et al., 2003). This panning process results in generation of new flavors and stabilization of the quality and characteristic of fermented tea leaves (Hojo, 2013). At the end of this process, green odor is eliminated and strong pleasant (fragrant) aroma will be emitted. After this step, the stems and veins of the leaves become more flexible than before due to the leaves moisture loss, and leaves become less vulnerable to breaking in the next step (Taiwan.gov, 2014).

1.11 Flavor composition of Oolong Tea

The perceived quality of oolong teas is evaluate based on appearance of leaves, the color, taste and aroma of the brew and features of infused young shoots. The term flavor of food is used to describe as taste and aroma of food. In tea, volatile compounds are responsible for aroma while non-volatile compounds give the taste. The flavor of tea is controlled by key chemical components which are volatile compounds, caffeine,

organic acids and polyphenols (Borse et al., 2002). The most important non-volatile chemical components that influence the taste of tea infusion are polyphenols, flavonols, caffeine, sugars, organic acids, amino acids ornithine and theanine (Seetohul et al., 2006). Polyphenols are example of non-volatile components that play important roles in perception of taste of tea. Nakagawa (1975) showed catechins (especially gallated flavonols) and other phenolic compounds and some amino acids are responsible for the astringency and bitterness of tea infusions. The umami taste comes from some amino acids such as theanine, serine, etc. Wang et al. (2010) found that the taste quality score positively correlated with the concentration of total free amino acid and theanine. Unlike black tea where theoflavins contribute significantly to astringency, the content of theoflavins is very low in light or medium fermented oolong tea (Chaturvedula & Prakash, 2011). However, thearubigin contents formed via oxidation of EGC and EGCG have impacts similar to black tea in oolong tea flavor (Takayangi et al., 1984). Other secondary polyphenolic compounds such as theasinensin, and oolongotheanine were formed in the infusion that contributes in the taste of tea (Nonaka et al., 1983; Nagabayashi et al., 1992). Therefore, the oolong tea infusion's sweetness and mellowness are the integrated taste combinations of non-oxidized catechins, secondary polyphenolic compounds, thearubigins, caffeine, free amino acids and related sugars (Chaturvedula & Prakash, 2011). Overall, the sweetness of oolong tea is stronger and the astringency is lower than green tea.

Volatile organic compounds are in trace amount about 0.01% of the total dry weight of tea, but due to their low threshold value have a high impact on the flavor (Fanaro et al., 2012). Analysis of volatile compounds is important for variety

authentication and evaluation of the quality of oolong tea. Some oolong varieties appear similar in flavors and appearances, so, correct differentiation is only possible for experts. This is important since some premium oolong varieties (e.g., Tie Guan Yin, Da Hong Pao) are sold at a premium price in the market compared to other inferior varieties (Lin et al., 2013). Chemical methods can significantly help in the quality assessment and variety identification of oolong tea. Oolong tea volatiles can be divided into two groups consisting terpenoid and non-terpenoid compounds. The detected terpenoid components in tea are monoterpene alcohols. The flavor volatiles compounds in teas with different quality and variation is due to different environmental conditions and the method of tea processing (Pripdeevech & Machan, 2011). Generally, the volatile organic components can be classified into compounds which that are derived from glycosides of terpenoid-related compounds and have sweet flowery aroma, and the compounds that are the products of lipid breakdown which have undesirable grassy odor (Ravichandran & Parthiban, 1998).

As explained by Reineccius (2004), several enzymatic reactions are responsible for formation of tea aroma in the fermentation process. The main precursors for tea aroma are amino acids, carotenoids, including β-carotene, lutein, neoxanthin, and violaxanthin (Yamanishi, 1977). A primary oxidation results in the significant reduction of carotenoids, particularly β carotene, resulting in the formation of ionone and terpenoid carbonyls (Yamanishi, 1977). By oxidation and secondary epoxidation reactions, other carotenoids give rise to ionone, linalool and substituted hydroxy- and epoxy-ionones (Sanderson & Grahamm, 1973). Fermentation can eliminate green flavor and stimulate fermentation of the fruity and floral aroma. The content of some compound such as

indole is significantly increased at the beginning of fermentation, but slowly decrease when the process continues (Wang et al., 2008). On the other hand, methyl salicylate can only be found in the medium degree- fermented teas and cannot be found in lightly fermented or green teas (Wang et al., 2008). Volatile compounds respond differently toward fermentation (Wang et al., 2008). There are several studies available on effects of fermentation on volatile compounds. Wang et al. (2008) found the total concentration of trans-2-hexenal, benzaldehyde, methyl-5-hepten-2-one, methyl salicylate and indole can distinguish unfermented from fermented teas. Trans- 2-hexenal and methyl salicylate may classify the semi- and fully-fermented teas. However, in one study, the content of cis-jasmone, trans-nerolidol and indole increased dramatically whilst the green fresh aroma of hotrienol decreased rapidly which can be used in differentiation of semi-fermented tea from non-fermented tea (Pripdeevech & Machan, 2011). Zhang et al. (2013) showed the content of (E)-geraniol, (E)-beta-damascenone, linalool oxide B and benzaldehyde increase with the increase of degrees of fermentation.

According to Wang et al. (2010) nerolidol, indole, benzeneacetaldehyde, linalool, linalool oxide I, hexanal, benzyl nitrile, geraniol and 1-penten-3-ol were the most common volatile compounds detected in most oolong tea samples. They also suggested that these compounds along with methyl salicylate, methyl jasmonate, phenylethyl alcohol, benzyl alcohol, cis-jasmone and β-ionone are possibly the most important contributor to fragrant flowery aroma of oolong tea infusions. These compounds may be generated during tea processing, in which β-glucosidase (primeverosidase) hydrolyze their glycosides and primeverosides (Wang et al., 2001). Pripdeevech & Machan (2011) used SDE and found hotrienol, geraniol and linalool were the major components in Green

Oolong tea while Chin Shin Oolong tea was dominated by linalool, indole and cis-jasmone. The major flavor volatiles of Chin Hsuan oolong tea were trans-nerolidol, cis-jasmone and geraniol. Indole, geraniol and cis-jasmone were detected as the main constituents in Four Season oolong tea.

Wang et al. (2010) study reported that perceived aroma score positively correlated with concentration of benzyl alcohol, benzeneacetaldehyde, linalool, phenylethyl alcohol, linalool oxide, indole, cis-jasmone, nerolidol, methyl jasmonate. However, the total quality score positively correlated with concentration of benzyl alcohol, geraniol, benzeneacetaldehyde, indole and toluene, but negatively correlated with the concentration of (E, E)-2,4-heptadienal (Wang et al., 2010). The same authors concluded that perceived aroma quality is less function of abundance but more the ratios between volatile compounds (Wang et al., 2010). In other studies, it was found that compounds including jasmine lactone, 1H-indole and alpha-farnesene, have a higher correlation with the aroma of oolong tea (Wang et al., 2011; Zhang et al., 2013). Compounds with a similar molecular structure and aroma such as (Z)-jasmone and methyl jasmonate are in higher concentration in oolong tea compare to other varieties of tea (Zhang et al., 2013). Alpha- Farnesene is also reported previously as the main oolong tea flavor (Kawakami et al., 1995; Wang et al., 2011).

Some studies have focused on the effects of different processing techniqueson flavor volatiles of oolong tea. In a study on comparison of volatile compounds of unbaked oolong tea and baked oolong tea, it was shown that baking significantly increased the compounds that were products of Maillard reactions such as pyrazines, pyrroles and some other nitrogen-containing compounds (Yu et al., 1999). They also

observed significant increases in total content of the volatile compounds in oolong as a result of thermal treatments. In addition, the content of some floral or woody type volatile compounds, such as trans-geraniol, cis-jasmone, linalool, linalool oxide, and β-ionone, decreased after thermal treatments (Yu et al., 1999). Irradiation is another processing method that has been shown to have significant impact on oolong tea flavor profile. About 40% of new compounds were identified after this process, but the irradiation at doses up to 20 kGy did not interfere with consumer perception (Fanaro et al., 2011).

A discussed earlier, most studies on health benefits of tea have focused on the non-volatile constituents, such as catechins, but much less studies exist on the biological activity of volatile chemicals from tea. Yanagimoto et al. (2003) the antioxidant properties of teas are in part due to the contributions of volatile compounds; consequently, drinking tea may help to prevent *in vivo* oxidative damage due the presence of various volatile compounds with antioxidant properties.

1.12 Water Chemistry

Drinking water is not chemically pure H_2O and the composition of water varies widely with geological conditions. Water contains small amounts of gases, minerals and organic matter of natural origin (Kozisek, 2005). Many substances are dissolved in water that is considered good quality water. Maximum acceptable concentrations of these substances and microorganisms have been established nationally and internationally to assure the safety of drinking water. In the US, municipal drinking water is regulated by the Environmental Protection Agency (EPA). The quality of municipally supplied tap water can vary by location (EPA, 2014). Most cities process water at treatment plants and are tested for EPA compliance and then piped to residential homes and industries. Federal

and state regulations require that the tap water that is piped to consumers meets health-based standards (International Bottle Water Association, 2014). Although attributes like water hardness have no effects on human health, it is considered a nuisance water problem because it interferes with cleaning. Since many of water attributes do not directly affect public health (e.g. sensory qualities), they are not regulated in the U.S. However, the EPA has set secondary standards that, though not enforced, serve as a guide (EPA, 2014).

1.13 Water Hardness

Water hardness is the traditional measure of the capacity of water to react with soap, hard water requires considerably more soap to produce lather. Water hardness is the measurement of the amount of ions which have lost two electrons (divalent cations) dissolved in the tested water which is related to total dissolved solids (Wurts, 2014). The more divalent cations dissolved in the water the "harder" the water. Basically, the total water hardness is the sum of the molar concentrations of Ca^{2+} and Mg^{2+}, in mol/L or mmol/L units. Although water hardness usually measures only the total concentrations of calcium and magnesium (which are the two most prevalent divalent metal ions), iron, aluminum, and manganese can also be present at elevated levels in some locations (Global Water, 2014). Generally, the other divalent cations contribute little to no appreciable additions to the water hardness measurement. In case of stream or river water, hardness reflects the geology of the catchment's area and is usually influenced by human activity in a watershed. For example, locations near mines often have higher concentrations of iron ions in the water resulting in a higher hardness (Global Water, 2014).

Water hardness is often not expressed as a molar concentration, but rather in various units, such as degrees of general hardness (dGH), English degrees (°e, e, or °Clark), grains per gallon (gpg), German degrees (°dH), parts per million (ppm, mg/L, or American degrees), or French degrees (°F). The various units represent an equivalent mass of calcium carbonate ($CaCO_3$) or calcium oxide (CaO) that, when dissolved in a unit volume of pure water, would result in the same total molar concentration of Ca^{2+} and Mg^{2+} (Frank, 1997) . The different conversion factors arise from the fact that equivalent masses of calcium carbonates and calcium oxide differ, and that different mass and volume units are used (Frank, 1997). The units are as follows (CMRIT, 2014):

- **Parts per million (ppm)** is defined as 1 mg/L $CaCO_3$. It is equivalent to mg/L without chemical compound specified.
- **Grains per Gallon (gpg)** is defined as 1 grain (64.8 mg) of calcium carbonate per U.S. gallon (3.79 litres), or 17.118 ppm.
- **mmol/L** is equivalent to 100.09 mg/L $CaCO_3$ or 40.08 mg/L Ca^{2+}.
- **A Clark degree** (°Clark) or English degrees (°e or e) is defined as one grain (64.8 mg) of $CaCO_3$ per Imperial gallon (4.55 litres) of water, equivalent to 14.254 ppm.
- **A French degree** (°F or f) is defined as 10 mg/L $CaCO_3$, equivalent to 10 ppm.
- **A degree of General Hardness** (dGH or 'German degree (°dH, deutsche Härte) is defined as 10 mg/L CaO or 17.848 ppm.
  Hardness of water is the precise mixture of minerals dissolved in the water along with the water's pH and temperature, which determine the behavior of the hardness. A single-number scale does not adequately describe hardness. However, the United States Geological Survey uses the following classification into hard and soft water (USGC, 2014).

Table 1.3 Classification of water hardness in different units (Wikipedia, 2014).

Classification	Hardness in mg/L	hardness in mmol/L	hardness in dGH/°dH	hardness in gpg
Soft	0–60	0–0.60	0.3-3.00	0-3.50
Moderately Hard	61–120	0.61–1.20	3.72-6.75	3.56-7.01
Hard	121–180	1.21–1.80	6.78–10.08	7.06-10.51
Very Hard	≥ 181	≥ 1.81	≥ 10.14	≥ 10.57

1.14 Iron

Iron is an element present in public and private water supplies and can result in poor tasting. When iron-rich waters mix with tea, coffee, or alcoholic beverages, they assume a black, inky appearance with an unpleasant taste (Colter & Mahler, 2006). According to the EPA, iron is an aesthetic problem rather than health hazard at concentrations commonly found in drinking water. There are four types of iron in water: Ferrous, Ferric, iron bacteria and organic iron. Ferrous (clear-water) iron is the most common form. In deep wells or aquifers, because oxygen content is low, iron is dissolved in water and water remains clear and colorless (Colter & Mahler, 2006). Tap water may remain clear, but it can precipitate and create rust colored particles if it sits for a while producing ferrous sulfate ($FeSO_4$), which has a metallic taste and use as a reference standard in food sensory evaluation. The metallic flavor of ferrous sulfate is come from its odor rather than taste (Hettinger et al., 1990). However, when ferrous iron is exposed to the atmosphere, iron begins to oxidize and reddish-brown-to-black particles begin to

form, and form ferric (WHO, 2006). Ferric iron is insoluble in water. Iron stains laundry and plumbing fixtures above 0.3 mg/l. Although color and turbidity may develop, there is usually no obvious taste at iron concentrations below 0.3 mg/l (WHO, 2006). Iron bacteria are nonpathogenic and exist in soil, groundwater, and surface waters. These bacteria gives water an unpleasant taste cause yellow stains on laundry and clog water systems. Organic iron exist as an organic complex in shallow wells and surface water and is usually yellow or brown (Colter & Mahler, 2006).

1.15 Copper

Copper in water usually come from the corrosive action of water leaching copper from copper pipes in buildings (Dietrich et al., 2004; WHO, 2006). High levels of dissolved oxygen can hasten copper corrosion. With time, concentrations of copper can significantly increase when standing in contact with the pipes. Copper concentrations can exceed health-based standards and can change flavor and increase health concerns (Dietrich et al., 2004, 2005). According to the WHO (2006), copper above concentrations of 1 mg/l, can stain sanitary ware and laundry may occur. At concentrations above 5 mg/l, copper also can add an undesirable color and bitter taste to water. WHO (2006) recommends a limit of 2 mg/l Cu to prevent adverse health effects from copper exposure. However, copper in drinking water can be an important source of dietary copper for humans (Zacarias et al., 2001). According to the EPA, the maximum contaminant level for copper is 1.3 mg/ l Cu in drinking water (USEPA, 1991) and there is an aesthetic based standard of 1 mg/l Cu (Cuppet et al., 2006).

1.16 Chlorine and Chloramine

Chlorine is widely used to disinfect drinking water in order to control bacteria and odors (WHO, 1996). Chlorine is used as disinfectant and bleach for both domestic and industrial purposes. Chlorine can be tasted in drinking-water at concentrations well below 4-5 mg/l (the health-based guideline value), and some people detect chlorine at levels as low as 0.3 mg/l. Chloramines are formed from the reaction of ammonia and chlorine. Chloramine is a broad term that is used to describe monochloramine (NH_2Cl), dichloramine ($NHCl_2$) and trichloramine (NCl_3). Monochloramines are the desired product for the disinfection process. Initially, chloramines were used because of its low impact on water flavor; however, it was observed that chloramines were more stable than free chlorine in the distribution system and were more effective for preventing bacterial regrowth (EPA, 1999). The increased interest in chloramines is mainly because they form very few disinfection byproducts. Higher chloramines, particularly trichloramine, can cause noticeable taste and odor (WHO, 2006). For monochloramine, no flavor was reported at concentrations between 0.5 and 1.5 mg/l (WHO, 2006). Overall, chloramine is a less effective disinfectant than chlorine, but it lasts longer in the water system. There are some concerns that chloramines may form high level of toxic disinfection byproducts (DBPs) in water which are more toxic than chlorine and has adverse health effects on the body (Food and Water Watch, 2010).

1.17 Effects of Water Compositions on Tea infusions

Composition of water plays an important role in tea leaves' chemical extraction and stability of the extracts in the infusion. Several factors may affect catechins, especially EGCG, stability including pH, temperature, metal ions, antioxidant level,

oxygen level, and the concentration of catechins in tea (Chen et al., 2001; Su et al., 2003; Sang et al., 2005; Wang et al., 2006). Stability of catechins is pH-dependent and pH values of tea infusions highly depend on the buffer capacity of waters. Catechins are relatively stable in acidic solution, whereas they are very unstable and decompose in a few minute in alkaline solution (Zhu et al., 1997). It has been shown that among catechins, EC and ECG are more unstable than EGCG and EGC with respect to infusion pH. In a study by Su et al. (2003), it was observed that catechins were susceptible to increased temperature and pH. In addition, catechins' stability is affected more by the ions present in the water than by the pH of the water. Nevertheless, for the same ionic environment, the catechins are less stable at higher pH values (Su et al., 2003). At the same conditions, catechins such as EGC and EGCG are also less stable at higher temperatures (Wang & Helliwell, 2000)**.** Boiling tea affects all of the catechins in the same way and degrades them in a similar manner.

The antioxidant activity of the teas depends on their total phenolic content and metal-chelating activity (Venditti et al., 2010). The rate of autoxidation of EC, EGC, ECG, and EGCG was found to increase with pH and can be inhibited by superoxide dismutase (SOD) and catalyzed by Cu^{2+} (Roginsky & Alegria, 2005). Chelating activity is always higher in hot teas than in cold teas especially in oolong tea and white tea (Venditti et al., 2010). However, at room temperature in sodium phosphate buffer at pH of 7.4, they showed varying stability: EGCG and EGC being completely degraded in 6 h of incubation, EC and ECG were degraded by less than 35% (Lun Su et al., 2003). The reason for this difference might be due to the three vicinal hydroxyl groups at positions 3′, 4′ and 5′ in EGCG and EGC which are more susceptible to degradation and producing

semiquinone free radicals than the two vicinal hydroxyl groups at positions 3′ and 4′ in ECG and EC (Yoshioka et al., 1991). Chen et al. (2001) by analyzing the composition of commercial bottled and canned tea beverages found that the EGCG, EGC, EC and ECG were mainly converted to their corresponding epimers and were present in low quantities and that pH affect the stability of these compounds.

It has been found by Wang & Helliwell (2000) that epimerization of catechins occurs more easily in tap water than in purified water. Mossion et al. (2007) showed that the high mineral content in water will result in the lower the extraction yield of total polyphenols, aluminum and total organic carbon. Chen et al. (1997) found that Fe (II), Fe (III), Cu (II), and Ca (II) at the concentration of 20, 20, 5 and 200 ppm, respectively can significantly decrease concentration of polyphenols in oolong tea infusions. The presence of metal ions enables a metal-catalyzed auto-oxidation of EGCG (Sang et al., 2005).

Extraction mechanisms of organic and inorganic compounds were described by Spiro & Price (1987a): first, water is up taken by leaves and then, elements and molecules diffuse from tea leaves to the infusion. When tea is brewed in highly mineralized water, during the first step, calcium uptake by leaves could take place and calcium could be complexed by pectins present in cell wall (Spiro et al., 1987b). Calcium is well known to modulate gelification of pectins (Capel et al., 2006) and this phenomenon along with other modification can limit the extraction of organic but also inorganic compounds. Tea cream is a precipitate that formed and it happens when tea cools down and it is result of complexation between caffeine and theaflavins or thearubigins (Mossion et al., 2007); it is controlled by several parameters such as pH, extraction temperature and leaf–water ratio (Chao & Chiang, 1999a) and is accelerated

by calcium addition (Jöbstl et al., 2005). Tea scum is another phenomenon which is defined as "surface film composed of calcium, hydrogenocarbonates and organic matter" (Mossion et al., 2007). Tea scum happens only in infusions prepared with hard water and is produced by the oxidation of organic compounds induced to the presence of calcium carbonate (Spiro and Jaganyi, 1994). Previously it has been shown that in water containing a high amount of calcium, 1.46 g L^{-1}, the extraction rates of theaflavins and caffeine are less than in ultrapure water (Spiro et al., 1987b). Moreover, calcium and magnesium appear to be the major elements involved in tea cream and scum formation (Spiro & Jaganyi, 1993). In water containing 10–150 mg Ca L^{-1}, tea leaves can uptake between 1 and 2.5 mg Ca g^{-1} leaves (Anderson et al., 1971). Anderson et al. (1971) study have shown that the effect of this uptake on polyphenol and caffeine extraction is insignificant, which different from the Spiro and Price (1987a) report.

Tea bushes are aluminum accumulating plant (Stagg & Millin, 1975), therefore, aluminum is highly extracted during brewing due to its strong affinity to organic matter (Mossion et al., 2007). Tea infusions could represent a primary source of aluminum daily uptake for consumers because of the high amount of aluminum. Those works have shown that, in tea infusions, aluminum is mainly bound to organic matter, the nature of which is not exactly known (Flaten, 2002). For a minor part, aluminum could be bound to oxalate (Flaten, 2002) or to fluoride (Erdemoglu et al., 2000). Declining pH due to the presence of weak acids would increase aluminum extraction from leaves because of competition between aluminum and proton for complex formation and presence of more soluble aluminum cation mostly for pH below 5 (Sigg et al., 2006).

1.18 Conclusions and Research Objectives

There is a lack of information about effects of water compositions on caffeine and catechins in tea in literatures. Moreover, there is not much information available on flavor volatile components of oolong tea and the method of flavor analysis of tea. In addition, effects of panning on flavor volatiles of oolong tea have not been studied before. Therefore, the objectives of this study were to:

1- Study effects of brewing time, chlorine, chloramines, iron, copper, pH and water hardness at EPA maximum contaminant levels for drinking water on extraction of EGCG and caffeine in green tea and oolong tea aqueous infusions.

2- Optimize solvent (dichloromethane, diethyl ether) and time of extraction (20, 40, 60 minutes) for the isolation of flavor compounds present in oolong tea using SDE technique. And, to compare two extraction techniques, SPME and SDE for the isolation and identification of flavor compounds present in oolong tea using GC-MS and GC-O.

3- Investigate effects of panning on flavor volatile compositions of oolong tea and to determine changes in aroma active compounds of panned compared to unpanned oolong tea using Gas Chromatography- Mass Spectrometry (GC-MS) and Gas Chromatography- Olfactrometry (GC-O).

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CHAPTER II

Water chemistry Effects on EGCG and Caffeine Extraction in Green and Oolong Tea

Abstract

In this study, effects of brewing time, chlorine, chloramine, iron, copper, pH and water hardness were investigated for their effects on extraction of epigallocatechine gallate (EGCG) and caffeine in green tea and oolong tea aqueous infusions. The levels of EGCG and caffeine were determined using high performance liquid chromatography (HPLC) with diode array detection (DAD). The extraction of EGCG and caffeine were lower when green tea was brewed in hard water compared to distilled water. Iron, copper and pH over the range of 6.3 to 8.3 did not significantly affect the extraction of EGCG or caffeine in green tea infusions. The extractions of these two compounds were significantly increased by brewing time. Brewing green tea and oolong tea in tap water resulted in higher extraction of caffeine but had no effect on EGCG compared to distilled water. The extraction of EGCG and caffeine were significantly increased ($P<0.05$) when green tea and oolong tea were brewed in the chlorinated water at 4.0 mg free chlorine per liter. Water chemistry affects extraction of caffeine and EGCG from tea.

Keywords: water chemistry, green tea, EGCG, caffeine, water hardness, chlorine, chloramine, iron, copper.

2.1 Introduction

Water has a number of unique properties that are essential to life which determine it's chemical behavior. The characteristics of water such as its chemical composition used in the production and preparation of food are quite important. Excessive amounts of some elements can result in imbalances in water chemistry. Hence, the importance of water quality cannot be underestimated by food manufacturers. The composition of water plays a vital role, both as a critical ingredient in ensuring food quality and as a key to efficient production. In addition, water can dissolve many different substances, giving water varying tastes and odors. Tea is the most consumed drink in the world after water. Water quality affects tea's taste and aroma, as well as health considerations (Goncalves, Paterson & Lima, 2006). Tea antioxidants have drawn increased attention in recent years due to their potential health benefits, not only as antioxidants but also as anti-microbial, anti-carcinogenic, and anti-arteriosclerotic compounds (Crespy & Williamson, 2004). Tea polyphenol is the leading functional component and an important parameter of tea quality. It is mainly composed of catechins with a proportion up to 70–80%. The main catechins in green tea are catechins, epicatechin (EC), epicatechin gallate (ECG), epigallo catechins (EGC), (-) epigallocatechin gallate (EGCG). EGCG is the most abundant catechins in tea. Tea catechins undergo many chemical changes during the manufacturing process and also brewing processes. Teas can be divided into three categories based on the tea fermentation process: green, oolong and black. Oolong tea is a semi-fermented tea that is allowed to oxidize more than a non-fermented green tea but less than a fully-fermented black tea.

Many factors affecting the extraction of tea in water have been reported, such as extraction time (Kyle, Morrice, McNeill & Duthie, 2007), extraction temperature (Labbe, Tremblay & Bazinet, 2006). Tea polyphenols, amino acids, saccharides and caffeine are the main factors which influence the quality of tea infusion (Danrong, Yuqiong & Dejiang, 2009). Epigallocatechin gallate (EGCG) and caffeine are usually isolated by extraction with organic solvents, and the extraction conditions such as solvent, temperature, duration of extraction, pH, and composition ratio of solvent to material can have a variety of effects on the extraction efficiency of EGCG and caffeine (Gadkari & Balaraman, 2013).

Few studies have been conducted on water chemistry effects on quality of teas. Water with high concentration of iron used in brewing tea and coffee can interact with tannin and giving the infusion a black inky appearance with a metallic taste (Dvorak, Prasai, Skipton, & Wildt, 2014). Calcium and magnesium also found to be the major elements involved in tea cream and scum formation (Spiro & Jaganyi, 1993). The quality of green tea beverage is greatly influenced by characteristics of water such as hardness (Horie, Yamauchi & Kohata, 1998). For example, iron and manganese from soil or pipes can affect both taste and appearance of tea (Dvorak et al., 2014). A 2009 report showed any hardness in excess of 200 ppm can cause clouding in iced tea (Bunn-O-Matic Cooperation, 2009). Arai & Kawamura (2006) showed tea infusions prepared with cathode water had significantly higher concentrations of EGCG, EGC and EC then that prepared with tap water.

Chemical taste or odor caused by chlorination of municipal water and the presence of hydrogen sulfide in the water can also detract from tea flavor. Chlorine is the

most common disinfectant used to treat drinking water. However, many consumers complain about the taste and odor problems associated with chlorine. Chloramine, a mixture of chlorine and ammonia, is a disinfectant used sometimes in place of chlorine treated water. Water treated with chloramine does not have the taste and odor problems of chlorine. Chloramine is a more stable compound which is the main advantageous of using chloramine in drinking water. It also does not produce the dangerous disinfection by-products, trihalomethanes and trihaloacetic acid, that can be produced in chlorine treated water.

The objectives of this study was to study effects of brewing time, chlorine, chloramines, iron, copper, pH and water hardness at maximum levels in Environmental Protection Agency (EPA) guideline for drinking water on extraction of epigallocatechine gallate (EGCG) and caffeine in green tea and oolong tea aqueous infusions.

2.2 Materials and Methods

2.2.1 Tea Sources

In this study, Xihu Longin green tea was purchased from Chinatea (Hangzhou, China), Dragon Well green tea (Lung Ching), Imperial Huangshan Maofeng green tea and Ice peak oolong tea (Dong Ding oolong) were purchased from EnjoyingTea (San Francisco, CA, USA).

2.2.2.1 Copper and Water Hardness

We investigated the effects of copper at EPA Maximum Contaminant Levels (MCL) (1.3 mg/L) and water hardness on extraction of EGCG and caffeine as affected by time in green tea Xihu Longin aqueous infusions. The Dortmund, Germany water (which

classified as very hard water) was the model for the hard water for this experiment which was obtained from BeerSmith^{TM} 1.2 software (BeerSmith, USA). The hard water was prepared adding 0.9 g calcium sulfate ($CaSO_4$) (Fisher Scientific, Fair Lawn, NJ, USA), 1.1 g magnesium sulfate ($MgSO_4$) (Sigma-Aldrich, St. Louise, MO, USA), 0.7 g calcium chloride (CaCl) (Carlson Company, Kent, Ohio, USA), 1.2 g sodium bicarbonate ($NaHCO_3$) (Fisher Scientific, Fair Lawn, NJ, USA), 1.7 g calcium carbonate ($CaCO_3$) (Acros Organics, Fairlawn, NJ, USA) into 3.78 L (1 gallon) distilled water. The solution was heated and stirred for 30 min until the chemicals were dissolved in water. Then, 1.0 mg/L cupric sulfate ($CuSO_4$. $5H_2O$) (Fisher Scientific, Fair Lawn, NJ, USA) was added to 200 mL of distilled and hard water individually and a stock solution of copper was used to prepare model systems with copper ions in distilled water and hard water. The results were four water solutions: 1) distilled water, 2) distilled water with copper, 3) hard water, 4) hard water with copper.

2.2.2.1.1 Statistical Analysis

The statistical design was a randomized complete block design (RCBD) with three replications to determine if differences existed in extraction of EGCG and caffeine among different water solutions. Data were analyzed by JMP 9.0 (SAS, Cary, NC, USA) and means were compared by using Fisher's least significant difference (LSD) method with significance at $P<0.05$.

2.2.2.2 Iron, Copper, pH and Water Hardness

We investigated the effects of iron and copper, individually, in distilled water or hard water at different pH on extraction of EGCG and caffeine in green tea Xihu Longin

aqueous infusions. The same method employed in the previous experiment was used to prepare hard water and distilled water. Ten mM phosphate buffer was added to hard water to obtain pH of 6.3 and 8.3 (EPA secondary regulations for drinking water). The results were six water solutions: distilled water at pH of 6.3, 7.0 and 8.3 and hard water at pH of 6.3, 7.0 and 8.3. A stock solution of iron was prepared using ferrous sulfate ($FeSO_4$) (Fisher Scientific, Fair Lawn, NJ, USA) by adding 0.3 mg to 200 mL distilled water and hard water and used to prepare model systems with iron ions in order to obtain the EPA maximum level (0.3 mg/L) (EPA secondary regulations for drinking water). The same method as experiment 1 was used for preparation of a stock solution of copper in hard water and distilled water separately.

2.2.2.2.1 Statistical Analysis

The statistical design was a balanced incomplete block design (BIBD) with three replications to determine if differences existed in extraction of EGCG and caffeine among different water solutions. Data were analyzed by JMP 9.0 (SAS, Cary, NC, USA) and means were separated by using Fisher's LSD method with significance at $P<0.05$.

2.2.2.3 Tap Water Vs Distilled Water

Extraction of EGCG and caffeine as affected by time in Xihu Longin green tea and Imperial Huangshan Maofeng green tea aqueous infusions that were prepared in tap water were compared to extraction of these two compounds in distilled water. The tea leaves were brewed individually in Blacksburg (VA, USA) tap water and distilled water. Blacksburg water composition was as follows: Na^+ 10 mg/L, K^+ 5 mg/L, Cu^{2+} 8 mg/L,

Mg^{2+} 6 mg/L, Total hardness ($CaCO_3$) 45 mg/L, SO_4^{2-} 2 mg/L, Cl^- 15 mg/L, CO_3^{2-} 6 mg/L, HCO_3^- 47 mg/L, and total dissolved solids (TDS) 100 mg/L.

2.2.2.3.1 Statistical Analysis

The statistical design was a completely randomized design (CRD) with three replications to determine if differences existed in extraction of EGCG and caffeine between tap water and distilled water. Data were analyzed by JMP 9.0 (SAS, Cary, NC, USA) and means were compared by using Fisher's LSD method with significance at $P<0.05$.

2.2.2.4 Chlorine and Chloramines

Effects of brewing time, chlorine, chloramines on extraction of EGCG and caffeine in Xihu Longin green tea aqueous infusions were investigated. Thirty mg/L ammonia-N stock solution was prepared and the pH was adjusted at 8.0 using 0.1 N sodium hydroxide (NaOH) to obtain EPA MCL for chloramine (4.0 mg/L). The solution was mixed on a stir plate, and bleach (6% sodium hypochlorite) (Clorox, Oakland, CA, USA) was slowly added to water solution to increase chlorine concentration to 120 mg/L. The concentration of total chlorine and ammonia was measured by using a portable spectrophotometer (Hach *DR 2700*™, Loveland, CO, USA).The ratio of Cl:N was adjusted to 4:1. For preparing the free chlorine water solution, 60 μL bleach (6% sodium hypochlorite) was added to 1000 mL distilled water to obtain 4.00 mg free chlorine/L (EPA MCL). Then, free chlorine was measured with portable Spectrophotometer Hach *DR 2700*™ to confirm.

2.2.2.4.1 Statistical Analysis

The statistical design was a CRD with three replications to study effects of free chlorine and chloramines on extraction of EGCG and caffeine. Data were analyzed by JMP 9.0 (SAS, Cary, NC, USA) and means were compared by using Fisher's LSD method with significance at $P<0.05$.

2.2.2.5 Free Chlorine

To confirm the result from previous experiment, effects of brewing time and free chlorine at EPA MCL, on extraction of EGCG and caffeine in four varieties of green tea and oolong tea aqueous infusions were investigated. The totals of four separate experiments were conducted for each variety of tea. Xihu Longin green tea, Imperial Huangshan Maofeng green tea, ice peak oolong tea (Tung Ting oolong), Dragon Well green tea (Lung Chin) were used for this experiment. The effects of water with free chlorine in comparison with distilled water were investigated on each variety of tea separately. The same method as previous experiment for preparation of water with free chlorine was used. The tea leaves were brewed in water with free chlorine and distilled water.

2.2.2.5.1 Statistical Analysis

Completely randomized design (CRD) with three replications was used for each experiment and means were separated using Fisher's LSD method with significance at $P<0.05$.

2.2.3 Infusion Preparation, and EGCG and Caffeine Extraction

Each infusion was made by pouring 200 mL boiling water (98 ^{o}C) over 4 g tea leaves and brewed for 5 min while stirring with magnetic stirring bar. After 5 min, 4 mL was extracted at 98 ^{o}C. Approximately 2 mL tea was placed into 10 mL conical screw cap tubes and centrifuged for 10 min at 300xg. After centrifugation, the supernatant was transferred into auto sampler vials for high performance liquid chromatography (HPLC) and placed in the auto sampler with refrigeration at 4 ^{o}C.

2.2.4 HPLC-DAD Analysis

The HPLC method used in this experiment was obtained from Goto, Yoshida, Kiso & Nagashima (1996). A HPLC-DAD (Model 1260 Infinity, Agilent Technology Inc, Santa Clara, CA) equipped with a 250 mm x 4.60 mm Luna 5µ C18 (Phenomenex, Torrance, CA, USA) column thermostated at 40 ^{0}C with a diode-array detector (DAD) (AgilentTechnologies, Wilmington, DE.) was used. Water –acetonitrite with two step linear gradients of acetonitrite concentration was used to separate EGCG and caffeine within 30 min. A binary mobile phase was used: A) water: acetonitrite, phosphoric acid (95.45:4.5:0.05, v/v/v); B) water: acetonitrite: O-phosphoric acid (49.95:50.0:0.05, v/v/v). The solvent composition started at 90% solvent A and 10% solvent B and was maintained for 5 min, then linearly increased to 30% solvent B over 3 min. This condition was maintained for 2 min followed by a linear increase of solvent B to 80% in 5 min. The final conditions were held for an additional 5 min. The initial conditions were regenerated and column flushed with 10 column volumes solvent A before the next sample.

2.3 Results and Discussion

In the experiment 2.2.1, there were significant differences ($P<0.05$) between different waters (Table 2.1). The extraction of EGCG and caffeine were lower ($P<0.05$) when green tea were brewed in hard water compared to distilled water; however, copper did not significantly ($P>0.05$) affect the extraction of EGCG or caffeine. In addition, extraction of EGCG and caffeine were increased ($P<0.05$) by brewing time. In the experiment 2.2.2, iron at 0.3 mg/L, copper at 1.3 mg/L and pH at range of 6.3-8.3 in distilled water and hard water did not significantly ($P>0.05$) affect the extraction of EGCG or caffeine in green tea infusions. The effects of these compounds at EPA MCL concentration were not significant on extraction of EGCG and caffeine.

Previously, it has been found that metal ions such as iron and copper form complexes that catalyze the oxidation of catechins by activating oxygen in water (Chen, You, & Chen, 1997). Chen et al. (1997) found that the concentrations of Fe^{2+}, Fe^{3+}, Cu^{2+}, and Ca^{2+} necessary to cause a significant decrease in polyphenols in oolong tea infusions were 20, 20, 5 and 200 ppm, respectively; in our study the level of these metal ions were less than these amount and similarly resulted in no significant effects on extraction of EGCG.

The source of iron in water can be rock and soil as well as iron pipes. Iron may react with tannins in coffee, tea and some alcoholic beverages to produce a black sludge, which affects both taste and appearance (Dvorak et al., 2014). In our study, iron at EPA MCL did not affect the extraction of EGCG or caffeine. Flavonoids can easily interact with a variety of metal ions and create complex compound, hence green tea catechins have the potential to affect absorption and metabolism of iron (Mira, Fernandez, Santos,

Rocha, Florencio & Jennings, 2002). Other studies examined relations between tea catechins and iron absorption in humans and showed tea catechins can diminish iron absorption, particularly in groups at risk of iron deficiency (Samman et al., 2001; Nelson & Poulter, 2004; Chacko; Thambi, Kuttan & Nishigaki, 2010), but their effects on other ions are poorly understood. At higher dose, iron might similarly affect the extraction of EGCG in green tea infusions, but in our study, we did not find any effects of iron on the EGCG extraction. It might be important for the future studies to measure the chelating activity of the tea infusions, since it measures how effective the compounds in tea can compete for ferrous ion (Venditti, Bacchetti, Tiano, Carloni, Greci & Damiani, 2010).

Tan (1985) found the tea leaves are able to remove substantial amounts of Cu^{2+} ions from aqueous solution. In our experiment, extraction of EGCG and caffeine were not significantly affected by copper in hard water or distilled water at pH range of 6.3-8.3 which is different to Tan (1985) results that showed the tea leaves Cu^{2+} absorbance capability depends on pH. Tan showed the Cu^{2+} absorption capability of tea leaves also dependent to metal concentration, physical nature of substrate and ionic strength. A study by Deng, Tao, He & Chen (1998) showed green tea ingestion over a long period had no apparent effect on absorption of copper, whereas it decreases that of zinc and increases that of manganese (Deng et al., 1998). Moreover, it has been suggested that EGCG acts as an antioxidant by chelating metal ions, such as copper and iron, to form complexes (Kashima, 1999; Nanjo, Goto, Seto, Suzuki, Sakai & Hara, 1996; Kelly, Geigerman, & Loo, 2001; Sutherland, Rahman & Appleton, 2006). The rate of autoxidation of EC, EGC, ECG, and EGCG was found to be decreased by superoxide dismutase and catalyzed by Cu^{2+} (Roginsky & Alegria, 2005).

A study by Lee & Lee (2008) showed the amount of caffeine extracted from green and black tea slightly decreased as the pH of the aqueous solution increased, but it sharply decreased when the pH was more than 7.0. Danrong et al. (2009) also found low pH (pH < 7.0) was helpful for conserving catechins in green tea extract and catechins concentrations were greater in green tea extracts prepared with distilled water. In this study, caffeine extraction was not significantly affected by pH in the range of 6.3, 7.0 and 8.3. This may be caused by the isomerization of caffeine due to the addition of OH in order to increase the pH of the aqueous solution (Vinchurkar, Rao, Mohan, Mittal, Schmidt & Jonah, 1997). Danrong et al. (2009) reported the amount of EGCG extracted from green and black tea was nearly constant when the pH was in the range of 3-9, which is similar to result of our study Catechins also found to be more stable under acidic condition rather than alkaline (Su, Leung, Huang, & Chen, 2003). Polyhydroxy characteristic of tea polyphenol structure is responsible for the solubility of polyphenols in water. Tea polyphenols can reversibly polymerize through the benzene ring and phenolic hydroxyl with hydrogen bonds and hydrophobic bonds (Danrong et al., 2009). EGCG and caffeine are usually isolated by extraction with organic solvents, and the extraction conditions (solvent, temperature, duration of extraction, pH, and composition ratio of solvent to material) can have a variety of effects on the extraction efficiency of EGCG and caffeine. The extraction decreases under conditions of small molecules, low pH and high temperature (Danrong et al., 2009).

Hard water contains a high concentration of cations, including Ca^{2+} and Mg^{2+}. These elements occur naturally in all water supplies. Polyphenol particles are electronegative, so they will be more stable if few electrolytes and small molecule

dispersants exist in tea solution. It is shown that a substantial part of water calcium (1-2.5 mg Ca g^{-1}) is taken up by the tea leaf during the preparation of infusions (Anderson, Hollins & Bond, 1971). Our results showed brewing tea in hard water decrease the extraction of EGCG compared with distilled water. This result is similar to Danrong et al. (2009) who showed the contents of tea polyphenols were higher in green tea extracts prepared with distilled water, owing to the weak acid environment and reduction of structure viscosity caused by the small organic molecules. Previous studies also showed water containing high amount of calcium (1.46 g L^{-1}) decreasing the rates of extraction of theaflavins and caffeine compare to distilled water (Spiro, Price, Miller & Araamin, 1987). In addition, the difference in pH among tested waters similarly influenced the effectiveness of extraction of tea polyphenols. Spiro & Price (1987) suggested two steps for the mechanism of extraction of organic and inorganic compounds from tea: first, water is absorbed by leaves and second molecules diffuse to the infusion. Calcium uptake by leaves could take place during the first step and could be complexed by pectins present in cell wall (Spiro et al., 1987). Previous studies showed calcium can cause jellification of pectins and these modifications (Capel, Nicolai, Durand, Boulenguer & Langendorff, 2006) and could then limit the extraction of organic but also inorganic compounds (Mossion, Potin-Gautier, Delerue, Le Hécho, & Behra, 2008).

As shown in Table 2.2, the extraction of caffeine was significantly ($P<0.05$) higher in tap water in comparison to distilled water in both green tea and oolong tea infusions (Study 2.2.3). There were no significant differences ($P>0.05$) in extraction of EGCG between tap water and distilled water in green tea and oolong tea infusions. Sang et al. (2005) found that epimerization and auto-oxidation are the two major reactions

causing the instability of EGCG and the temperature, pH, partial pressure of oxygen, level of antioxidants, concentration of EGCG, and other components of tea affect the rates of these reactions. They also suggest the instability of EGCG is due to superoxide-mediated auto-oxidation of EGCG. EGCG dimer is the major product of auto-oxidation in tea (Wang & Helliwell, 2000). Individual catechins can epimerize at high temperatures. In green tea infusions, the main change appears to be epimerization from the epistructure to the nonepistructure (Wang & Helliwell, 2000). Wang & Helliwell (2000) found that epimerization of catechins happen more easily in tap water than in purified water and therefore they rapidly degrade. The difference in pH between tap and purified water and the different ions present in the tap water might be the main factors influencing this phenomenon. However, among these factors, the ions present in the water affect the stability of catechins more than the pH of the water. EGCG stability is less in tap water at higher temperature than purified water (Wang & Helliwell, 2000). The solubility of catechins was associated with the polarity of catechins. Danrong et al. (2009) found non-ester catechins were greater in green tea that extracted in polar liquids such as deionized water, distilled water, reverse osmosis water, and ultra-pure water in comparison with activated carbon adsorbed water. Activated carbon adsorbed water had higher contents of catechins ester since activated carbon selectively adsorbed polar material and resulted in a weak polar liquid.

In our study, we hypothesized that because of the oxidative characteristic of some of the disinfectants such as chlorine and chloramine, their presence in tap water might also be the reason that we observed higher extraction of EGCG and caffeine compare to distilled water in study 2.2.4. We found significant differences ($P<0.05$) in extraction of

EGCG and caffeine in green tea infusions among treatments (Table 2.3). Caffeine and EGCG extraction were higher ($P<0.05$) in water with free chlorine compared to distilled water or water with chloramines. The caffeine content in tea brewed in distilled water was higher ($P<0.05$) than the tea brewed in chloramine water. There was no differences ($P>0.05$) in extraction of EGCG between distilled water and chloramine water. As shown in Table 2.4, there were significant differences ($P<0.05$) between free chlorine water and distilled water (study 2.2.5). EGCG and caffeine extraction from Xihu Longin green tea in water with free chlorine were higher ($P<0.05$) than distilled water. Results from brewing Imperial Huangshan Maofeng green tea, ice peak oolong tea and Dragon Well green tea in free chlorine water and distilled water showed the extraction of caffeine was higher ($P<0.05$) in chlorine water compared to distilled water. However, there were no differences ($P>0.05$) in extraction of EGCG between free chlorine water and distilled water.

Results showed free chlorine in brewing water affected the extraction of EGCG and caffeine. Free chlorine increased the caffeine extraction in all four verities of teas; however, only EGCG extraction from Xihu Longin green tea was higher in free chlorine water than distilled water. Perhaps free chlorine accelerates epimerization of catechins to EGCG. Studies on tea and coffee suggest that caffeine is produced from the purine nucleotides AMP, GMP, and/or IMP and that theo- bromine is the immediate precursor of caffeine (Suzuki, Ashihara, & Waller, 1992; Fujimori & Ashihara, 1994). Free chlorine also enhances the formation of the purine ring of caffeine from precursors.

2.4 Conclusions

Our study showed the composition of water can affect extraction of EGCG and caffeine in green and oolong teas. Water hardness and chlorine at EPA MCL significantly affected the extraction of these two compounds.

Table 2.1 Effects of copper and water on extraction of caffeine and EGCG in Xihu Longin green tea.

Treatment	Caffeine (mg/mL)	EGCG (mg/mL)
Distilled water	0.65[a]	0.83[a]
Hard water	0.58[b]	0.57[b]
Pooled Standard Error	0.02	0.03
Copper	0.62 [a]	0.71[a]
Non-copper	0.61 [a]	0.69[a]
Pooled Standard Error	0.02	0.03

[a-b] Means ± SD within a column with the same letter are not different (p>0.05).

Table 2.2 EGCG and caffeine extraction in Xihu Longin green tea and Imperial Huangshan Maofeng green tea subjected to different water (tap and distilled).

Tea	Treatment	Caffeine (mg/mL)	EGCG (mg/mL)
Xihu Longin green Tea	Tap Water	1.60[a]	1.07[a]
	Distilled water	1.54[b]	1.07[a]
	Pooled Standard Error	0.03	0.02
Imperial Huangshan Maofeng green tea	Tap Water	0.9[a]	0.63[a]
	Distilled water	0.88[b]	0.64[a]
	Pooled Standard Error	0.03	0.02

[a-b] Means ± SD within a column with the same letter are not different (p>0.05).

Table 2.3 Effects of chlorine and chloramines on extraction of EGCG and caffeine in Xihu Longin green tea.

Treatment	Caffeine (mg/mL)	EGCG (mg/mL)
Distilled water	1.48[b]	0.93[b]
Free Chlorine water	1.73[a]	1.03[a]
Chloramine water	1.41[c]	0.92[c]
Pooled Standard Error	0.07	0.04

[a-c] Means ± SD within a column with the same letter are not different (p>0.05).

Table 2.4 Effects of free chlorine on extraction of EGCG and caffeine in Xihu Longin green tea, Imperial Huangshan Maofeng green tea, Ice peak oolong tea (Tung Ting oolong) and Dragon Well green tea (Lung Chin) in caparison to distilled water.

Tea	Treatment	Caffeine (mg/mL)	EGCG (mg/mL)
Xihu Longin green tea	Free chlorine	1.32[a]	0.87[a]
	Distilled	0.97[b]	0.80[b]
	Pooled Standard Error	0.04	0.01
Imperial Huangshan Maofeng green tea	Free chlorine	0.99[a]	0.65[a]
	Distilled	0.96[b]	0.65[a]
	Pooled Standard Error	0.04	0.02
Ice peak oolong tea (Tung Ting oolong)	Free chlorine	1.07[a]	0.71[a]
	Distilled	0.97[b]	0.69[a]
	Pooled Standard Error	0.04	0.03
Dragon Well green tea (Lung Chin)	Free chlorine	1.16[a]	0.36[a]
	Distilled	0.64[a]	0.35[a]
	Pooled Standard Error	0.02	0.03

[a-b] Means± SD within each tea variety with the same letter are not different ($p>0.05$).

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CHAPTER III

Comparison of SDE and SPME for Analysis of Flavor Compounds in Jin Xuan Oolong Tea

Abstract

Simultaneous Distillation-Extraction (SDE) and Solid Phase Micro Extraction (SPME) are common procedures for the isolation of flavor compounds in foods. The purpose of this study was to optimize SDE conditions (solvent and time) and to compare SDE with SPME for the isolation of flavor compounds in Jin Xuan oolong tea using GC-MS and GC-O. The concentration of volatile compounds isolated with diethyl ether was higher ($P<0.05$) than for dichloromethane and concentration was higher at 40 min ($P<0.05$) than 20 or 60 minutes extraction. For SDE, 128 volatiles were identified using GC-MS and 45 aroma active compounds using GC-O. *Trans* nerolidol was the most abundant compound in oolong tea. The number of volatiles identified using GC-MS was lower in SPME than SDE. For SPME, 59 volatiles and 41 aroma active compounds were identified. The composition of the volatiles isolated by the two methods differed considerably but provided complementary information.

Keywords: Oolong tea, SDE, SPME, GC-O, GC-MS

3.1 Introduction

Tea (*Camellia sinensis*) is the second most consumed beverage in the world, after water. Teas are divided in three categories based on their fermentation level: green (non-fermented), oolong (semi-fermented) and black (fully-fermented). The purpose of tea fermentation is to enhance the flavor, which is the most important element for tea evaluation (Wang, Park, Chung, Baik & Park, 2004). Oolong tea is mostly produced and consumed in Taiwan and southern China; however, in recent years, consumption of oolong tea is becoming more popular in the world, especially in China and Japan (Wang et al., 2010). The Jin Xuan tea bush, also known as No. 12 or "milky" oolong tea, has become popular recently as consumer look for new varieties in oolong tea.

Few studies are available examining the flavors of Jin Xuan oolong tea. The quality of oolong teas is traditionally assessed by tea masters in processing facilities according to the tea leaf appearance, color, taste and aroma before and after brewing. There have been limited studies on oolong teas with emphasis on taste and aroma composition properties (Huang, Shi, Shi, Gu, Chen, & Gong, 2003; Wang, Lee, Chung, Baik, So & Park, 2008; Chen, Jiang, Duan, Shi, Xue & Kakuda, 2010). In addition, studies on oolong tea flavor have not used Gas Chromatography-Olfactrometry (GC-O) analysis for identification of aroma active compounds. Use of GC-O in flavor studies is especially important since the human nose can have higher sensitivity than GC detectors; some compounds important in food flavors are not detectable with GC or GC-MS instruments (Benn & Peppard, 1996; Ferreira, Lopez, Escudero & Cacho, 1998).

The flavor of tea consists of volatile compounds that contribute to the aroma and nonvolatile compounds that contribute to the taste (Hara, Luo, Wickremasinghe &

Yamanishi, 1995; Scharbert & Hofmann, 2005; Wang et al., 2010). Although tea's volatile compounds are present in low quantities (around 0.01% of the total dry weight), they have a high impact on the flavor because of their low odor detection concentrations. The perceived aroma quality in tea (or any food for that matter) is not just a function of abundance, but primarily ratios between the volatile compounds and their odor thresholds (Wang et al., 2010).

Volatile compounds of tea are classified into main two groups: non-terpenoids and terpenoids. Non-terpenoids include products of lipid oxidation, which impart undesirable grassy odors, and terpenoids such as linalool and geraniol, which are responsible for sweet and flowery aromas of tea (Fanaro, Duarte, Araújo, Purgatto & Villavicencio, 2011) and are mainly derived from glycosides of terpenoid related compounds (Ravichandran & Parthiban, 1998).

Two very common methods of flavor analysis of foods are simultaneous distillation and extraction (SDE) and solid-phase microextraction (SPME). SPME is a simple, fast, inexpensive and solvent-free technique that provides a fraction suitable for GC–MS analysis (Kataoka, Lord & Pawliszyn, 2000). The SPME fiber consists of a fused-silica fiber coated with a polymeric film that is absorbs volatile compounds in the sample headspace by partition, adsorption or mixed mechanisms. Desorption of volatiles from the SPME fiber into the injection port of a GC allows the chromatographic analysis of this volatile fraction without matrix interferences (D'Agostino, Sanz, Martínez-Castro, Giuffrè, Sicari, & Soria 2014).

SDE is another common procedure for the isolation of flavor compounds in foods and is used for its simplicity and versatility. SDE combines solvent extraction and vapor

distillation extraction using a Likens-Nickerson apparatus. The advantages of this extraction method include simplification of the extraction procedures, savings in organic solvent use and decreased loss of samples during the transfer process (Zhang & Li, 2010). Because headspace sampling selects only the most volatile molecules, SDE remains a very common method as it avoids less volatile compounds that may chromatograph poorly in a GC. SDE has been used successfully for isolation of volatiles from spices (Zawirska-Wojtasiak & Wąsowicz, 2002), fruits (Pino, Marbot & Bello, 2002) or food subjected to boiling (Majcher & Jelen´, 2009). However, the solvents and extraction times reported in the different studies vary widely. High temperature applied during extraction using this technique can potentially lead to breakdown of some flavor compounds and create artificial compounds which do not normally exist. Hence, using a proper method of extraction is essential for the effectiveness of SDE. There are few studies that have used flavor extraction using SDE for tea (Sawai, Yamaguchi & Tanaka, 2004; Rawat, Gulati, Kiran Babu, Acharya, Kaul & Singh, 2007; Pripdeevech & Machan, 2011; Zeng, Wu, Huang & Wu, 2012).

The purposes of this study were two fold. For SDE, to optimize solvent (dichloromethane, diethyl ether) and time of extraction (20, 40, 60 minutes) for the isolation of flavor compounds present in oolong tea using GC-MS. Second, to compare two extraction techniques, SPME and SDE for the isolation and identification of flavor compounds present in oolong tea using GC-MS and GC-O.

3.2 Materials and Methods

3.2.1 Materials

Replicate samples of Jin Xuan (*Chin-Hsuan*, or Zhu Shan) oolong tea (milky tea) samples were purchased from Tea of Life ® Health Inc. in Rosedale, NY. Diethyl ether (HPLC grade) and ethyl decanoate (internal standard) were purchased from Sigma-Aldrich Co (St. Louis, MO). Dichloromethane (HPLC grade) and anhydrous sodium sulfate were purchased from Fisher Scientific (Pittsburg, PA).

3.2.2 Volatile Extraction by Simultaneous Distillation and Extraction (SDE)

Tea leaves (50 g) was placed in a 1 L flat-bottom flask containing 400 ml of boiling distilled water and immediately attached to the SDE apparatus. Diethyl ether or dichloromethane (100 ml) were taken into a 250 ml flat-bottomed flask along with 0.5 mL of 100 ppm ethyl decanoate as an internal standard. The flat-bottom flask containing tea was on one side of the Likens-Nickerson apparatus (SDE method), and the flask with solvents was on the other side (sides were switched depending on whether the aqueous phase was more or less dense than organic). The solvent and tea infusions were heated via hot plates. Oolong tea volatile compounds were extracted using diethyl ether or dichloromethane for 20, 40 or 60 min with three replications (2x3x3). After extraction, the extracts were dried over anhydrous sodium and filtered. Then, the extract was concentrated to 2 ml using a rotary evaporator set to 35 °C. This concentrate was used for gas chromatography-mass spectrometry (GC-MS) or gas chromatography flame ionization detector- olfactometry (GC- FID/O) analysis.

3.2.3 Volatile Extraction by SPME for GC-MS Analysis

For SPME, the extraction of volatiles conducted on tea infusions which make the results more comparable to SDE technique and enhance the extraction of volatiles. Tea infusion was made by pouring 200 mL boiling water (98 ^{o}C) over 4 g tea leaves and brewed for 5 min. Then, the tea infusion was filtered with Whatman No. 4 paper and approximately 5 mL of the tea infusion and 1g of NaCl were placed into 10 mL headspace vials with Teflon-lined silicon septa (Chromacol, Fisher Scientific). An AOC-5000 Plus (Shimadzu Scientific, Columbia, MD) SPME auto-sampler was used for extraction and injection to GC-MS. Samples were equilibrated for two minutes prior to extraction. A 2 cm 50/30 um divinylbenzene/carboxen/polydimethylsiloxane (DVM/Carboxen/PDMS) SPME fiber (Supelco, Bellefonte, PA) was exposed to the headspace above the tea in headspace vials for 30 minutes at 40°C with an agitation speed of 250 rpm.

3.2.4 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

For the identification of the volatile compounds, data was collected using Shimadzu GCMS-QP2010 Ultra mass selective detector (Shimadzu, Columbia, MD, USA) equipped with GCMS Solutions software. Samples were injected into the GC injection port at 200 °C for five minutes and all injections were made in splitless mode. Volatile compounds were separated on a nonpolar (SHRXI-5MS, Shimadzu, 30m x 0.25mm id x 0.25 μm film thickness) column. Helium used as a carrier gas in constant flow mode (0.68 mL/min). The initial oven temperature was set to 50 ºC and held for 5 minutes, and then increased to final temperature of 250 ^{o}C at a rate of 4º C/min and maintained for 6 minutes. The mass spectrometer scans were made from m/z 40-400 and

were performed every 0.3 seconds. The ion source and quadruple were at 230 and 200 ^{o}C. Chromatographic peaks were identified by combined matching standardized retention time (LRI/Kovats values) from DB-5 column (Flavornet and Pherobase), and fragmentation spectra of standards from NIST 11 (Scientific Instrument Services, Ringoes, NJ) and the Wiley 2010 libraries (John Wiley and Sons Inc.). Confirmation of the identification was sought by matching the mass spectra of the compounds with the reference mass spectra present in the NIST 11 and Wiley libraries (acceptable similarity index was above 90% index).

3.2.5 Statistical Analysis for GC-MS Data

The results from GC-MS analysis were analyzed by JMP 11.0 (SAS, Cary, NC, USA). Two-way ANOVA was used to find significant differences in total concentration of volatile compounds between solvents and extraction times with 3 replications for SDE. Means were compared by using Fisher's least significant difference (LSD) method with significance at $P<0.05$.

3.2.6 Volatile Extraction by Solid Phase Microextraction (SPME) for GC-FID/GC-O Analysis

For volatile extraction using SPME and GC-O, 5 ml of tea aqueous infusion which was prepared similarly to GC-MS analysis was placed in a 15 mL glass vial with a Teflon-lined septum. The sample was heated at 40°C using an 'RTC basic' heater with an ETS D4 Fuzz Controller (IKA Werke, Wilmington, NC) while being stirred using a 4 mm stir bar. An SPME fiber (50/30 μm DVB/CAR/PDMS) on a 2 cm StableFlex fiber (Supelco Bellefonte, PA) was manually inserted into the vial and was exposed

approximately 1 cm above the headspace for 30 minutes at 40°C while a magnetic bar continued to stir the sample.

3.2.7 Gas Chromatography-Olfactrometry (GC-O) Analysis

The GC-O analysis was conducted using a HP 5890A GC (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame ionization detector (FID) split with a sniffing port (ODOII; SGE Inc. Austin, TX). Volatile and semi-volatile compounds were desorbed in the injector port. A DB-5ms column (30m x 0.25-mm i.d. x 0.25 µm film thickness; J&W Scientific, Folsom, CA) using hydrogen as the carrier gas with a flow rate of 1.0 ml x min^{-1} (linear flow velocity ~ 25 cm/sec) was used. The effluent coming from the column was split 1:1 between the FID and the sniffing port using deactivated fused silica capillaries (1m length x 0.32 µm i.d.). The injector temperature was set to 250°C. The injection port and detector was set to 250°C and 275 °C, respectively and injections were made in splitless mode. The initial oven temperature was 50 °C and increased at 10 °C/min until reaching a final temperature of 200°C. Chromatograms were recorded using a HP 3396A integrator (Hewlett-Packard Co., Palo Alto, CA).

3.2.8 Time-Intensity Olfactrometry Data Acquisition

This study approved by Institutional Review Board (IRB) at Virginia Tech (IRB 13-580). Two experienced panelists were selected for GC-O analysis. Panelists were trained for 20 hours before the study began. Based on the previous studies and our preliminary studies, 33 pure aroma compounds (standards) associated with tea were selected and used to train the panelists (Appendix D). The aromas selected were: spice, sweet, nutty, earthy, musty, roasty, green pepper, cherry, waxy, smoky, herbal, woody,

floral, minty, buttery, pungent, fruity, green, banana, pineapple, citrus, vanilla and rancid. All these aromas were diluted in distilled water in a way that did not irritate panelists' nose. The concentrations used to make the solutions were varied for different aromas.

In the first step of training, panelists were trained to detect and describe the aroma characteristics of each compound individually, and each session followed with group discussion and comparison of the answers to aroma description that reported for each compound in database of Flavornet (http://www.flavornet.org/flavornet.html), Pherobase (http://www.pherobase.com/) and The Good Scents Company (http://www.thegoodscentscompany.com). During this training, compounds randomly passed to the panelists. Panelists were asked to sniff each sample for few seconds (<5 Sec) and then write the aroma descriptions. Between each sample, panelist refreshed their noses by smelling coffee beans. This step took approximately 7 hours. In the next step, discrimination testing, panelists were trained to discriminate the different odors and this took 5 hours. The compounds were randomly assigned to the panelists (about 20 aromas per session and each session took an hour). The panelists individually assessed 5 compounds at the time and then it followed with group discussion. Then, panelists proceeded to evaluate the next 5 compounds and the same procedure was repeated. In the third step, 12 compounds were diluted in three concentrations from low to high concentration. Panelists were asked to identify and score intensity of each aroma from 1 to 5 where 1 represents the lowest intensity and 5 was the highest. At the end of evaluations, the results of panelists were compared and discussed. The goal of this step was to unify panelists' approach in scoring the intensity of the aromas and help make their scores consistent. This step took 6 hours. In the last two hours of training, the

panelists were asked to sniff actual samples from sniffing port of GC-O for 20 minutes. Panelists were asked to write the aroma description, time and intensity (1-5) for each detected aroma. The results were discussed with them following of each sniffing session. For the actual test, similar to the training, two assessors sniffed tea extracts from SDE and SPME methods for 20 minutes three separate times. Panelist also scored the intensity of each aroma in scale of 1 to 5, where 1 was the lowest score and the 5 was the highest.

Mean aroma intensities for each odorant were calculated by averaging the reported intensity by panelists. Aroma-active compounds were defined as ones that were detected by two assessors at least fifty percent of the time with shared similar descriptions, as well as similar retention times or those that had an intensity of more than 3 by the panelists. Identification of volatile compounds was based upon odor descriptions and RI values from DB-5 column. Values were also compared to literature. A mixture of n-parafins (C5 – C26) ASTM D2287 quantitative calibration solution in carbon disulfide (Suplico, Bellefonte, PA, USA) was used in determining the RI values for the volatile compounds eluted by the GC-O. Solutions of hydrocarbons were analyzed in the same manner on DB-5 column to calculate RI:

$$LRI = 100[(t - t_n)/(t_{n+1} - t_n)+n]$$

Where the t is the retention time of component, n is the carbon number of preceding n-alkane and n+1= carbon number of subsequent n-alkane. The databases Flavornet (http://www.flavornet.org/flavornet.html) and Pherobase (http://www.pherobase.com/) were used to aid in identifying the compounds based on standardized retention and aroma.

3.3 Results and Discussion

3.3.1 Comparison of Time and Solvents in SDE Extraction

The results of two-way ANOVA analysis of GC-MS data from SDE extraction showed that the total concentration of isolated volatile compounds extracted with diethyl ether were significantly higher ($P<0.05$) than dichloromethane. The concentration of volatile compounds at 40 min extraction was higher ($P<0.05$) than for 20 or 60 minute extraction times. Overall, extraction with diethyl ether for 40 min resulted in higher ($P<0.05$) concentration of volatile compounds compared to other combination of solvents and extraction times. In addition, there were interactions ($P<0.05$) between "time and solvents" and "time, solvents and compounds". A total of 128 compounds were identified from SDE extraction using GC-MS consisting of: 29 alcohols, 20 aldehydes, 24 ketones, 4 acids, 5 esters and 19 terpenes. Changes within the six chemical groups as affected by solvents and extraction time are shown in Figure 3.1 and 3.2, respectively. Alcohols comprised the largest group of volatiles (total concentration). The concentration of alcohol compounds increased by extraction time. Terpenes concentration was the highest at 40 min. Figure 3.2 shows the effect of different extraction solvent on extraction of volatile compounds. The percentage of peak area for terpenes and alcohols were higher in diethyl ether extraction compared to dichloromethane; however, ketones concentration was higher in dichloromethane extraction. Overall, the isolation of compounds is significantly influenced by the isolation procedure. These results are similar to those who report the using of different extraction techniques and different solvents might affect the composition and contents of the isolated compounds (Lopez & Gomez, 2000; Nonato,

Carazza, Silva, Carvalho, & Cardeal, 2001; Bonino, Schellino, Rizzi, Aigotti, Delfini, & Baiocchi, 2003).

3.3.2 GC-MS Identifications

The identified volatiles in this study, their identification methods, observed LRI and references where previously identified in oolong tea are shown in Table 3.1. Sixty five of these volatile compounds have been reported in previous studies on oolong tea. In general, the differences between the identified compounds in different studies might be due to the different extraction method SPME or SDE and sample preparation dry tea or brewed tea (Zhang, Zeng, Zhao, Kong, Lu & Xu, 2013). Moreover, previous studies showed that different compositions of aroma precursors and different aroma precursors of the same aroma components are likely to be present in tea leaves of different tea bush varieties (Ogawa et al., 1997).

Compounds identified belonged to several different chemical classes: 29 alcohols, 20 aldehydes, 24 ketones, 4 acids, 5 esters, 19 terpenes and 27 miscellaneous. Alcohols (45.9%), terpenes (20%) and ketones (16.4%) account for 82.3% of volatile compounds identified in oolong tea. *Trans*-nerolidol (45.6% of total alcohols), indole (14.3%), phenylethyl alcohol (4.8%) were three major alcohols identified. Ketones are another major group of compounds present in oolong tea. Among a total 24 identified ketones, jasmine lactones (17% of total ketones), *trans*-β-ionone (14.3%) and sulcatone (11.3%) were the major ketones in oolong tea.

Among the 19 detected terpenes, α-farnesene (35.8% of total esters), geraniol (21.2%) and linalool (12.7%) were the major terpenes compounds. The presence of terpenes is important in flavor of tea (Rawat et al., 2007) and is known to contribute in

floral aroma of oolong tea (Ogawa et al., 1997).The ratios between the volatile compounds are important in perceived aroma quality in tea (Wang et al., 2010). Most of the sesquiterpene aroma compounds in oolong tea are present as diglucosides, such as β-acuminoside, β-primeveroside, and β-vicianoside. In tea leaves, glycosidase can hydrolyse the diglucosides to liberate various aroma compounds in oolong tea and black tea during manufacturing process (Guo, Ogawa, Yamauchi, Watanabe, Usui & Luo, 1996). Wang, Kubota, Kobayashi, & Juan (2001) reported that the high concentrations of the glycosides in oolong tea could be obtained during the manufacture though biosynthesis. During the fermentation process, the tea leaves are injured, leading to increase in enzyme activity on the substrates, which results in higher concentrations of aromatic alcohols (Ma, Qu, Zhang, Qiu, Wang, & Chen, 2014).

Among 128 compounds that were identified with GC-MS using a combination of retention index and mass spectral matching against library standards, *trans*-nerolidol (16.8%), α-farnesene (9.8%), indole (7.4%), geraniol (4.3%), linalool (2.5%), 3-hexen-1-ol benzoate, (2.3%), benzeneacetaldehyde (1.8%), benzyl nitrile (1.7%) and hexanal (1.6%) were found at the highest concentrations in our work. All of these volatile compounds have been previously reported in other studies on oolong tea. Pripdeevech & Machan (2011) used SDE to extract volatile compounds in Jin Xuan (Chin Hsuan) oolong tea grown in Thailand, and they identified 68 volatile compounds. The most dominant volatile compounds in their research were reported as *trans*-nerolidol, *cis*-jasmone, geraniol, hotrienol, linalool, and *trans*-linalyl oxide (pyranoid). However, only *trans*-nerolidol, cis, jasmine, geraniol, linalool were found in our experiment. It is

unclear whether methodological differences or differences in teas grown in Thailand or Taiwan (our tea) contributed to this difference.

Trans-nerolidol was the most concentrated volatile compounds in SDE extraction. Nerolidol is a sesquiterpene present as an essential oil in many plants (AbouLaila, Sivakumar, Yokoyama & Igarashi, 2010) and it is used as a fragrance ingredient (Lapczynski, Bhatia, Letizia & Api, 2008). Many health benefits of nerolidol have been recognized such as anti-ulcer (Klopell et al., 2007), antioxidant (Pacifico et al., 2008), antibacterial properties (Braca, Siciliano, D'Arrigo, & Germano, 2008), antitumor effects (Ryabchenko, Tulupova, Schmidt, Wlcek, Buchbauer, & Jirovetz, 2008). Nerolidol is a volatile compound that gives a flowery aroma (Lapczynski et al., 2008). It exists in variety of teas, especially in oolong tea at a relatively high concentration, can be considered one of the key odorants and is used as an indicator for the high quality oolong tea flavor (Wang et al., 2001; Kai, Yoshida, Kageyama, Saito, Ishigaki & Furukawa, 2008; Pripdeevech & Machan, 2011; Zou et al., 2011). The nerolidol content can be considered an important factor determining the quality of oolong tea (Ma et al., 2014). Nerolidol concentration is low in fresh leaves, but the content is greatly increased during the manufacturing process, mainly in the fermentation stage which reached its highest levels (Ma et al., 2014). The same authors also observed nerolidol content decreased during the fixation, shaping and drying processes (Ma et al., 2014).

Wang et al. (2008) found the total concentration of five flavor volatile compounds, *trans*-2-hexenal, benzaldehyde, methyl-5-hepten-2-one, methyl salicylate and indole, are important to distinguish unfermented teas from fermented teas, while *trans*-2-hexenal and methyl salicylate could be used to classify the semi-fermented from

fully-fermented teas. In our experiment, all of these compounds were identified in oolong tea except methyl-5-hepten-2-one. However, Pripdeevech & Machan, (2011) used *cis*-jasmone, *trans*-nerolidol and indole to differentiate semi-fermented tea from non-fermented tea. This study showed that the content of these components increased dramatically whilst the green fresh aroma of hotrienol (which was not identified in our study) decreased rapidly during fermentation. Whereas, others (Kawakami, Ganguly, Banerjee & Kobayashi, 1995; Wang et al., 2008; Wang et al., 2011; Zhang et al., 2013) reported compounds such as (E)-geraniol, (E)-β-damascenone (not identified in our study), linalool oxide B and benzaldehyde have higher concentrations with increased of degrees of fermentation. Some studies reported that (E,E)-2,4-heptadienal and (Z)-3-hexenol increase with the degrees of fermentation (Wang et al., 2008; Wang et al., 2011). We were unable to detect these last two compounds in our study.

Indole was another volatile compound found at relatively high concentrations in our experiment. In green teas, the content of indole is very low, but its level increases quickly at the beginning of fermentation in oolong tea and then slowly decreases with continuing fermentation. Eventually, there is no detectable amount of indole found in the most heavily fermented oolong teas and all black teas (Wang et al., 2008). It is important to note that fermentation does not make all of the aroma compounds change in the same direction. For instance, contrary to indole, methyl salicylate with a sweet and spicy odor appears only in teas that have at least a medium degree of fermentation, but cannot be detected in the unfermented and lightly fermented teas (Wang et al., 2008).

Wang et al. (2011) reported that perceived aroma score positively correlated with concentration of benzyl alcohol, benzeneacetaldehyde, linalool, phenylethyl alcohol,

linalool oxide, indole, *cis*-jasmone, nerolidol, and methyl jasmonate. In addition, they found that the total quality score positively correlated with concentration of benzyl alcohol, benzeneacetaldehyde, geraniol, indole and toluene, but negatively correlated with the concentration of (E, E)-2, 4-heptadienal. All of these compounds except toluene were identified in our experiment. Zhang et al. (2013) also found that (E)-β-damascenone (rose-like flavor) and benzaldehyde (almond, sweet) play important roles in the aroma of oolong tea. Zhang et al. (2013) and others (Wang et al., 2008; Wang et al., 2011) indicated that a few compounds, including jasmine lactone and α-farnesene, have high correlations with the aroma quality of oolong tea. Jasmine lactone with a floral and fruity odor is noticeably higher in oolong tea because of the special manufacturing process (Wang et al., 2001). α-Farnesene is also reported as the main oolong tea flavor (Kawakami et al., 1995), and has a higher content in this variety of tea (Wang et al., 2011).

A total of 59 volatile compounds were identified using SPME. Oolong tea volatiles were divided into 10 alcohols, 12 aldehydes, 9 ketones, 7 acids, 8 terpenes and an ester. Among 13 alcohols, 2-ethyl-1-hexanol (55.0% of total alcohols) was the major alcohol. Among 8 detected terpenes, geraniol (21.0% of total terpenes) and linalool (20.0%) were the major terpenes. Among the volatile compounds that were extracted with SPME, indole (11.1%), 2-ethyl-1-hexanol (9.0%), an unknown (5.7%), butyrolactone (5.3%), 5-(hexadecyloxy)-2-pentadecyl-*cis*-1,3-dioxane (4.1%), geraniol (2.6%), linalool (2.4%), and α-terpinyl acetate (2.3%) had the highest peak areas.

In a study by Lin, Zhang, Pan, Xu, Luo and Wang (2013b), the main compounds extracted by SPME in the five oolong varieties were α-farnesene, nerolidol and indole.

They also reported that (E)-β-ocimene, 2-ethenyl-1, 1-dimethyl-3-methylidene-cyclohexane, linalool, benzeneacetaldehyde, benzene ethanol and benzyl cyanide were abundant. In our study, benzene ethanol and benzyl cyanide were not detected by either of the SPME or SDE techniques and 2- ethenyl-1,1-dimethyl-3-methylidene-cyclohexane was only detected with SDE.

When comparing the two extraction methods, the number of identified volatile compounds was significantly higher in SDE, but 38 compounds were identified in both methods. The number of identified volatile compounds in each of the six chemical categories was also higher with SDE. SPME was able to extract only eight terpenes which compare to 19 in SDE. The eight terpenes that were identified using SPME were also identified with SDE. Terpenes are important chemical class that significantly contributes to aroma of teas. The compounds with the highest peak areas were not the same in both extraction techniques, but the volatile compounds with the highest peak areas in SDE were also identified in SPME. However, this situation was not the same vice versa. 2-Ethyl-1-hexanol (9.0%), 2, unknown (5.7%), butyrolactone (5.3%), 5-(hexadecyloxy)-2-pentadecyl-*cis*-1,3-dioxane (4.1%) were not detected using SDE.

Previous studies have also indicated that the volatile extracts of tea differ greatly between different extraction methods (Kawakami et al., 1995; Zhu, Li & He, 2008). For example, it was shown that SPME using a PDMS/DVB fiber coating has good selectivity for hydrocarbon compounds (Lin, Dai, Guo, Xu, and Wang, 2013a). On the other hand, with SDE, the semi-volatile compounds have higher recovery than other compounds (Zhang et al., 2013). The compounds with high volatility may be lost in the processing steps, and the compounds with low volatility may be difficult to be extracted using SDE.

Although, recovery results were not equally satisfactory for all compounds, the good repeatability illustrates that SDE is suitable for extraction of volatile components of tea (Zhang et al., 2013). Previously, Garcia-Esteban, Ansorena, Astiasaran, Martin & Ruiz (2004) reported that SPME was more efficient for extracting highly-volatile, low-molecular weight compounds, while SDE was more appropriate for extracting compounds with low volatility that could not be extracted by SPME. Madruga, Elmore, Dodson & Mottram (2009) suggested that none of these techniques should be rated as superior to another and both extraction techniques could be regarded as techniques that provide complementary information.

3.3.3 GC-O Analysis

The 45 aroma active volatiles that were tentatively identified based on the combination of LRI and odor descriptors in oolong tea from SDE extraction are listed in Table 3.2. From these 45, nine were confirmed with GC-MS. With SPME, results showed 41 of these compounds have aroma activity based on their LRI and odor descriptors, but only six of them were confirmed with GC-MS (Table 3.3). Seventeen aroma active compounds were common in both extraction techniques from GC-O analysis: ethyl acetate, methylbutanal, methyl butanoate, pentanal, (E) 2-hexenal, butanediol, furfural, isoamyl acetate, dimethyl pyrazine, dimethylthiazole, 5-methylfurfural, 3,5-octadien-2-one, ethylmethylpyrazine, linalool oxide, (+)-cis-rose oxide, 4-ethyl-6-hepten-3-one and one compound that was not identified.

In our analysis, we identified *trans*-2-hexenol (green/grass aroma). This compound is a lipid degradation product and can be found in oolong tea with inferior quality. Usually, a higher amount of *trans*-2-hexenol is found in non-fermented teas

(0.22-0.27%) whereas other teas produced by semi-fermentation tea processing showed significantly lower amounts (0.04–0.08%) (Wang et al., 2010; Pripdeevech & Machan, 2011). We also detected 2,5-dimethylpyrazine, which is a thermally generated compound (Wang et al., 2010). This compound exhibits toasted flavor, and is known as a reaction product from amino acids and sugars (Kato & Shibamoto, 2001). Jin Xuan oolong is well known for its milky aroma. A buttery characteristic of diacetyl (2, 3-butanedione) that was detected with the GC-O analysis might contribute to the perception of milky aroma in Jin Xuan oolong.

Panelists scored the intensity of each aroma on a 5 point scale where 1 was the lowest aroma intensity score and 5 was the highest score. The identified compounds were grouped into six categories based on their aroma description: fruit, sweet, floral, nut/must, green and smoke/bad smell. The aroma intensities of compounds for each of the six groups and two extraction methods are shown in Figure 3.3. The most important feature of this figure is the difference in intensity of nut/must and green aromas for the two extractions. Panelists did not detect any green aroma with the SPME extract and they detected more nutty aroma in the SDE extract compared to SPME. Generally, fermentation processing can cause the loss of grassy or green flavors, whereas formation of the fruity/floral and other fermented characters increases (Wang et al., 2008). A study on GC-O analysis of extruded potato snacks using SDE and SPME showed that SPME extraction was not appropriate for the isolation of high molecular compounds or for those with a strong affinity to the matrix, such as ethyldimethylpyrazine, which similarly was not detected in SPME GC-O analysis in our study (Majcher & Jelen´, 2009). On the other hand, SDE could extract all of the active aroma components, including those with high

and low molecular mass and low volatility; however, some identified aroma compounds with SPME cannot be detected in distillation extraction techniques due to the presence of solvent (Majcher & Jelen´, 2009).

The most intense odorants in oolong tea from SDE extraction were isoamylacetate (fruity), dihydromethylcyclopentapyrazine (nutty) and γ-cadinene (smoke, roasted meat) and an unidentified compound with a nutty aroma. However, the most intense odorant in SPME were pentanone (fruity), (Z)-2-penten-1-ol (smoky, plastic), 3, 5-octadienone (fat, meaty), (-)-*cis*-rose oxide (sweet) and an unidentified compound with earthy aroma. 3, 5-Octadien-2-one has previously reported in several studies as an important aroma active compound in oolong tea (Pripdeevech & Machan, 2011, Wang et al., 2008, Zhang et al., 2013).

3.4 Conclusions

In this study, extraction with diethyl ether for 40 min resulted in higher ($P<0.05$) concentration of volatile compounds compared to other combination of solvents and extraction times. A total 200 volatile compounds found with both method of extraction using GC-MS and GC-O. Many of the identified volatile compounds of oolong tea were also reported in previous studies. Overall, more volatiles were extracted using SDE compared to SPME; however, each method was able to identify compounds that the other could not. Some of these differences might also results of different exposure time of tea leaves to the boiling water which was much higher for SDE. Overal, the identified volatile compounds from SPME represent the tea beverage and the ones that identified

from SDE represent the tea leaves and tea beverage. We suggest that the data from both methods will be used together for analysis of flavor compounds in food products.

Table 3.1 Identified oolong tea volatiles with method of identification and supporting data and literature comparisons.

#	Compounds	LRI	SDE	SPME	Previously reported
1	n.i.	566-606*		O	
2	n.i.	534-559*		O	
3	2-Methylpropanal	550*	O		4
4	Diacetyl	596*	O		
5	Butanenitrile, 3-methyl-	626	MS		
	3-Penten-2-one, (E)-	633	MS		
6	Ethyl acetate	634*	O	O	
7	n.i.	643-651		O	
8	2-Pentenal, (E)-	648	MS		
9	Methylbutanal	653*	O	O	2
10	Isobutanol	653		O	
11	1-Pentanol	660	MS		4
12	2-Penten-1-ol, (Z)-	664	MS		4
13	n.i.	679-693*	O	O	
14	Pentanone	696*	O		
15	Hexanal	698	MS, O		2, 4, 8
16	2-Hexenal	700		MS	
17	α,γ-Dimethylallyl alcohol	704*	O		
18	1,2-Propanediol, 3-methoxy-	713	MS		
19	Ethyl propionate	715*		O	
20	Methyl butanoate	723*	O	O	
21	Diethyl acetal	727*		O	
22	3-Methyl-1-butanol	732*	O		4
23	Pentanal	738*	O	O	2, 3, 4
24	2-Pentanone, 4-hydroxy-4-methyl-	740	MS,O		
25	Methyl-2-butenal	749*		O	
26	2-Hexenal, (E)-	749	MS, O	MS, O	3, 8
27	3-Hexen-1-ol, (Z)-	753	MS	MS	
28	Fucoserratene	761*	O		
29	Pentanol	763*		O	
30	Hexane-1-ol	765	MS		
31	Isobutyl acetate	774*		O	
32	(Z)-2-Penten-1-ol	776-881*		O	
33	4-Methyl-3-penten-2-one	790**		O	
34	2-Heptanone	790	MS		1,2
35	Ethyl butyrate	796*		O	
36	Butanediol	797*	O	O	
37	4-Heptenal, (E)-	799	MS		2
38	Heptanal	801	MS	MS	2, 3, 4
39	Oxime-, methoxy-phenyl-	804		MS	
40	Propyl propanoate	810*		O	
41	Butyrolactone	812		MS	
42	1,7-Octadiene, 3,6-dimethylene-	819		MS	
43	Furfural	829*	O	O	1,2, 8
44	Ethoxypropanol	832*		O	
45	Ethyl methylbutyrate	840*		O	
46	Isopropyl butanoate	846*		O	
47	2-Heptenal, (E)-	856	MS		
48	Benzaldehyde	859	MS, O	MS	1, 2, 3, 6, 7, 8
49	2,4-Dimethylbenzophenone			MS	
50	Spiro[2.4]heptan-4-one	861	MS		
51	1-Heptanol	871	MS, O		
52	2-Hexene,3,5,5-trimethyl-	874	MS		
53	1S-β-pinene	875	MS		
54	Isoamyl acetate	876*	O	O	
55	2-Hexenol	880*	O		1
56	1-Octen-3-ol	880	MS		
57	2,3-Octanedione	885	MS		
58	Sulcatone	887	MS	MS	2, 3, 4, 7

59	2-Methylbutyl acetate	890-898*	O		
60	Furan, 2-pentyl-	892	MS		4
61	2,4-Heptadienal, (E,E)-	896	MS, O	MS	2, 3
62	n.i.	901-906*	O		
63	Dimethyl pyrazine	904*	O	O	1,2
64	Octanal	904	MS		
65	3-Hexen-1-ol, acetate, (Z)-	908	MS		
66	Dimethylthiazole	912-926*	O	O	
67	2-Chlorocyclohexanol	930	MS		
68	Mesitylene	932	MS		
69	o-Cymene	935	MS		
70	α-Thujene	936-942*	O		
71	1-Hexanol, 2-ethyl-	942		MS	
72	D-Limonene	942	MS	MS	
73	Benzyl alcohol	948	MS		1,2 , 3,
74	3,5-Octadien-2-ol	956	MS		
75	Ethyl isohexanoate	959	O		
76	Benzeneacetaldehyde, diethyl acetal	961	MS	MS	1, 3, 7, 8
77	1H-Pyrrole-2-carboxaldehyde, 1-ethyl-	967	MS		
78	β-Ocimene	970	MS	MS, O	6, 7
79	5-Methylfurfural	977*	O	O	6, 8
80	cis-3-Hexenyl isovalerate	977	MS		
81	2-Octenal, (E)-	982	MS		
82	trans-2-Pinanol	982	MS		
83	Isophorone	985	MS		1, 2, 7
84	Octadien-3-ol	993	MS,O		
85	3,5-Octadien-2-one	997	MS, O	MS, O	1, 2, 8
86	Ethylmethyl pyrazine	1000*	O	O	2, 6
87	2-Nonyne	1000	MS		
88	Octanone	1005		O	
89	2,4-Heptadienal	1005*	O		6, 8
90	α-Methyl-α- 4-methyl-3-Pentenyl]oxiranemethanol	1006		MS	
91	Linalool oxide	1009	MS		1, 2, 3, 8
92	Dimethylheptenal	1053*		O	
93	Linalool oxide(furanoid**)**	1063	MS, O	O	1, 2, 3, 7, 8
94	3,5-Octadien-2-one	1076	MS		
95	2-Pentylthiophene	1080-1096*	O		
96	1,2-Heptanediol	1082	MS		
97	Ethyldimethylpyrazine	1096*	O		1, 2
98	R-Linalool	1101	MS	MS, O	1, 2, 3, 4, 6, 7
99	3,4-Dimethylcyclohexanol	1105	MS	MS	
100	Nonanal			MS	
101	(+)-cis-Rose oxide	1109*	O	O	
102	Phenylethyl Alcohol	1114	MS	MS	1, 3, 5, 6, 7, 8
103	4-Ethyl-6-hepten-3-one	1115*	O	O	
104	3,7-Nonadien-2-ol, 4,8-dimethyl-	1117		MS	
105	n.i.	1118		MS	
106	Cyclohexane, 2-ethenyl-1,1-dimethyl-3-methylene-	1118	MS		7
107	1,2-Dihydro-8-hydroxylinalool	1130	MS		
108	2-Piperidinone, N-[4-bromo-n-butyl]-	1133	MS		
109	Benzyl nitrile	1139	MS	MS	1, 3, 8
110	Lilac aldehyde B	1145	MS		
111	Lilac aldehyde D	1151	MS		
112	(R,S)-5-Ethyl-6-methyl-3E-hepten-2-one	1148	MS		
113	Acetic acid, 2-ethylhexyl ester	1152	MS		
114	Cyclohexanone, 2-(3-oxobutyl)-	1157	MS		
115	2-Nonenal, (E)-	1161	MS		
116	3-Isopropylidene-5-methyl-hex-4-en-2-one	1165	MS		
117	2,2,7,7-Tetramethyl-4,5-dimethyleneoctane	1171	MS		
118	2H-Pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl-	1171	MS		
119	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-	1180	MS		
120	Naphthalene	1185	MS		
121	3,7-Octadiene-2,6-diol, 2,6-dimethyl-	1190	MS		8
122	α-Terpineol	1193	MS	MS	1

123	Methyl salicylate	1196	MS	MS	1, 2, 3, 6, 8
124	Ethyl octenoate	1196-1205*	O		
125	Decanal	1203	MS	MS	
126	2,4-Nonadienal, (E,E)-	1207	MS		6
127	Benzofuran, 2,3-dihydro-	1210	MS		8
128	β-Cyclocitral (1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-)	1212	MS	MS	2, 6
129	Nerol	1216	MS	MS, O	
130	cis-3-Hexenyl-α-methylbutyrate	1216	MS	MS	
131	n-Valeric acid cis-3-hexenyl ester	1218	MS		
132	(Z)-Piperitol	1222	O		
133	Citral (2,6-Octadienal, 3,7-dimethyl-, (Z)-)	1223	MS	MS, O	2
134	D-Carvone	1224	MS		
135	Acetic acid, 2-phenylethyl ester	1229	MS	MS	
136	Geraniol	1229	MS	MS	1, 2, 3, 6
137	2-Decenal	1231	MS		6
138	1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	1232	MS	MS	
149	Nonanoic acid	1235		MS	
140	Cyclopentane-1-carboxylic acid, 2-hydroxy-1,2,3-trimethyl-, ethyl ester	1239		MS	
141	Cholestan-22(26)-epoxy-3,16-dione	1240	MS		
142	Dihydromethylcyclopentapyrazine	1243*	O		
143	trans-2-(2-Pentenyl)furan	1240	MS		
144	Ethyl phenylacetate	1247*		O	
144	Benzothiazole	1248*	O		
146	Indole	1250	MS	MS	1, 2, 3, 7, 8
147	Formic acid, (2-methylphenyl)methyl ester	1252	MS	MS	
148	Cyclopentanepropanoic acid, 3-oxo-, ethyl ester	1253		MS	
149	1-Oxaspiro[4.5]dec-6-ene, 2,6,10,10-tetramethyl-	1254	MS	MS	
150	Methylnonanedione	1258*		O	1
151	2,4-Decadienal, (E,E)-	1260	MS	MS	8
152	Linalyl acetate	1271*	O		
153	2,4,4-Trimethyl-3-(3-methylbutyl)cyclohex-2-enone	1274		MS	
154	α-Terpinyl acetate	1278	MS	MS	
155	Safrole	1279*	O		
156	Eugenol	1281	MS	MS	1, 6
157	2-Undecenal	1283	MS		
158	2-Octenal, 2-butyl-	1289	MS		
159	Decanoic acid, ethyl ester	1292	MS	MS	
160	Tetradecane	1300		MS	
161	cis-Jasmone	1401	MS		
162	2-Pentadecanone, 6,10,14-trimethyl-	1405	MS		
163	1,3-Dioxane, 5-(hexadecyloxy)-2-pentadecyl-, cis-	1409		MS	
164	Dodecanal	1409	MS, O		
165	2-Buten-1-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	1419	MS		1
166	cis-Thujopsene	1420	MS		
167	Caryophyllene	1427	MS		7
168	α-Ionone	1432	MS		1, 2
169	Coumarin	1441	MS		1, 5
170	β-Phenylethyl butyrate	1444	MS		
171	2-Methyltetracosane/Tetracontane, 3,5,24-trimethyl	1450	MS		5
172	(E)-Geranyl acetone	1455	MS	MS	
173	cis- β-Farnesene	1460	MS		7
174	2(3H)-Furanone, 5-heptyldihydro-	1470	MS		1, 8
175	1-Dodecanol	1475	MS	MS	
176	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	1488	MS		
177	Bornyl butyrate	1491	O		
178	trans- β-Ionone	1491	MS	MS	1, 2, 3, 6, 8
179	Isopiperitone	1496*	O		
180	Jasmin lactone	1497	MS	MS	8
181	Bicyclo[2.2.1]heptane-2,5-diol, 1,7,7-trimethyl-, (2-endo,5-exo)-	1511		MS	
182	α-Farnesene	1513	MS	MS	7, 8
183	Butylated Hydroxytoluene	1519	MS		1

184	n.i.	1524	MS		
185	1,8(2H,5H)-Naphthalenedione, hexahydro-8a-methyl-, cis-	1531	MS		
186	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	1538	MS		1, 5
187	γ-Cadinene	1540-1541*	O		2
188	trans-Nerolidol	1572	MS	MS	1, 2, 3, 6, 7, 8
189	3-Hexen-1-ol, benzoate, (Z)-	1578	MS	MS	8, 9
190	Tetradecanal	1614		MS	
191	Farnesene epoxide, E-	1623	MS		
192	cis-3-Hexenyl phenyl acetate	1638	MS		
193	Methyl jasmonate	1656	MS		1, 3, 8
194	2H-1b,4-Ethanopentaleno[1,2-b]oxirene, hexahydro-, (1a- α-1b- β-4, β,4a- α,5a-α)-	1661	MS		
195	α-Cadinol	1665	MS		1
196	cis-3-Hexenyl salicylate	1676			
197	13-Heptadecyn-1-ol	1702	MS		
198	Pentadecane, 2,6,10,14-tetramethyl-	1707		MS	
199	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-	1726	MS		
200	Benzyl Benzoate	1773	MS		1, 6, 8

1= (Pripdeevech & Machan, 2011), 2= (Wang et al., 2008), 3= (Wang et al., 2010), 4= (Kim et al., 2011), 5= (Chen et al., 2013), 6= (Fanaro et al., 2011), 7= (Lin et al., 2013), 8= (Zhang et al., 2013), 9= (Ma et al., 2014)
*= Confirmed LRI from GCO analysis

Table 3.2 Oolong tea aroma active compounds using time-intensity GC-O with SDE extraction.

#	Compound	LRI	Confirmed LRI [a]	Aroma Description	Intensity [b]
1	2-Methylpropanal	550	550	Fruity, sweet	3
2	Diacetyl	596	593	Butter	2
3	Ethyl acetate	634	634	Fruity, pineapple	3
4	Methylbutanal	653	641	Almond, chocolate	2
5	n.i.[c]	679-693	-	Nutty, cookie	2
6	**Pentanone**	696	711	Fruity, ether	2
7	α,γ-Dimethylallyl alcohol	704	712	Green	2
8	Methyl butanoate	723	723	Real ripe fruit, ether	2
9	3-Methyl-1-butanol	732	736	Smoky, burnt	2
10	Pentanal	738	732	Nutty, almond, pungent	2
11	Fucoserratene	761	760	Green, plastic	2
12	**Hexanal**	796	801	Green, grass	2
13	Butanediol	797	806	Fruity, apple	1
14	Furfural	829	828	Nutty, almond, sweet	3
15	n.i.	838	-	Smoky, earthy, fishy	4
16	Isoamyl acetate	876	876	Fruity, sweet, banana	4
17	**2-Hexenol**	880	880	Green, fruity	2
18	2-Methylbutyl acetate	890-898	880	Fruity, sweet	1
19	n.i.	901-906		Waxy, nutty	4
20	Dimethyl pyrazine	904	905	Nutty, roasted nut, musty	3
21	Dimethylthiazole	912-926	928	Smoky	3
22	α-Thujene	936-942	938	Grass, green, floral, herb	2
23	**Benzaldehyde**	964	960	Nutty, almond	2
24	**Heptanol**	971	962	Cooked vegetable, green	2
25	Ethyl isohexanoate	959	968	Fruity	1
26	5-Methylfurfural	977	978	Nutty, almond, caramel	2
27	**1,5-Octadien-3-ol**	992	988	Earthy	2
28	Ethylmethyl pyrazine	1000	993	Fruity, sweet	3
29	**2,4-Heptadienal**	1005	1011	Nutty, musty	1
30	3,5-Octadien-2-one	1040	1040	Nutty	1
31	**(Z)-Linalool oxide**	1076	1070	Flower, sweet, nutty	2
32	Ethyldimethylpyrazine	1096	1084	Popcorn	3
33	2-Pentylthiophene	1080-1096	1089	Sweet	1
34	(+)-cis-Rose oxide	1109	1109	Green flower	3
35	4-Ethyl-6-hepten-3-one	1115	1120	Meaty, fish	2
36	Ethyl octenoate	1196-1205	1202	Musty, pungent	3
37	(Z)-Piperitol	1222	1220	Green, herb	1
38	Benzothiazole	1248	1240	Smoky, gasoline	2
39	Dihydro methyl cyclopentapyrazine	1243-1245	1248	Roast,nutty	4
40	Linalyl acetate	1271	1261	Sweet	3
41	Safrole	1279	1280	Sweet, spice	2
42	**Dodecanal**	1411	1409	Citrus, fruity	1
43	Bornyl butyrate	1491	1490	Celery, green	1
44	Isopiperitone	1496	1493	Fruity, sweet	1
45	γ-Cadinene	1540-1541	1540	Smoky	4

Bold Compounds were also detected with GC-MS

[a] LRI values confirmed with databases Flavornet and Pherobase to identify the compounds based upon standardized retention and aroma.

[b] The average aroma intensity score by panelist on a scale of 5 where 1= low intensity and 5= high intensity.

[c] Not identified compound

Table 3.3 Oolong tea aroma active compounds using time-intensity GC-O with SPME extraction

#	Compound	LRI	Confirmed LRI [a]	Aroma Description	Intensity [b]
1	n.i. [c]	534-559	-	Earthy, smoky	4
2	n.i.	566-606	-	Fruity, nutty	1
3	Ethyl acetate	640	628	Fruity, pineapple	1
4	n.i.	643-651	-	Fruity	2
5	Methylbutanal	650	641	Nutty, cocoa, almond	2
6	Isobutanol	653	647	fruit	2
7	Pentanone	698	711	Fruit juicy gum	4
8	Ethyl propionate	715	713	fruity	1
9	Methyl butanoate	723	723	Ether, fruit, sweet	2
10	Diethyl acetal	727	734	Fruity, cream	2
11	Pentanal	730	735	Nutty, almond	1
12	Methyl-2-butenal	749	753	Fruity, green	3
13	Pentanol	763	759	Fruity, sweet	1
14	Isobutyl acetate	774	776	Fruit, apple, banana	3
15	(Z)-2-Penten-1-ol	776-781	767	Smoky, plastic	4
16	4-Methyl-3-penten-2-one	790	798	Fruity, sweet	2
17	Ethyl butyrate	796	804	Sweet, apple	1
18	Butanediol	800	806	Fruity	2
19	Propyl propanoate	810	812	Fruity, sweet, pineapple	3
20	Furfural	822	829	Fruity, sweet	3
21	Ethoxypropanol	832	833	Sweet	1
22	Ethyl methylbutyrate	840	846	Fruity, apple	3
23	Isopropyl butanoate	846	846	Sweet, fruity	3
24	**2-Hexenal**	861	854	Apple, sweet	2
25	Isoamyl acetate	873	876	Fruity, banana	1
26	Dimethyl pyrazine	895	892	Nutty, peanut butter, cocoa, meat	1
27	Dimethyl pyrazine	906	905	Nutty, musty, cocoa, roasted nut,	1
28	Dimethylthiazole	931	928	Smoky, roast	1
29	5-Methylfurfural	982	978	Vanilla, almond	1
30	Ethylmethyl pyrazine	992	993	Fruity	1
31	Octanone	1005	999	Bad smell, smoky, gasoline	4
32	Dimethylheptenal	1053	1056	Fruity, green	1
33	**α-Ocimene**	1058	1056	Fruity	1
34	**(Z)-Linalool oxide**	1064	1070	Cherry, floral	2
35	**3,5-Octadienone**	1098	1095	Meaty, fat, smoky	4
36	(-)-cis-Rose oxide	1114	1117	Juicy, fruity gum, sweet	4
37	4-Ethyl-6-hepten-3-one	1115	1120	fish	2
38	**Nerol**	1237	1233	sweet	1
39	Ethyl phenylacetate	1247	1252	Floral, fruit, sweet	2
40	**Citral**	1253	1254	Fruity, lemon	1
41	Methylnonanedione	1258	1253	Apple banana	2

Bold Compounds were also detected with GC-MS

[a] LRI values confirmed with databases Flavornet and Pherobase to identify the compounds based upon standardized retention and aroma.

[b] The average aroma intensity scored by panelist on a scale of 5 where 1= low intensity and 5= high intensity.

[c] Not identified compound

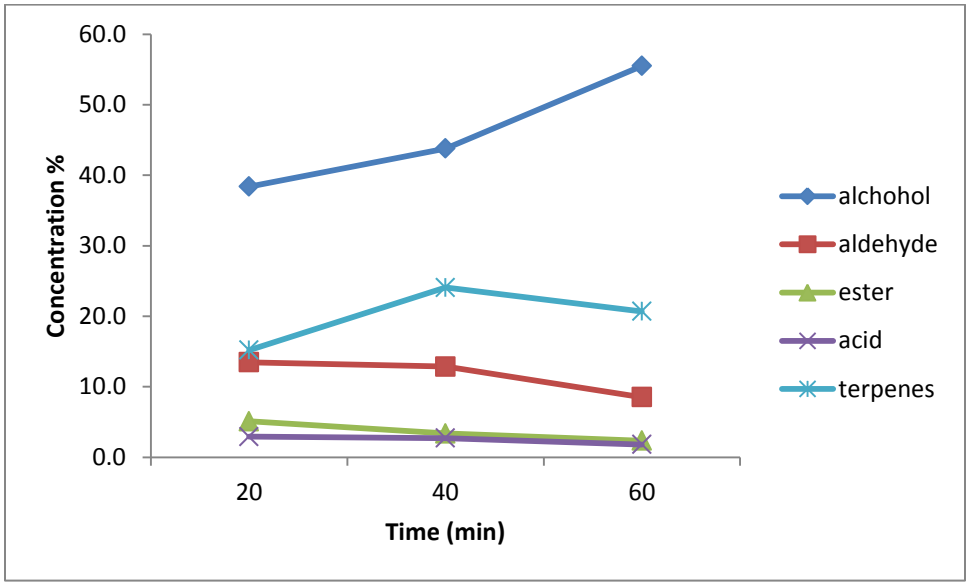

Figure 3.1 Effects of extraction time on chemical composition of identified volatiles using GC-MS.

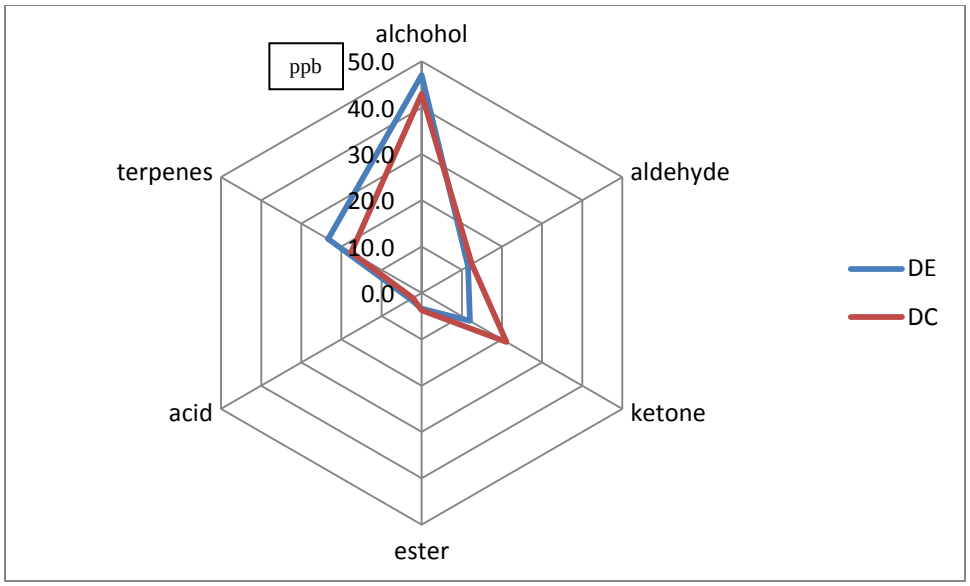

Figure 3.2 Comparison of chemical composition of extracted volatile compounds in diethyl ether (DE) and dichloromethane (DC) using GC-MS.

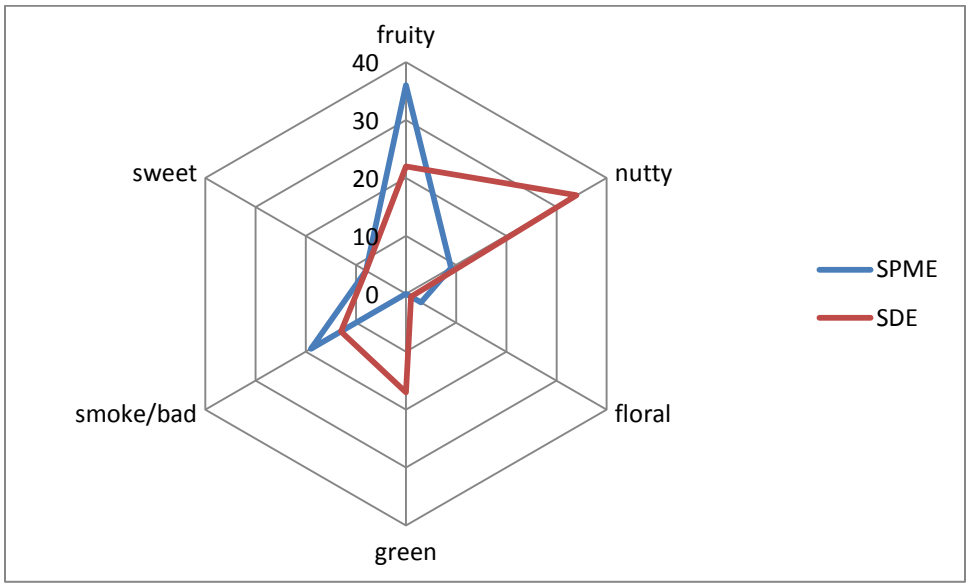

Figure 3.3 Radargram of aroma profile of oolong tea using two extraction methods obtained from grouping of identified compounds using GC-O with similar aroma characteristics.

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CHAPTER IV

Changes in Flavor Volatile Compositions of Oolong Tea after Panning in Tea Processing

Abstract

Panning is a processing step used in manufacturing of some varieties of oolong tea. There is limited information available on effects of panning on oolong tea flavors. The goal of this study was to determine effects of panning on flavor volatile compositions of oolong using Gas Chromatography- Mass Spectrometry (GC-MS) and Gas Chromatography- Olfactrometry (GC-O). SDE and SPME techniques were applied for extraction of volatiles in panned and unpanned teas. From this study, a total of 190 volatiles were identified from SDE and SPME extractions using GC-MS and GC-O. There were no significant differences ($P>0.05$) in aldehydes and terpenes contents of unpanned and panned tea; however, alcohols, ketones, acids and esters contents were significantly reduced by panning. Among major 12 volatiles used for identification and quality assessment of oolong tea in previous studies, *trans* nerolidol, 2- hexenal, benzaldehyde, indole, gernaiol, and benzenacetaldehyde contents have significantly decreased ($P<0.05$) by panning; however, panning increased ($P<0.05$) contents of linalool oxide, *cis* jasmone, methyl salicylate in oolong tea. The GC-O study also showed the increase of aroma active compounds with sweet descriptions and decrease of aroma active compounds with fruity and smoky descriptions by panning. In overall, panning significantly changes the volatile compositions of the tea and created new aroma active compounds. Results from this study can be used in quality assessment of panned oolong tea.

Keywords: Oolong tea, Flavor analysis, Panning, GC-MS, GC-O.

4.1 Introduction

Oolong tea is manufactured predominantly in southeast China and Taiwan (Lee, Dou, Chen, Lin, Lee & Tzen, 2008). Less than 2% of tea manufactured in the world is semi-fermented oolong tea (Hara, Luo, Wickremashinghe & Yamanishi, 1995); however, due to the complex processing steps and the limited supply, oolong teas usually have a higher unit price than green and black teas in the international tea market. Current increases in consumption of oolong tea in the world might be results of the recent studies on tea polyphenols and their health benefits, and also unique taste and aroma of this variety of tea.

Oolong tea categorized as a semi-fermented tea. The tea fermentation refers to natural browning reactions induced by oxidative enzymes in the cells of tea leaves. This process is mainly the oxidative polymerization of catechins and endogenous polyphenol oxidase and peroxidase catalyzed this reaction (Chaturvedula, & Prakash, 2011). Oolong tea is generally fermented from 20% to 60% in order to create a taste and color somewhere between green and black teas. Fermentation is responsible for creation of many flavor compounds. During fermentation process, the tea leaves are injured and consequently, it causes increase in enzyme activity on the substrate, and creation of more aromatic alcohols (Ma, Qu, Zhang, Qiu, Wang & Chen, 2014). Wang, Lee, Chung, Baik, So & Park (2008) found that fermentation can cause the loss of grassy or green flavors, whereas formation of the fruity/ floral and obtaining black tea's sweet and bold flavor. There are no standard recipes or procedures on how to manufacture oolong tea. The processing and the level of oxidation are decided by each tea garden or tea master.

Panning is a processing step in the manufacturing of some varieties of commercially available oolong tea that performed after fermentation step. Panning is the exposure of tea leaves to heat in order to stop fermentation and destroy enzymes responsible for fermentation (Zhen, 2003, Info Taiwan, 2014). The purpose of panning is inactivating the enzymes by high temperature to inhibit further fermentation and develop a unique flavor in oolong tea (Hui, Meunier-Goddik, Josephsen, Nip & Stanfield., 2003). During this process, tea leaves lose high amount of moistures and thus are softer making the rolling into string shapes and dehydration easier (Hui et al., 2003). Panning can be done using rotary pan or panning machine, convection oven, or via pan-frying. The exact temperature and time depends on various teas and determined by tea masters. The panning period also depends on the variety of tea leaf and loading quantity. Although during this process, most of the enzymes are inactivated and not much oxidation of polyphenols takes place after this process, still some flavor forming enzymes are not deactivated (Hui et al., 2003). Panning is known to eliminate grassy odor while leaving a nutty smell and taste. Panning also prevents tea leaves from breaking before they are rolled and the result is soft and flexible leaf texture with a strong pleasant aroma (Info Taiwan, 2014). However, there is limited information available on panning effects on volatile compounds of oolong tea and the compounds responsible for the aroma of panned tea are still in need of clarification.

Flavor analysis of oolong tea is important in variety authentication and quality assessment of oolong tea. Some of fine oolong varieties are sold at a premium price in the market compared to other low-grade varieties. Some oolong varieties are very similar in appearances and flavor that accurate identification and differentiation is only possible for

tea experts or experienced tea tasters (Zhang, Zeng, Zhao, Kong, Lu & Xu, 2013). Thus, there is significant interest in developing accurate chemical methods for quality assessment and identification of oolong tea varieties. In our previous study, we found that composition of the volatile compounds extracted by the two commonly used extraction methods SDE and SPME differed considerably and it is necessary that two methods used together for effective volatile analysis (Sheibani, Duncan, Kuhn, Dietrich & O'Keefe, 2014).

Non-volatile components are generally responsible for the taste, while volatile components give the aroma (Rawat, Gulati, Kiran Babu, Acharya, & Singh, 2007). The unusual taste of oolong tea infusion depends on the various fermentation degrees. Nonaka, Kawahara & Nishioka (1983) (as cited in Chaturvedula & Prakash, 2011) reported the fruity and sweet taste of oolong tea infusion are the integrated taste of non-oxidized catechins, thearubigins, some secondary polyphenolic compounds, caffeine, free amino acids and related sugars, and volatile compounds. Compare to green tea, the astringency of oolong tea is lower and the sweetness taste is stronger). Volatile flavor compounds of tea are mostly compose of non- terpenoids or terpenoids, which responsible for sweet flowery aroma to tea (Rawat et al., 2007). Previous studies showed volatile compounds such as *trans*-nerolidol, *trans*-2-hexenal, benzaldehyde, methyl-5-hepten-2-one, methyl salicylate, indole(Wang et al., 2008; Pripdeevech & Machan, 2011), *cis*-jasmone (Pripdeevech & Machan, 2011), (E)-geraniol, (E)-β-damascenone, linalool oxide B, benzaldehyde (Kawakami, Ganguly, Banerjee & Kobayashi, 1995; Wang et al., 2008; Wang et al., 2011; Zhang et al., 2013), (E,E)-2,4-heptadienal and (Z)-

3-Hexenol (Wang et al., 2008; Wang et al., 2011) are the key odorant and indicator for the high quality of oolong tea.

The objectives of this study were to investigate effects of panning on flavor volatile compositions of oolong tea and to determine changes in aroma active compounds of panned compared to unpanned oolong tea using Gas Chromatography- Mass Spectrometry (GC-MS) and Gas Chromatography- Olfactrometry (GC-O).

4.2 Materials and Methods

4.2.1 Panning Process

Three batches of unpanned Jin Xuan (*Chin-Hsuan*, or Zhu Shan) oolong tea samples were purchased from Tea of Life® Health Inc. in Rosedale, NY before the experiment and stored at room temperature. To pan the tea leaves, 680 grams of oolong tea was placed on a metal baking dish. Then, the dish was heated/panned in a convection oven at 120 ^{o}C for 6 hrs. The method and selected condition for the panning process was based on our preliminary study and literatures. After this period, the tea leaves were cooled down to room temperature. The panning and flavor analysis were performed for the three batches separately.

4.2.2 Volatile Extraction by SDE Method

Previously, we obtained optimal condition for SDE technique including solvent and extraction time for flavor analysis of tea and we applied the same extraction condition in this study (Sheibani et al., 2014). The SDE apparatus, Likens-Nickerson apparatus, was used. Tea leaves (50 g) were placed in a 1 L round bottom flask containing 400 ml of boiling distilled water. 100 ml of HPLC grade diethyl ether (Sigma-

Aldrich Co., St. Louis, MO) with 0.5 mL of 100 ppm ethyl decanoate (internal standard) (Sigma-Aldrich Co., St. Louis, MO) were placed into a 250 ml extraction flask. Two electric heating were used to maintain boiling the tea and solvents. In SDE apparatus, the volatiles were steam-distilled and extracted into diethyl ether for 40min. After extraction, the extracts were dried over anhydrous sodium (Fisher Scientific, Pittsburg, PA) and filtered. Then, the extract was concentrated to 2 ml in a vacuum rotary evaporator and nitrogen gas. The concentrates were injected into Gas Chromatography- Mass spectrometry (GC-MS) and Gas Flame Ionization Detector- Olfactometry (GC- FID/O) for volatile analysis.

4.2.3 Volatile extraction by SPME for GC-MS analysis

Four grams of tea leaves were placed in 200 mL of boiling distilled water (98 ^{o}C) and brewed for 5 min. Then, 5 mL of the filtered tea infusion and 1g of NaCl were placed into 10 mL headspace vials with Teflon-lined silicon septa (Chromacol, Fisher Scientific). SPME method and injection to GC-MS was conducted using an AOC-5000 Plus (Shimadzu Scientific, Columbia, MD) SPME auto-sampler. Samples were equilibrated for two minutes prior to extraction. A DVM/Carboxen/PDMS SPME fiber (2 cm 50/30 um) (Supelco, Bellefonte, PA) was exposed to the headspace above the tea extract in headspace vials for 30 minutes at 40 °C with an agitation speed of 250 rpm.

4.2.4 Volatile extraction by SPME for GC-FID/GC-O analysis

The extraction and injection were performed manually for GC-O analysis. 5 ml of tea aqueous infusions which was prepared similar to GC-MS analysis was placed in a 15 mL glass vial with a Teflon-lined cap. A ‘RTC basic’ heater with an ETS D4 Fuzz

Controller (IKA Werke, Wilmington, NC) was used to heat samples at 40°C while being stirred using a 4 mm stir bar. A 50/30 μm SPME fiber (DVB/CAR/PDMS) on a 2 cm StableFlex fiber (Supelco Bellefonte, PA) was inserted into the vial and was exposed approximately 1 cm above the headspace for 30 minutes while a magnetic bar continued to stir the sample.

4.2.5 GC-MS Analysis

The volatile constituents of each sample were analyzed using Shimadzu GCMS-QP2010 Ultra mass selective detector-(Shimadzu, Columbia, MD, USA) equipped with GCMSsolution and capillary nonpolar column (SHRXI-5MS, Shimadzu, 30m * 0.25mm id * 0.25 μm film thickness). The oven temperature was initially held at 50 ºC for 5 min and then increased at 4º C/min to final temperature of 250 ^{o}C. The injector temperature was 200 °C and injections were made in splitless mode. Ultra high purity Helium used as a carrier gas at a flow rate of 0.69 mL/min. The mass spectra were collected at m/z 40-400 and were performed every 0.3 seconds. The ion source and quadruple were set at 230 and 200 ^{o}C, respectively. Identification of the volatile components was performed by combined matching standardized retention time (LRI values) from DB-5 column (Flavornet and Pherobase), and fragmentation spectra of standards from NIST 11 (Scientific Instrument Services, Ringoes, NJ) and the Wiley 2010 libraries (John Wiley and Sons inc.). Confirmation of the identification was sought by matching the mass spectra of the compounds with the reference mass spectra present in the NIST 11 and Wiley libraries (acceptable similarity index was above 90%). The results were compared with our control unpanned samples.

4.2.6 GC-O Analysis

This study approved by Institutional Review Board (IRB) at Virginia Tech (IRB 13-580). A GC-O analysis was carried out using a HP 5890A GC (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame ionization detector (FID), a sniffing port (ODOII; SGE Inc. Austin, TX), and a DB-5ms column (30m x 0.25-mm i.d. x 0.25 µm film thickness) (J&W Scientific, Folson, CA). The detector was set to 250°C and 275 °C respectively; and all injections were made in the splitless mode. The initial oven temperature was 50 °C and increased at 10 °C/min until reaching a final temperature of 200°C. Chromatograms were recorded using a HP 3396A integrator (Hewlett-Packard Co., Palo Alto, CA). Hydrogen was used as the carrier gas with a flow rate of 1.0 $ml.min^{-1}$ (linear flow velocity ~ 25 cm/sec). The GC column effluent was split 1:1 between the FID and the Olfactrometer using deactivated fused silica capillaries (1-m length x 0.32 µm i.d.). Two trained assessors (the training procedure explained in Chapter 3) were selected for GC-O analysis. Two assessors sniffed tea extracts from SDE or SPME method for 20 minutes from each batch. Aroma descriptions, times and intensity were recorded for every sample. The assessors indicated aroma intensity in scale 1-5 where 1 was the lowest intensity and 5 was the highest.

Mean aroma intensities for each odorant were calculated by averaging the reported intensity by panelists. Aroma-active compounds were defined as the ones that were detected by the panelists fifty percent of the time with similar descriptions and retention times or those scored higher than 3 by panelist. Kovats or Linear Retention Index (LRI) values were determined using a series of alkanes (C5-C26) which were run under the same condition. Identification of volatile compounds was based upon their odor

descriptions and RI values from DB-5 column. The databases Flavornet (http://www.flavornet.org/flavornet.html) and Pherobase (http://www.pherobase.com/) were used to aid in identifying the compounds based upon standardized retention and aroma.

4.2.7 Statistical Analysis

We previously conducted the similar experiments on unpanned oolong tea with three replications and the results from GC-MS and GC-O were compared with the panned tea from this study. The data from GC-MS were analyzed by JMP 11.0 (SAS, Cary, NC, USA).Two way analysis of variance (ANOVA) and mean comparisons at using Tukey's test 5% significance were conducted on different compound categories: alcohols, aldehydes, ketones, terpenes, acids and ester results from SDE and SPME techniques of panned and unpanned tea.

One way ANOVA was also used to find significant differences in 12 volatiles that previously reported as major flavor compounds in oolong tea extracted with SDE and SPME techniques between panned and unpanned tea with 3 replications. Means were compared by using Fisher's least significant difference (LSD) method with significance at $P<0.05$.

4.3 Results and Discussion

4.3.1 GC-MS Analysis

A total 0f 190 volatile compounds were identified from SDE and SPME using GC-MS and GC-O. Previously, we identified 200 volatile compounds in panned oolong tea which only 79 of these compounds were shared with the panned oolong tea (Table

4.1) which shows the significant impacts of panning on flavor volatiles of oolong tea. We also found that the information from SDE and SPME are different but can complement each other. Therefore, we used the same approach to analyze and discuss our results from GC-MS and GC-O.

A total of 121 volatiles were extracted from panned oolong tea with SDE technique. Among these 129 compounds, 18 alcohols, 11 aldehydes, 16 ketones, 23 terpenes and 13 acids were detected. The most abundant compounds were furfural (10.8%), *trans*- nerolidol (8.5%) , α.-farnesene (4.8%), 1H-pyrrole-2-carboxaldehyde 1-ethyl- (3.9%), benzyl nitrile (3.5%), 2-furancarboxaldehyde, 5-methyl- (2.8%), indole (2.5%), benzenamine, 4-methoxy-2-methyl- (2.3), butanenitrile, 3-methyl- (2.1%) and ethanone, 1-(2-furanyl)- (2.1%). Only *trans*- nerolidol, α.-farnesene, indole and benzyl nitrile also appeared as most abundant compounds in unpanned tea. Similar to unpanned tea, *trans*- nerolidol (43.9% of total alcohols) and indole (13.4%) were two major alcohols. Ethanone, 1-(2-furanyl) - (21.5% of total ketones) and 3(2H)-Furanone, dihydro-2-methyl (11.0%) were two major ketones; however, the major ketones in unpanned tea were jasmine lactones and *trans*-, β-ionone. These two compounds were identified in panned tea but in much lower concentration. Furfural (49.5% of total aldehydes) was the most abundant aldehyde in panned tea, but hexanal and benzeneacetaldehyde were the most abundant aldehydes in unpanned tea. Among 23 identified terpenes in panned tea, α.-farnesene (24.2%) and linalool oxide (9.1%) were the compounds with highest peak area. For unpanned tea, similarly .α.-farnesene was the most abundant terpenes and followed by geraniol and linalool. Sesquiterpenes in oolong tea present as diglucosydes that can be hydrolyze to form various aromatic compounds in

manufacturing process (Guo, Ogawa, Yamauchi, Watanabe, Usui, & Luo, 1996). These glucosydes can be obtained by biosynthesis during the manufacturing process (Wang, Kubota, Kobayashi, & Juan, 2001).

From SPME technique, a total of 48 volatile compounds were detected including 8 alcohols, 8 aldehydes, 4 ketones, 7 acids, and 11 terpenes. The compounds with highest peak area were indole (9.3%), furfural (7.1%), 1H-pyrrole-2-carboxaldehyde, 1-ethyl- (5.5%), benzyl nitrile (4.1%), 1, 1, 5-trimethyl-1, 2-dihydronaphthalene (TDN) (3.4%), benzenamine, 4-methoxy-2-methyl- (2.9%), spiro[3.6]deca-5,7-dien-1-one,5,9,9-trimethyl (2.8%), oxime-, methoxy-phenyl (2.5%), 3,4-dimethylcyclohexanol (2.4%), and 3-amino-4-methylbenzyl alcohol (2.4%). Indole (44.7% of total alcohols) 1-hexanol, 2-ethyl- was the most abundant alcohol for panned and unpanned, respectively. Among 8 identified aldehydes, similar to SDE furfural (31.6% of total aldehydes) had the highest peak percentage but for unpanned tea was 2, 4-decadienal, (E,E)- hotrienol (18.5% of total terpens) had the highest peak areas among terpenes and this compound was not identified in unpanned tea. The most abundant terpenes in the unpanned tea was geraniol. Overal, the identified volatile compounds from SPME represent the tea beverage and the ones that identified from SDE represent the tea leaves and tea beverage.

Results from ANOVA showed there was significant difference ($P<0.05$) between the peak percentages of alcohols of panned and unpanned teas (Figure 4.1). The percentage of alcohols in unpanned tea were significantly higher ($P<0.05$) than panned tea from SDE; however, there was no significant difference ($P>0.05$) in alcohols percentages between unpanned and panned tea in SPME. Fermentation results in increased activity of enzyme on the substrate that leads to creation of aromatic alcohols

(Ma et al., 2014); however, during panning, many of these enzymes might be destroyed by heat in order to stop fermentation and consequently results in formation of less aromatic alcohols. There were no significant differences ($P>0.05$) in aldehyde percentages of panned and unpanned tea in both extraction techniques. Analysis of ketones showed significant differences ($P<0.05$) in panned and unpanned tea in both extraction techniques. Additionally, the peak percentages of panned tea for ketones were higher ($P<0.05$) in SDE compared to SPME. No esters were identified in panned tea. There was no differences ($P>0.05$) between the acids contents of panned and unpanned tea in both SDE and SPME. The percentage of terpenes in both extraction techniques were not different ($P>0.05$) between panned and unpanned tea; however, terpenes percentages of unpanned tea in SDE were higher ($P<0.05$) than SPME.

Most of the available studies on oolong tea volatiles have investigated major compounds that either differentiate oolong with fully fermented teas or non-fermented teas or studied the compounds that are indicator of quality in oolong tea. In fermentation, several enzymatic reactions are responsible for formation of tea aroma. The main precursors for tea aroma are amino acids and carotenoids, including β-carotene, lutein, neoxanthin, and violaxanthin (Yamanishi, 2012). During fermentation a primary oxidation results in the significant reduction of carotenoids, particularly β carotene resulting in the formation of ionone and terpenoid carbonyls (Yamanishi, 2012). By oxidation and secondary epoxidation reactions, other carotenoids give rise to ionone, linalool and substituted hydroxy- and epoxy-ionones (Sanderson & Grahamm, 1973). Generally, grassy or green flavors are diminished during fermentation, but fruity, floral and other fermented characters are known to be increased (Wang et al., 2008).

Pripdeevech & Machan (2011) used *cis*-jasmone (woody, herbal), *trans*-nerolidol (floral), indole (pungent) and hotrienol to differentiate semi-fermented tea from non-fermented tea. They showed the content of the first three volatiles were increased significantly while hotrienol (green, sweet) was decreased. In our study, the content of *cis*-jasmone was significantly higher ($P<0.05$) in panned tea while the content of indole and *trans*-nerodiol were significantly decreased ($P<0.05$) by panning. The content of indole is very low in non-fermented tea, but its level increased quickly at the beginning of fermentation in oolong tea and then slowly decreased by continuing fermentation (Wang et al., 2008). Indole precursor might be destroyed by the heat treatment from panning to stop fermentation process, and led to changes in indole content in the panned tea. GC-MS analysis was able to identify hotrienol only in the panned tea. This may suggest that heat treatment resulted in formation of hotrienol in oolong tea.

Other studies showed different compounds are important to distinguish oolong from other variety of teas. Other than indole, Wang et al. (2008) found flavor compounds such as *trans*-2-hexenal (green), benzaldehyde (almond) and methyl salicylate (peppermint) are important to distinguish unfermented teas from fermented ones. *Trans*-2-hexenal and methyl salicylate also may be used to classify the semi from fully-fermented teas. Others also reported the content of compounds like (E)-geraniol (floral,rose) , (E)-β-damascenone (not identified in our study), and linalool oxide B (floral) increase with degrees of fermentation (Kawakami, Ganguly, Banerjee & Kobayashi, 1995; Wang et al., 2008; Wang et al., 2011; Zhang et al., 2013). In our study, *trans*- 2-hexenal, methyl salicylate and geraniol contents were decreased significantly ($P<0.05$) by panning but linalool oxide content were significantly ($P<0.05$) increased.

Trans-2-hexenol (grassy, green) is a product of lipid degradation and result in inferior quality to tea (Pripdeevech & Machan, 2011). Usually, a higher amount of *trans*-2-hexenal is detected in non-fermented tea whereas semi-fermented tea significantly lower amounts (0.04–0.08%) (Pripdeevech & Machan, 2011). Our results suggest that panning promotes some of the flavor characteristics of non-fermented as well as fermented teas in oolong tea.

There are few studies available that suggest volatile flavor compounds that affect the perceived quality of oolong tea. *Trans*-nerolidol has reported as one of the key odorants and can be considered as an indicator for the high quality oolong tea flavor (Kai, Yoshida, Kageyama, Saito, Ishigaki & Furukawa, 2008; Pripdeevech & Machan, 2011; Wang et al., 2011; Zou et al., 2011, Ma et al., 2014). Similarly, we found that *trans*-nerolidol was the most dominant volatile in unpanned oolong tea in our study; however, during panning the concentration of these compound significantly decreased ($P<0.05$) (Figure 4.2). Similar to our results, Ma et al. (2014) reported decreases in the nerolidol content during other thermal process steps in oolong tea manufacturing such as fixation, shaping and drying. Nerolidol has a floral aroma (Lapczynski, Bhatia, Letizia & Api, 2008) and exists at a relatively high concentration in variety of oolong tea. Even though the amount of this compound significantly decreased by panning, still it was the second most abundant compound in the panned tea in our study. The content of nerolidol is small in fresh leaves, but the content was greatly increased and reaches to its highest level during the fermentation stage of manufacturing (Ma et al., 2014).

Wang et al. (2011) reported that perceived aroma score positively correlated with concentration of benzyl alcohol (sweet, flower), benzeneacetaldehyde (honey, floral),

linalool (flower), phenylethyl alcohol (honey), linalool oxide (flower), indole (pungent), *cis*-jasmone (herbal, woody), nerolidol (flower), methyl jasmonate (flower). In addition, they found total quality score positively correlated with concentration of benzyl alcohol, benzeneacetaldehyde, geraniol, indole and toluene (not identified in our study) but negatively correlated with the concentration of (E,E)-2,4-heptadienal (identified with GC-O in our study). Other studies also showed benzaldehyde (almond) (Zhang et al., 2013), jasmine lactone (floral and fruity) (Wang et al., 2001; Wang et al., 2008; Wang et al., 2011; Zhang et al., 2013), and α-farnesene (woody) (Kawakami, 1995; Wang et al., 2008; Wang et al., 2011; Zhang et al., 2013) play important roles in aroma of oolong tea and have high correlation with the aroma of oolong tea. Compounds such as (E)-β-damascenone (Zhang et al., 2013) and methyl-5-hepten-2-one (Wang et al., 2008) also are reported as major flavor compounds but they did not detected in our study. In addition, phenylethyl alcohol was identified in high concentration in the unpanned tea but was not detected in the panned tea. There were significant reduction ($P<0.05$) in amounts of *trans*-nerolidol, 2-hexenal, benzaldehyde, indole, geraniol, and benzenacetaldehyde as a result of panning. However, panning caused significant increases ($P<0.05$) in contents of linalool oxide, *cis*-jasmone, and methyl salicylate. There were no significant difference ($P>0.05$) in content of linalool, jasmine lactone and benzyl alcohol in panned and unpanned tea.

Furfural was the most dominant volatiles in the panned tea in SDE extraction and also identified as the second most abundant compounds in SPME. Furfural was not identified in unpanned tea with both extraction techniques in GC-MS analysis but we identified it as aroma active compound with GC-O analysis. Furfural has been reported in

flavor profile of oolong tea in previous studies (Wang et al., 2008; Pripdeevech and Machan, 2011; Zhang et al., 2013). Furfural has been found in an extensive range of teas, coffees, fruits, and wine and has been used as an ingredient for flavor enhancements in food (Rega, Guerard, Delarue, Maire, & Giampaoli, 2009). Furfural odor is like baked bread, almond and sweet (Rega et al., 2009). Furfural is formed by the heat treatment or acid hydrolysis of polysaccharides, which contain hexose and pentose fragments (IARC, 1995). Panning can create compounds that are generated in the thermal degradation of cellulose and hemicellulose such as furfurals, 2-furancarboxaldehyde-5-methyl and other furans (Guillén & Manzanos, 1997; Sung, 2013).

Pripdeevech & Machan (2011) also indicated that process of steaming or panning at high temperature in non-fermented tea method may bring about higher appearance of the lipid degradation product such as heptanoic acid, 2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, and nonanoic acid that identified in our studies. In comparison to unpanned tea, many pyrrole compounds were identified in the panned tea such as pyrrole, 1H-pyrrole, 1-ethyl-, 1H-pyrrole-2-carboxaldehyde, 1-methyl-, ethanone, 1-(1H-pyrrol-2-yl)-, 2-acetylpyrrole and 1H-pyrrole-3-carboxylic acid, 2,4-dimethyl-, methyl ester. The generation of these nitrogen-containing heterocycles is assumed to be caused by a Strecker degradation of theanine and amino acids during the tea preparation, and it assumes to be responsible for the aroma of the heat treated teas (Yu, Yang, Lin & Chang, 1999).

4.3.2 GC-O Analysis

In SDE extraction, 47 aroma active volatiles were tentatively identified based on the combination of LRI and odor descriptors in panned oolong tea and 9 of these

compounds were also identified by GC-MS analysis (Table 4.2). Among these compounds, 17 compounds were previously identified by both extraction techniques in the unpanned tea. However, in SPME, we identified 42 compounds that possessed aroma activity and only 10 of these compounds were identical to extracted aroma compounds from SDE (Table 4.3). Among these 42 compounds, 17 compounds were shared with unpanned tea but only 6 of these 42 compounds were detected by GC-MS analysis.

The Identified aroma components of panned and unpanned tea for both extraction techniques were grouped in six categories based on their aroma description: fruity, sweet, floral, nutty, green and smoky/burnt. The total aroma intensities of identified aroma compounds from SDE and SPME for each aroma group are shown in Figure 4.3 and 4.4, respectively. The most important features that were consistent between these two figures were the increase of sweet aroma and decrease of fruity and smoky in the panned tea. Floral aroma was not considerably affected by panning. Green aroma was the most different feature between two figures. Previously, our panelists were unable to detect any green aroma in unpanned tea from SPME extraction; however, for the panned tea, the numbers of compounds responsible for green and grassy flavor were identified. On the other hand, in SDE, similar to previous report, green aroma was slightly decreased. The other inconsistent result was related to nutty aroma. In SDE, there were more intense aroma active compounds with nutty smell detected in the unpanned tea. In contrast to SDE results, total aroma intensity for nutty smell was increased by panning which is more consistent with the literatures. The differences in two methods' capabilities have been indicated in some flavor studies. A SPME extraction technique was found to be not suitable for the isolation of high molecular compounds or for those with a strong affinity

to the matrix; however, some compounds cannot be detected in SDE due to the presence of solvent (Majcher & Jelen´, 2009) and also some artificial compounds can be generated during the extraction.

Among the identified aroma active compounds in the panned tea from SDE, pentanal (somkey), dihydromethylcyclopentapyrazine (nutty), and one not identified compound (nutty) had the highest aroma intensity with intensity of 4/5 in the panned tea. Dihydromethylcyclopentapyrazine and the not identified compound both with nutty flavor were also detected as one of the most intense aroma in the unpanned tea. However, Limonene oxide (fruity) was the only compounds that scored 4 in SPME. We were able to identify (E, E)-2, 4-heptadienal (nutty) and (Z)-3-hexenol (green) in our GC-O which has been shown to be increased by degree of fermentation (Wang et al., 2008; Wang et al., 2011). Similar to the unpanned tea, *trans*-2-hexenol was identified which considered an off-flavor in oolong tea. We also detected pyrazines such as ethylmethyl pyrazine, dihydromethylcyclopentapyrazine, which are known as thermally generated product of amino acids and sugars (Kato & Shibamoto, 2001; Wang et al., 2010); however, both of these compounds were found in unpanned tea as well. Jin Xuan oolong is well known for its milky aroma. Previously, we identified dieactyl with butter aroma and suggested that the milky aroma of this variety of tea is associated with this compound. However, this compound was not detected by our panelists in the unpanned tea and panning might have resulted in elimination of the milky aroma in Jin Xuan oolong.

4.4 Conclusions

Despite a few similarities in the most abundant identified compounds from GC-MS analysis and aroma active compounds from GC-O analysis between the unpanned and panned tea, panning significantly changed the aroma volatile components of oolong tea. The abundance of alcohols, ketones, acids and esters were significantly changed by panning; however, there were no changes in contents of terpenes and aldehydes. Since over-heating/panning the leaves may result in a burnt odor and underpanning may result in a greenish odor and red central vein (Hui et al., 2003), optimization of time and temperature in panning to manufacture best quality tea need to be investigated for the future studies. Moreover, conducting sensory studies to better understand consumer perception of panning effects on quality of oolong tea is necessary for large scale manufacturing and commercialization of the panned tea.

Table 4.1 Identified volatiles in the panned oolong tea with method of identification, LRI and comparisons with the unpanned tea.

#	Compound	LRI	SDE	SPME	Unpanned
1	n.i.	561-580*	O		X
2	2- Methyl-3-buten-2-ol	620*	O	O	
3	Butanenitrile, 2-methyl-	637	MS		
4	Butanenitrile, 3-methyl-	646	MS		X
5	Methylbutanal	647*	O	O	X
6	2H-Pyran, 3,4-dihydro-6-methyl-	644	MS		
7	Pyrrole	646	MS		
8	Isobutyraldehyde	655*	O		
9	1-Pentanol	657	MS		X
10	Pentane, 1-chloro-	662	MS		
11	2-Penten-1-ol, (Z)-	661	MS, O		X
12	Methyl methylbutanoate	675*	O		
13	Pentenone	682*	O		X
14	Methyl 2-methylpropionate	687-688*		O	
15	Cyclopropane, 1,1,2,3-tetramethyl-	695	MS		
16	Hexanal	700		MS	X
17	Dimethylallyl	702*	O		X
18	3(2H)-Furanone, dihydro-2-methyl-	703	MS		X
19	1H-Pyrrole, 1-ethyl-	711	MS	MS	
20	Methylcyclohexane	715-723*	O		
21	Pyrazine, methyl-	717	MS	MS, O	
22	Maleic anhydride	724	MS		
23	Methyl butanoate	727*	O		X
24	Furfural	727	MS, O	MS, O	X
25	Pentanal	740*	O	O	X
26	Methyl-2-butenal	747*	O		X
27	2-Hexenal, (E)-	747	MS, O		X
28	2-Furranmethanol	750	MS	MS	
29	p-Xylene	764	MS		
30	Pentanol	765*		O	X
31	Benzene, 1,3-dimethyl-	767		MS	
32	Methyl-2-butenol	774*	O	O	
33	1,5-Heptadiene, 2,6-dimethyl-	781	MS		
34	1-Hexenol	792*		O	
35	3-Hexenal	798*	O		
36	n.i.	800-807*		O	
37	Oxime-, methoxy-phenyl-_	802	MS		X
38	Ethanone, 1-(2-furanyl)-	808	MS		
39	1-(3H-Imidazol-4-yl)-ethanone	810		MS	
40	Pyrazine, ethyl-	811	MS		
41	Propyl propanoate	817*		O	X
42	1H-Pyrrole-2-carboxaldehyde, 1-methyl-	824	MS	MS	
43	n.i.	830-837*	O	O	X
44	Isopropyl butanoate	854*		O	X
45	Furfuryl alcohol	855*	O		
46	Benzaldehyde	857	MS, O	MS	X
47	2-Hexenal	859-865*	O		X
48	2-Furancarboxaldehyde, 5-methyl-	860	MS	MS, O	
49	(Z)-3-Hexenol	861*		O	X
50	Methyl 2-furoate	873	MS		
51	1-Octen-3-ol	877	MS		X
52	Heptanal	882*	O		
53	2-Methylbutyl acetate	883-885*		O	X
54	Sulcatone	884	MS		X
55	β-Myrcene	889	MS	MS	
56	Pyrazine, 2-ethyl-6-methyl-	895	MS	O	X
57	2,4-Hexadienal	928*		O	
58	Bicyclo[2.2.1]heptane, 2-butyl-	909	MS		
59	Furan, 2-propyl-	910	MS		

60	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	920	MS		
61	Dimethylthiazole	928*		O	X
62	Mesitylene	930	MS		X
63	o-Cymene	931	MS	MS	X
64	D-Limonene	938	MS	MS	X
65	Benzyl alcohol	944	MS		X
66	trans-β-Ocimene	951	MS	MS, O	X
67	Benzeneacetaldehyde	957	MS	MS	X
68	1H-Pyrrole-2-carboxaldehyde, 1-ethyl-	965	MS	MS	X
69	Heptanol	970*		O	X
70	Ethyl isohexanoate	971*		O	X
71	Filbertone	972*	O		
72	Cyclohexene, 1-(3-ethoxy-1-propenyl)-, (Z)-	973	MS		
73	Ethanone, 1-(1H-pyrrol-2-yl)-	980	MS	MS	
74	1,5-Octadienone	983*		O	
75	Acetophenone	989	MS		
76	1-Octanol	997	MS		
77	Linalool oxide	1000	MS, O	MS, O	X
78	Acetylthiazole	1017*	O		
79	2,4-Heptadienal	1019-1023*		O	X
80	Pyrazine, 3-ethyl-2,5-dimethyl-	1019	MS		
81	1,8-Cineole	1025-1029*	O		
82	(E)-2-Heptenal	1041*	O		X
83	2-Acetylpyrrole	1051*		O	
84	Linalool oxide(furanoid)	1053	MS, O		X
85	2-Octenal	1055*	O		
86	α-Ocimene	1058*	O	O	
87	3,5-Octadien-2-one	1066	MS		X
88	Ethyldimethylthiazole	1073*		O	
89	p-Cresol	1076*	O		
90	R-Linalool	1091	MS	MS	X
91	3,5-Octadienone	1096*	O		X
92	Hotrienol	1102	MS	MS	
93	3,4-Dimethylcyclohexanol	1103		MS	X
94	Benzenamine, 4-methoxy-2-methyl-	1110	MS	MS	
95	1,5,9-Undecatriene, 2,6,10-trimethyl-, (Z)-	1115	MS		
96	Isophorone	1118	MS		X
97	2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)- E,Z-Alloocimene	1128	MS		
98	Limonene oxide	1129*		O	
99	γ-Heptalactone	1130**		O	
100	Benzyl nitrile	1136	MS	MS	X
101	1,3-Cyclopentadiene, 1,2,3,4-tetramethyl-5-methylene-	1151	MS		
102	1-[2-Aminoethyl]hypoxanthine	1154	MS		
103	2-Nonenal, (E)-	1158	MS		
104	3,5-Diethyl-2-methylpyrazine	1160*		O	
105	1H-Pyrrole-3-carboxylic acid, 2,4-dimethyl-, methyl ester	1161	MS		
106	Benzeneacetic acid, .α.-oxo-, ethyl ester	1163	MS		
107	2H-Pyran-3-ol, 6-ethenyltetrahydro-2,2,6-trimethyl-	1168	MS		X
108	Benzeneacetic acid, methyl ester	1176	MS		
109	3-Amino-4-methylbenzyl alcohol	1181	MS	MS	
110	Butanoic acid, 3-hexenyl ester, (E)-	1184	MS		
111	Isobutylmethoxypyrazine	1189*	O		
112	α-Terpineol	1190	MS	MS	X
113	Dihydrocarveol	1191*		O	
114	Methyl salicylate	1193	MS	MS, O	X
115	Ethyl octanoate	1197*		O	X
116	1,3-Cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-	1199	MS		
117	Decanal	1202	O	MS	X

118	(Z)-4-Decenal	1203*		O	
119	1H-Indene, 2,3-dihydro-1,1,5,6-tetramethyl-	1206	MS		
120	Benzene, (ethenyloxy)-	1208	MS		
121	1,3-Cyclohexadiene-1-methanol, 4-(1-methylethyl)-	1211	MS		
122	β-Cyclocitral	1211	MS		
123	4a(2H)-Naphthalenol, octahydro-, trans-	1213	MS		
124	Benzene, 1-(1,5-dimethylhexyl)-4-methyl-	1216	MS		X
125	Prop-2-en-1-one, 1-(6,6-dimethylbicyclo[3.1.1]hept-2-en-2-yl)-	1218	MS, O		
126	3-Phenylpropan-1-ol	1220*		O	
127	Citral	1221	MS		X
128	Geraniol	1227	MS	MS	X
129	Acetic acid, 2-phenylethyl ester	1229	MS	MS	X
130	Isocyclocitral	1230	MS		
131	Nonanoic acid	1234		MS	X
132	2,6-Octadienal, 3,7-dimethyl-, (E)-	1236	MS		
133	Nerol	1239*	O	O	X
134	2(1H)-Naphthalenone, 3,4,4a,5,6,7-hexahydro-1,1,4a-trimethyl-	1242	MS		
135	Ionone	1242	MS	MS	X
136	4-Acetamido-2-methallylphenol	1244	MS		
137	Benzothiazole	1246*	O		X
138	Indole	1248	MS		X
139	Isobornyl formate	1249*	O	O	
140	Formic acid, (2-methylphenyl)methyl ester	1250	MS		X
141	n.i.	1252*		O	
142	Pyrazine, 2,5-dimethyl-3-propyl-	1253	MS		
143	Dihydromethylcyclopentapyrazine	1255*	O		X
144	Cyclohexane, 1,2-diethenyl-4-(1-methylethylidene)-, cis-	1256	MS		
145	4-Hydroxy-3-methylacetophenone	1258	MS		
146	Spiro[3.6]deca-5,7-dien-1-one,5,9,9-trimethyl	1259	MS	MS	
147	2H-Pyran-3-ol, 2-ethoxy-3,4-dihydro-, acetate	1262		MS	
148	2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester	1263	MS		
149	6-Hydroxynicotinic acid di-methyl derivative	1264	MS		
150	Linalyl acetate	1264-1271*	O	O	X
151	Pentanoic acid, 4-methyl-, ethyl ester	1270		MS	
152	Benzene, 2-(2-butenyl)-1,3,5-trimethyl-	1277	MS		
153	1, 1, 5-Trimethyl-1, 2-dihydronaphthalene	1278	MS	MS	
154	Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-	1280	MS		
155	Safrole	1281*	O		X
156	Bicyclo[3.1.0]hexan-3-ol, 4-methyl-1-(1 methylethyl)-	1282	MS		
157	Phenol, 2-(1,1-dimethyl-2-propenyl)-3,6-dimethyl-	1284	MS		
158	cis-anti-cis-Tricyclo[7.3.0.0(2,6)]-7-dodecene	1286	MS		
159	Hexanoic acid, hexyl ester	1292	MS		
160	Decanoic acid, ethyl ester	1297	MS	MS	X
161	*cis*-Jasmone	1299	MS	MS	X
162	*cis*-Linalool pyran oxide	1401*	O		
163	Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl-	1402	MS		
164	n.i.	1417-1421*	O		
165	Cyclopropanecarboxylic acid, 2,2-dimethyl-3-(2-methyl-1-propenyl)-, 2-methyl-4-oxo-3-(2-pentenyl)-2-cyclopenten-1-yl ester, [1R	1423	MS		
166	α-Ionone	1428	MS		
167	6,7-Dimethyl-1,2,3,5,8,8a-Hexahydronaphthalene	1432	MS		
168	Coumarin	1437	MS, O		X
169	β-Phenylethyl butyrate	1440	MS		X

170	(E)-Geranyl acetone)	1451	MS		X
171	cis-β-Farnesene	1456	MS		X
172	Linalyl isovalerate	1477*	O		
173	4-(2,4,4-Trimethyl-cyclohexa-1,5-dienyl)-but-3-en-2-one	1484	MS		
174	*trans*-.β.-Ionone	1488	MS		X
175	Jasmin lactone	1493	MS	MS	X
176	1H-Benzocyclohepten-7-ol, 2,3,4,4a,5,6,7,8-Octahydro-1,1,4a,7-tetramethyl-, cis-	1499	MS		
177	Gamma.-Muurolene	1504	MS		
178	α-Farnesene	1508	MS	MS	X
179	Bicyclo[2.2.1]heptan-2-one, 1-(bromomethyl)-7,7-dimethyl-, (1S)-	1512	MS		X
180	Butylated Hydroxytoluene	1514	MS		X
181	cis-Thujopsene	1519	MS		X
182	Citronellyl butyrate	1536*	O		
183	Levomenol	1545	MS		
184	trans-Nerolidol	1564	MS, O	MS	X
185	3-Hexen-1-ol, benzoate, (Z)-	1572	MS		X
186	Benzoic acid, hexyl ester	1579	MS		
187	Farnesene epoxide, E-	1599	MS		X
188	Cyclopentaneacetic acid, 3-oxo-2-(2-pentenyl)-, Methyl ester, [1.α.,2.α.(Z)]-	1649	MS		
189	2-Furanmethanol, tetrahydro-.α.,.α.,5-trimethyl-5-(4-methyl-3-cyclohexen-1-yl)-, [2S-[2.α.,5.β.(R*)]]-	1660	MS		
190	Phytol	1836	MS		

*= Confirmed LRI from GCO analysis

Table 4.2 The aroma active compounds in the panned oolong tea using time-intensity GC-O with SDE extraction.

#	Compound	LRI	Confirmed [a]	Aroma Description	Intensity [b]
1	n.i. [c]	561-580		Nutty, Chocolate, caramel	4
2	Methylbutenol	620	620	Green, herb	2
3	Methylbutanal [1, 2]	647	641	Popcorn, nutty, chemical	2
4	Isobutyraldehyde	655	662	Green	2
5	Methyl methylbutanoate	675	674	fruity	2
6	Pentenone [1,2]	682	780	fruity	2
7	α,γ-Dimethylallyl alcohol	702	712	Green, sweet, fruit	3
8	Methylcyclohexane	715-723	716 [(2)]	Sweet, nutty, cookies	1
9	Methyl butanoate[1,2]	727	723	Sweet	3
10	Pentanal [1, 2]	740	732	Smoky, nutty	4
11	Methyl-2-butenal [2]	747	752	Green pepper	1
12	Methyl-2-butenol [1]	774	775	Celery, herb	2
13	3-Hexenal	798	800	Green, earthy	2
14	**Furfural** [1, 2]	825	829	Nutty, chocolate	2
15	n.i. [c, 1]	830-837		Waxy, smoky	1
16	**(E)-2-Hexenal**	851	844	Fruity	2
17	Furfuryl alcohol	855	851	Smoky, burnt	1
18	2-Hexenal [2]	859-865	854	fruity	2
19	Heptanal	882	885 [(2)]	Burnt plastic, smoky	2
20	**Benzaldehyde** [1]	964	960	Nutty	2
21	**(E)-2-Penten-1-ol**	965	973	Mushroom	2
22	Filbertone	972	972	nutty	2
23	**6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane**	984	981 [(2)]	Nutty, musty, sweet	2
24	Acetylthiazole	1017-1024	1020	Nutty, Waxy	2
25	1,8-Cineole	1025-1029	1032	Nutty, floral, sweet	2
26	(E)-2-Heptenal	1041	1041	Waxy, fatty	3
28	2-Octenal	1055	1060 [(2)]	Nutty,	2
29	α-Ocimene [2]	1058	1056	Fruity, floral	1
30	p-Cresol	1076	1075	Smoky	1
31	3,5-Octadienone	1096	1095	Citrus, fruity, sweet	2
32	**(E)-Linalool oxide**	1176	1172	Sweet, floral, fruity	2
33	Isobutylmethoxypyrazine	1189	1186	Green, smoky	2
34	Decanal	1211	1211[(2)]	Waxy	3
35	**Linalool oxide** [1,2]	1217	1212 [(2)]	Floral, fruity, earthy	2
36	Nerol [2]	1239	1233	Sweet	2
37	Benzothiazole [1]	1246	1240	Smoky	3
38	Isobornyl formate	1249	1245	earthy	2
39	Dihydromethylcyclopentapyrazine [1]	1255	1248	Nutty, smoky	4
40	Linalyl acetate [1]	1264- 1271	1261	Sweet, floral	1
41	Safrole [1]	1281	1280	Spicey, smoky	2
42	cis-Linalool pyran oxide	1401	1402	Citrus	2
43	n.i. [c]	1417-1421		Smoky, cooked meat	4
44	**Coumarin**	1433	1439	Sweet, waxy	3
45	Linalyl isovalerate	1477	1478	Fruity, waxy	2
46	Citronellyl butyrate	1536	1528	Fruity	3
47	**(Z)-Nerolidol**	1570	1565	Waxy	3

Bold Compounds were also detected with GC-MS

[1] Identified with SDE in GC-O analysis of unpanned tea

[2] Identified with SPME in GC-O analysis of unpanned tea

[a] LRI values confirmed with databases Flavornet and Pherobase to identify the compounds based upon standardized retention and aroma.

[b] The average aroma intensity score by panelist on a scale of 5 where 1= low intensity and 5= high intensity.

[c] Not identified compound

Table 4.3 The aroma active compounds in the panned oolong tea using time-intensity GC-O with SPME extraction.

#	Compound	LRI	Confirmed [a]	Aroma Description	Intensity [b]
1	Methylbutenol	609	620	Spice, herb	1
2	Methylbutanal [1]	654	641	Sweet, vanilla, almond	1
3	Methyl 2-methylpropionate	687-688	685[(2)]	Fruity, sweet	2
4	α,γ-Dimethylallyl alcohol	718	712	Green	1
5	Methyl butanoate [1, 2]	723	723	Fruity, sweet	3
6	Pentanal [1, 2]	728	732	Nutty	1
7	Pentanol [1]	765	759	Fruity	2
8	Methyl-2-butenol	788	779	Spicy, herb	1
9	1-Hexenol	792	789	Green pepper	3
10	n.i. [c]	800-807	-	Smoky, earthy	3
11	Propyl propanoate[1]	817	812	Sweet	1
12	**Furfural** [1, 2]	829	829	Nutty, earthy	2
13	**Methyl pyrazine**	833-835	828	Nutty, popcorn	1
14	n.i. [c, 2]	839-846	-	Chocolate, nutty	1
15	Isopropyl butanoate [1]	854	847	Fruity, floral	2
16	(Z)-3-Hexenol	861	858	Green	1
17	2-Methylbutyl acetate [2]	883-885	880	Sweet, fruit	3
18	2,4-Hexadienal	906	910	Grassy	2
19	Dimethylthiazole [1, 2]	928	928	Plastic, smoky	1
20	Heptanol [2]	970	962	Green	2
21	Ethyl isohexanoate [2]	971	968	Fruity	2
22	1,5-Octadienone	983	988	Musty	1
23	Ethylmethyl pyrazine [1, 2]	986	993	Fruity	2
24	2,4-Heptadienal [2]	1019-1023	1011	Nutty, sweet	1
25	**(E)-β-Ocimene**	1038	1038	Sweet, vanilla	3
26	2-Acetylpyrrole	1051	1045	Nutty	1
27	α-Ocimene	1062	1056	Apple, banana, fruity	3
28	Ethyldimethylthiazole	1073	1078	Earthy	1
29	Limonene oxide	1129	1132	Fruity	4
30	γ-Heptalactone	1130	1130	Nutty	2
31	3,5-Diethyl-2-methylpyrazine[1]	1160	1160[(2)]	Nutty, chocolate	1
32	Dihydrocarveol	1191	1190	Spicey, mint	1
33	Ethyl octanoate	1197	1198	Floral, fruity	3
34	(Z)-4-Decenal	1203	1200	Green, musty	1
35	**Linalool oxide** [2]	1214	1212	Green, floral	1
36	3-Phenylpropan-1-ol	1220	1219 [(2)]	Fruity	1
37	**5-Methyl-2-furancarboxaldehyde**	1222	1224[(2)]	Musty	1
38	Nerol	1226	1233	Sweet	1
39	**Methyl salicylate**	1234	1234	Nutty, floral	2
40	Isobornyl formate	1246	1245	Green	2
41	n.i.[c]	1252-1261	-	Nutty, chocolate	3
42	Linalyl acetate [2]	1263	1261	Sweet	2

Bold Compounds were also detected with GC-MS

[1] Identified with SPME in GC-O analysis of unpanned tea

[2] Identified with SDEE in GC-O analysis of unpanned tea

[a] LRI values confirmed with databases Flavornet and Pherobase to identify the compounds based upon standardized retention and aroma.

[b] The average aroma intensity score by panelist on a scale of 5 where 1= low intensity and 5= high intensity.

[c] Not identified compound

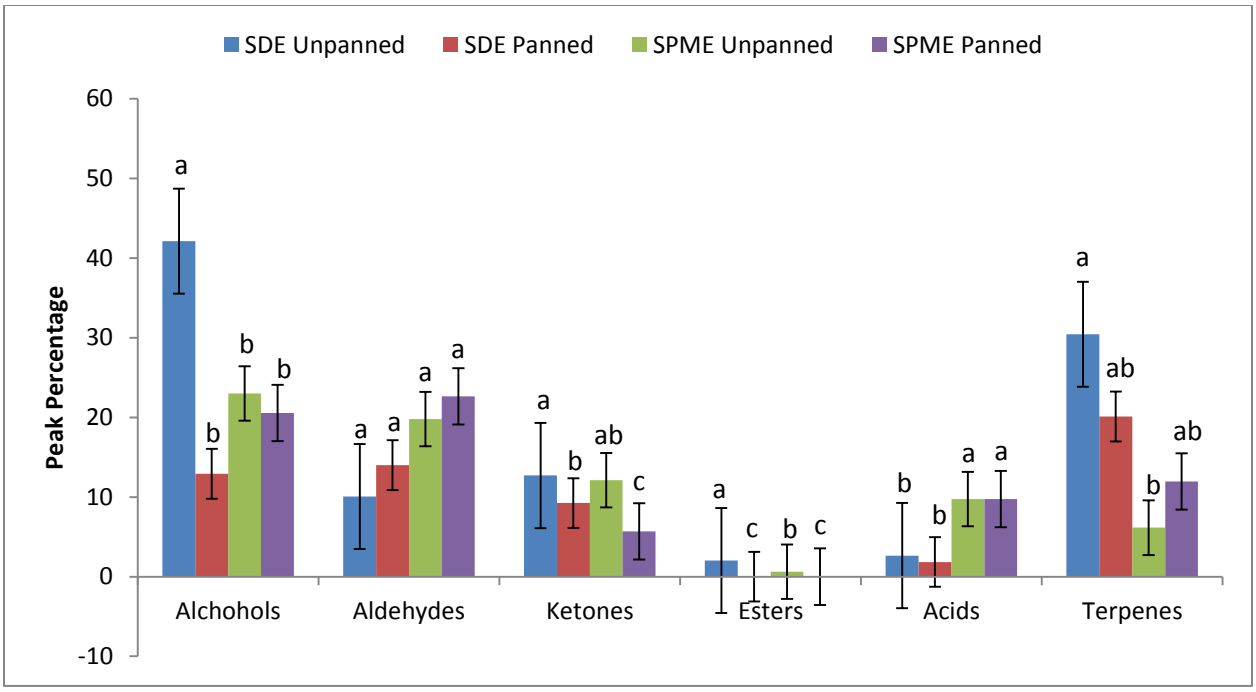

Figure 4.1 Mean comparison of peak percentages of chemical composition the unpanned and panned tea using SDE and SPME.[a-c] Means within a class of compounds with the same letter are not significantly different (P>0.05). Bars represent standard deviations.

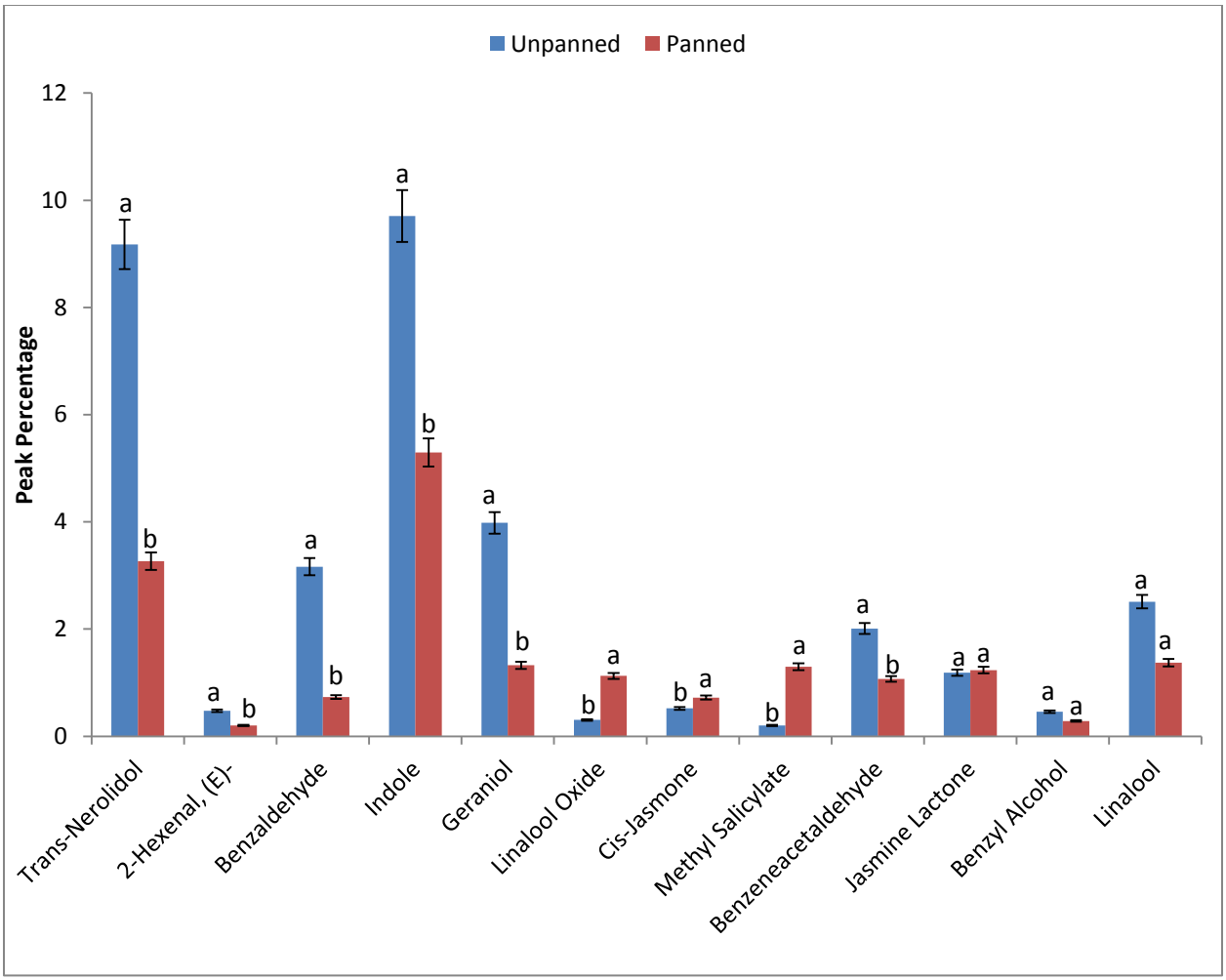

Figure 4.2 Mean comparison of peak percentage of 12 major volatiles in the panned and unpanned tea identified from SDE and SPME techniques. [a-b] Means with the same letter within each compounds are not significantly different (P>0.05). Bars represent standard deviations.

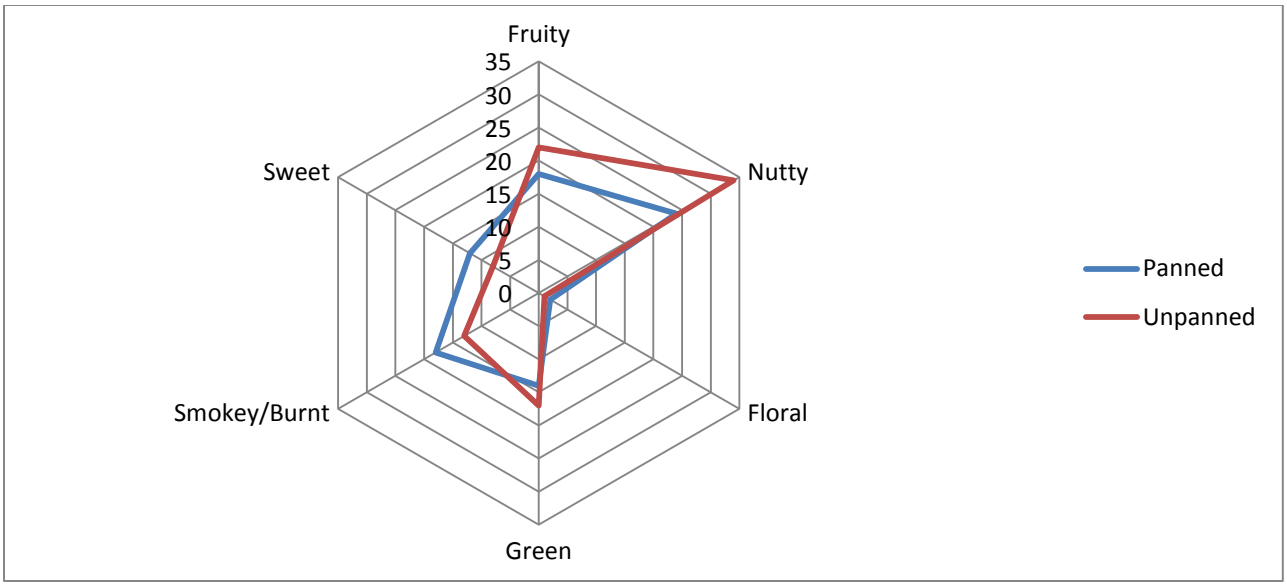

Figure 4.3 Radargram of aroma profile of panned oolong tea using SDE obtained from grouping of identified compounds using GC-O with similar aroma characteristics.

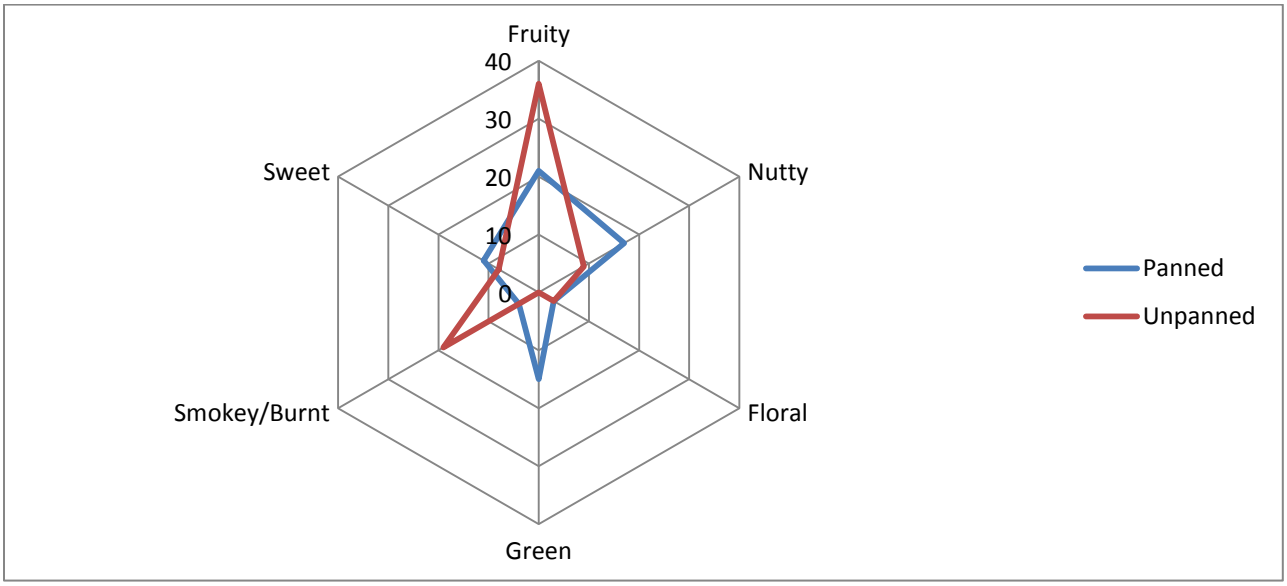

Figure 4.4 Radargram of aroma profile of panned oolong tea using SPME obtained from grouping of identified compounds using GC-O with similar aroma characteristics.

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CHAPTER V

CONCLUSIONS

Results from the first experiment showed the extraction of EGCG and caffeine were lower when green tea was brewed in hard water compared to distilled water. Brewing green tea and oolong tea in tap water resulted in higher extraction of caffeine but had no effect on EGCG compared to distilled water. The extraction of EGCG and caffeine were significantly increased ($P<0.05$) when green tea and oolong tea were brewed in the chlorinated water at 4.0 mg free chlorine per liter. Overall, our study showed the composition of water can affect he extraction of EGCG and caffeine in green and oolong teas. Further studies on effects of other chemicals in drinking water on polyphenols, caffeine and sensory properties of teas need to be conducted. The similar study can also be conducted to study effects of water chemistry on extraction of different important compounds in variety of products such as coffee, wine, beer, etc. Beverage industry can use the results of this experiment to increase the amount of caffeine and catechins in their products without adding more tea leaves into their formulations.

In the second experiment, results from optimization of Simultaneous Distillation-Extraction (SDE) technique showed the total concentration of isolated volatile compounds that extracted with diethyl ether were significantly higher ($P<0.05$) than dichloromethane. The total concentration of volatile compounds at 40 min extraction were higher ($P<0.05$) than other extraction times. In SDE, a total of 128 volatiles were identified using GC-MS and 45 aroma active compounds using GC-O. *Trans*- nerolidol is the most abundant compound in oolong tea. The number of extracted volatiles using GC-MS was much lower in SPME. SPME was able to identify 59 volatiles and 41 aroma active compounds were identified. Each method was able to identify compounds that the other could not. The composition of the volatile components extracted by the two

methods differed considerably; however, we suggest that the data from both methods will be used together for analysis of flavor compounds in food products. For the future, results from our study can be compared with other flavor extraction methods such as Solvent Assisted Flavor Evaporation (SAFE) and Purge and Trap. This is not only assisting us to verify many of the identified flavor volatiles in our study but also we can use this data to compare the effectiveness of these methods of extraction with SDE and SPME. Results from this study can provide important information about effectivness of these two methods of flavor extraction to scientists who work in area of flavor science.

In the third experiment, effect of panning on flavor volatile composition of Jin Xuan oolong tea was studied. A total of 190 volatiles were identified from SDE and SPME extractions using GC-MS and GC-O. Among 12 major volatiles that have identified in previous studies as indicator of quality of oolong tea, *tran* nerolidol, 2-hexenal, benzaldehyde, indole, gernaiol, and benzenacetaldehyde contents have significantly decreased ($P<0.05$) by panning; however, panning increased ($P<0.05$) contents of linalool oxide, *cis* jasmone, methyl salicylate in oolong tea. The GC-O study also showed the increase of aroma active compounds with sweet descriptions and decrease of aroma active compounds with fruity and smoky descriptions by panning. Despite there were few similarities in the most abundant identified compounds from GC-MS analysis and aroma active compounds from GC-O analysis between the unpanned and panned tea, panning significantly changed the aroma volatile components of oolong tea. Since over-heating/panning the leaves may results in a burnt odor and underpanning may results in a greenish odor and red central vein, optimization of time and temperature in panning to manufacture best quality tea need to be investigated for the future studies.

Moreover, conducting sensory study to better understanding of consumer perception of panning effects on quality of oolong tea is necessary for the large scale manufacturing and commercialization of the panned tea. Results from this study can also be used in quality assessment of panned oolong tea.

According to Mintel (2013) survey, oolong tea had 50% growth in appearance in restaurant menu in 2013. The reason for this increase in demand might be result of recent studies that associated presence of polyphenols in oolong tea with health benefits. The other reason for this increase in demand might be the unique taste of oolong tea compare to other varieties of tea. Since per capita tea consumption is increasing in the U.S. (Carter, 2014), and American (especially Generation Y) seeking for flavorful alternatives to current products in the market, oolong tea might become more popular in the near future. This will result in increase in production of oolong tea and its exports to the U.S and other countries. Consequently, food industry will be seeking for appropriate instrumental methods to measure the quality of oolong teas especially the flavors. Study of flavor volatiles of oolong tea can assist tea industry in quality assessment, variety identification, and ranking/rating of different oolong tea products.

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APPENDIX A

GC-MS Chromatograms

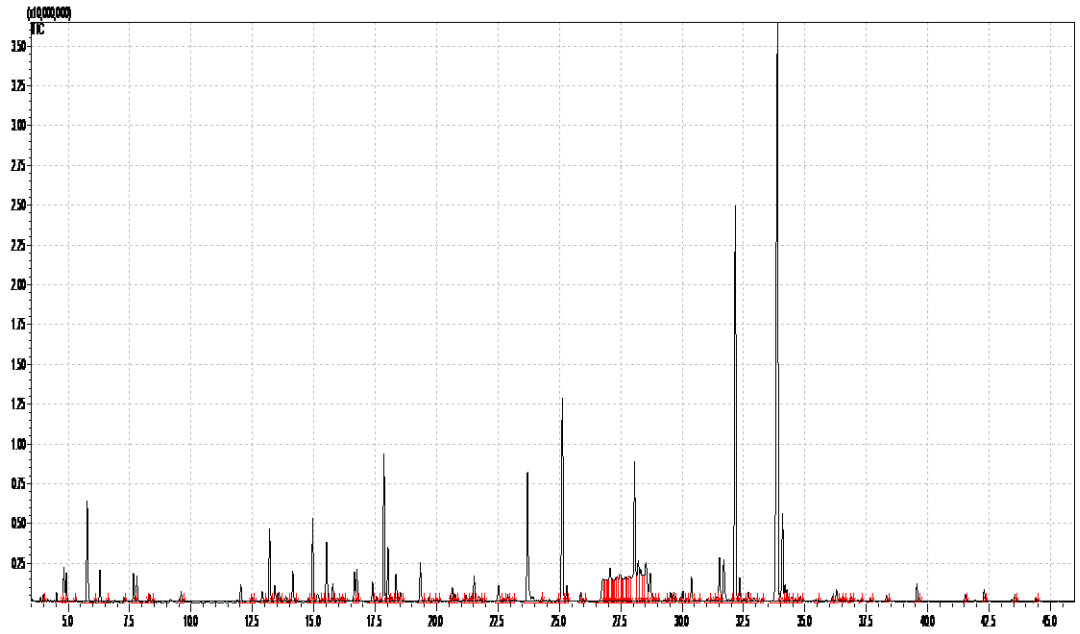

Figure A.1 GC-MS Chromatogram of unpanned Jin Xuan oolong tea after SDE extraction with diethyl ether for 20 min

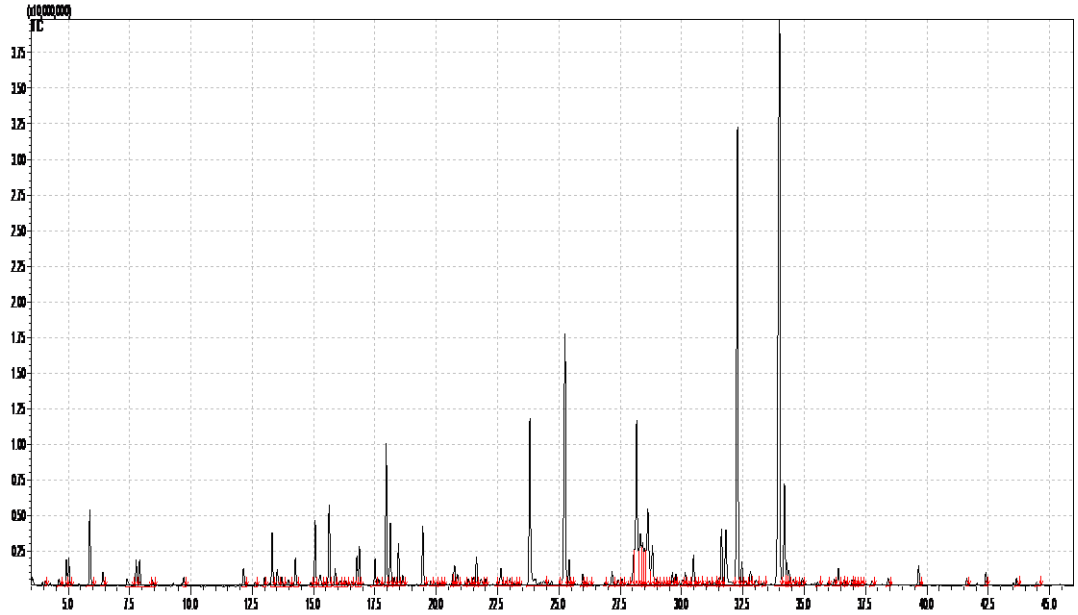

Figure A.2 GC-MS Chromatogram of unpanned Jin Xuan oolong tea after SDE extraction with diethyl ether for 40 min

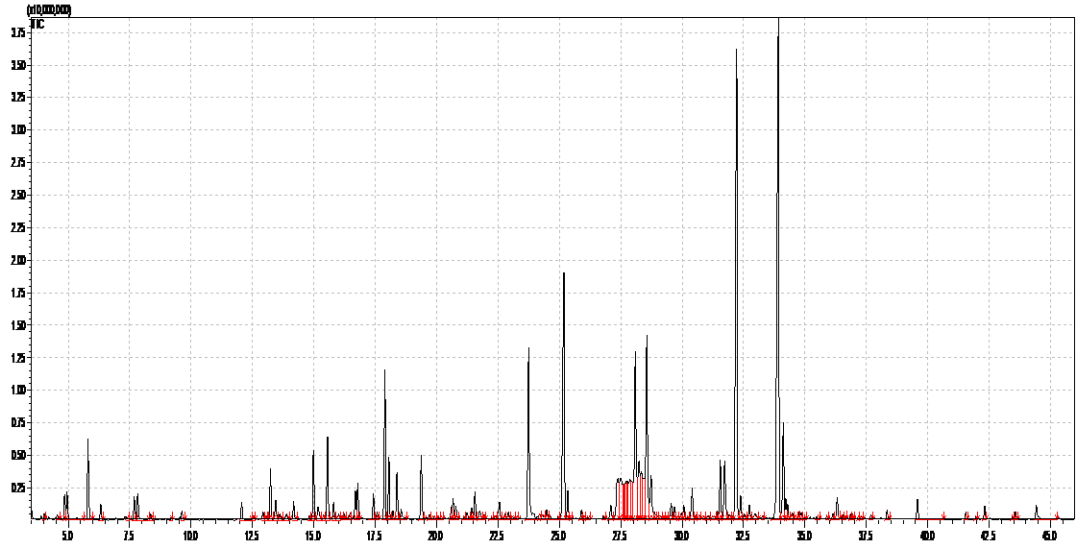

Figure A.3 GC-MS Chromatogram of unpanned Jin Xuan oolong tea after SDE extraction with diethyl ether for 60 min

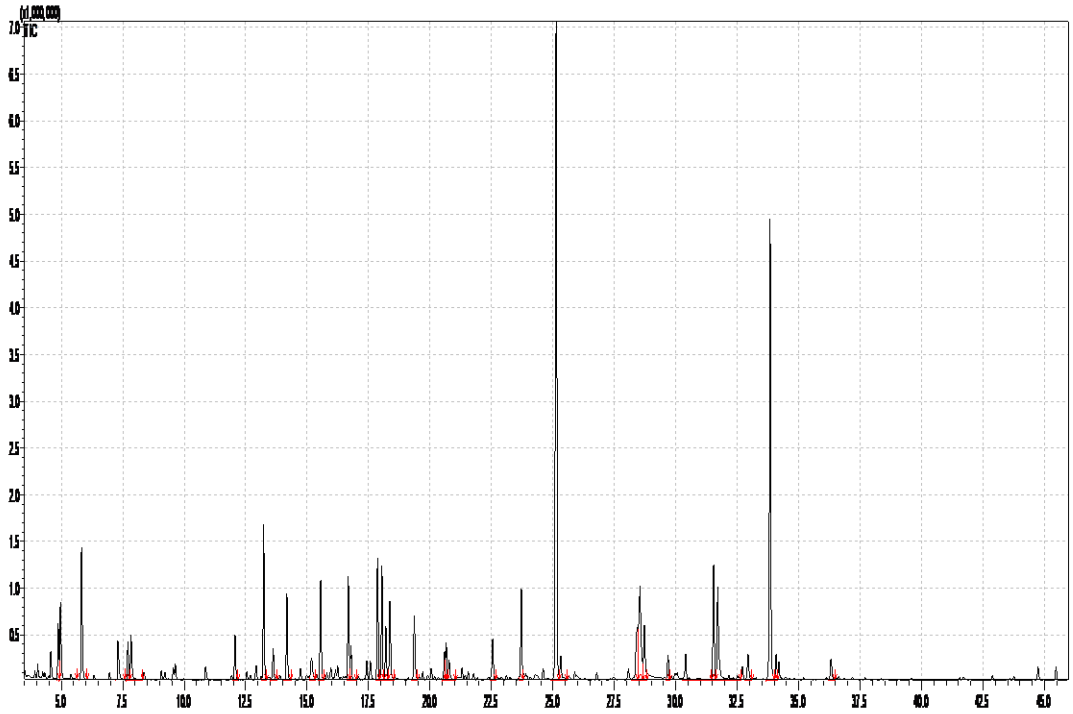

Figure A.4 GC-MS Chromatogram of unpanned Jin Xuan oolong tea after SDE extraction with dichloromethane for 20 min

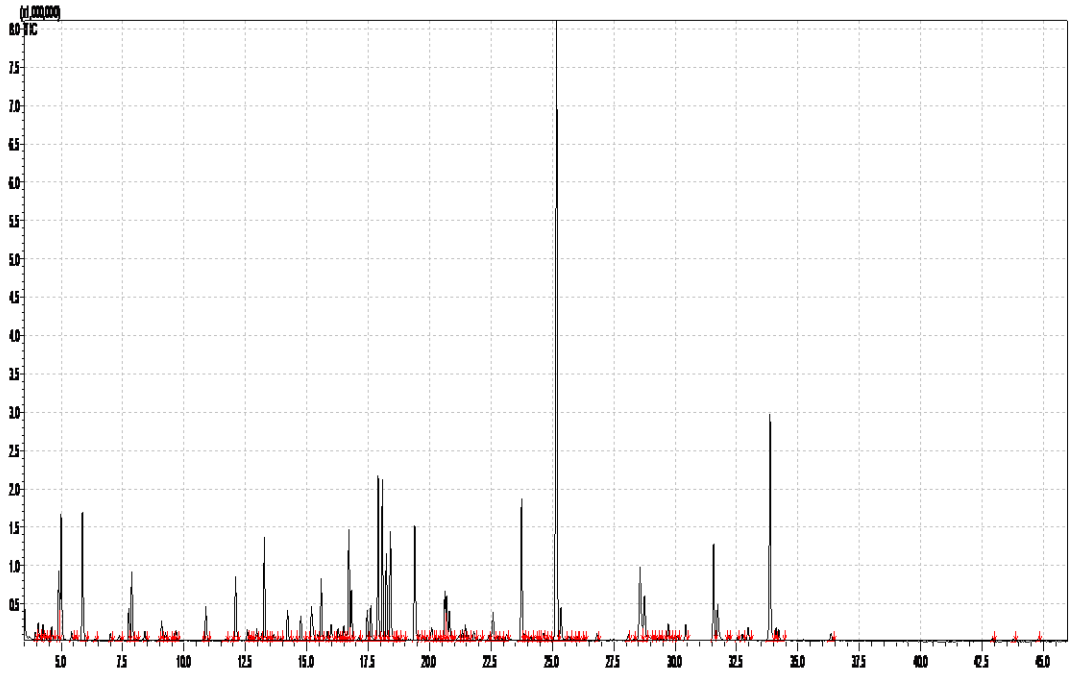

Figure A.5 GC-MS Chromatogram of unpanned Jin Xuan oolong tea after SDE extraction with dichloromethane for 40 min

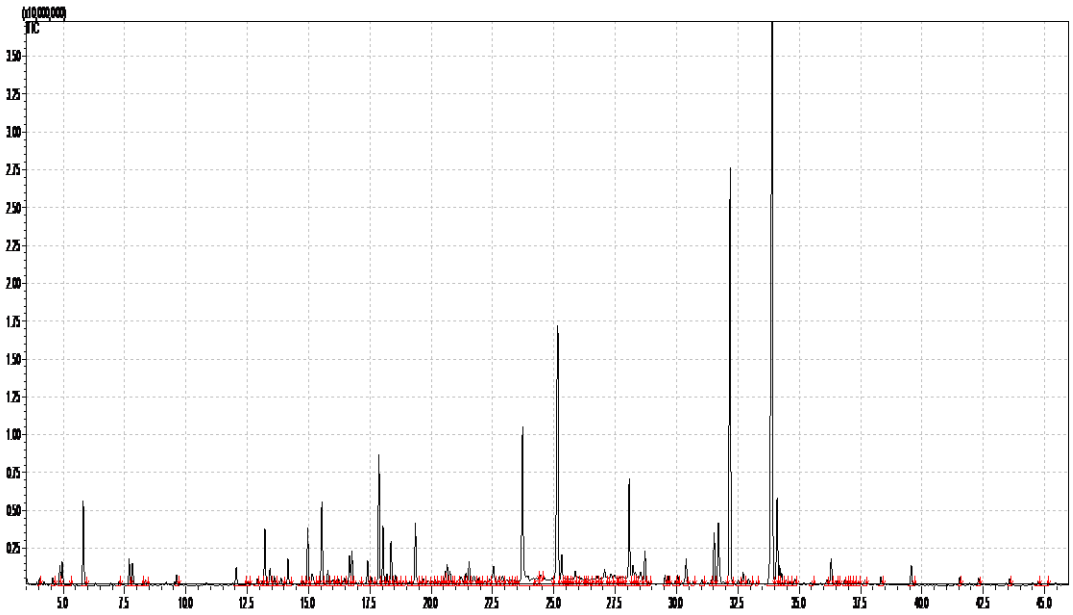

Figure A.6 GC-MS Chromatogram of unpanned Jin Xuan oolong tea after SDE extraction with dichloromethane for 60 min

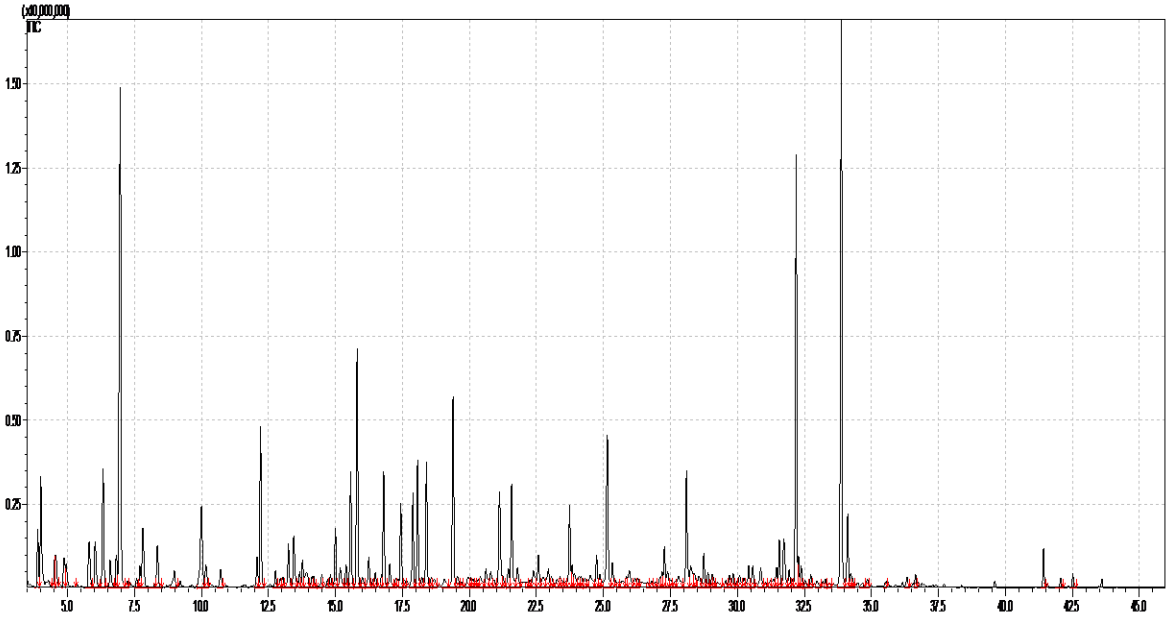

Figure A.7 GC-MS Chromatogram of panned Jin Xuan oolong tea after SDE extraction with dichloromethane for 40 min

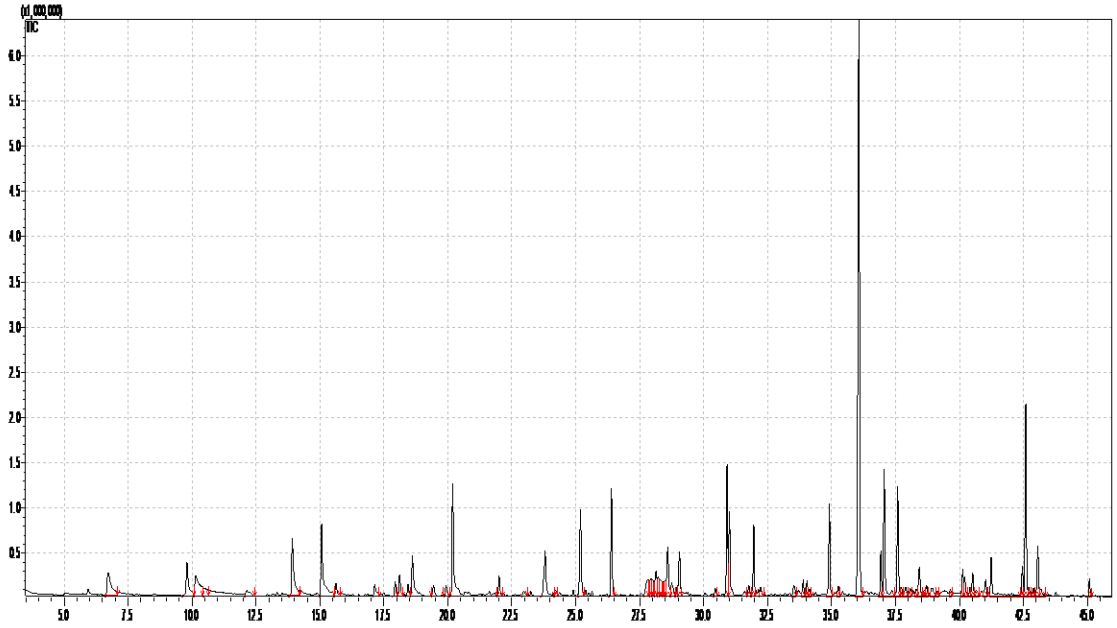

Figure A.8 GC-MS Chromatogram of unpanned Jin Xuan oolong tea from SPME extraction

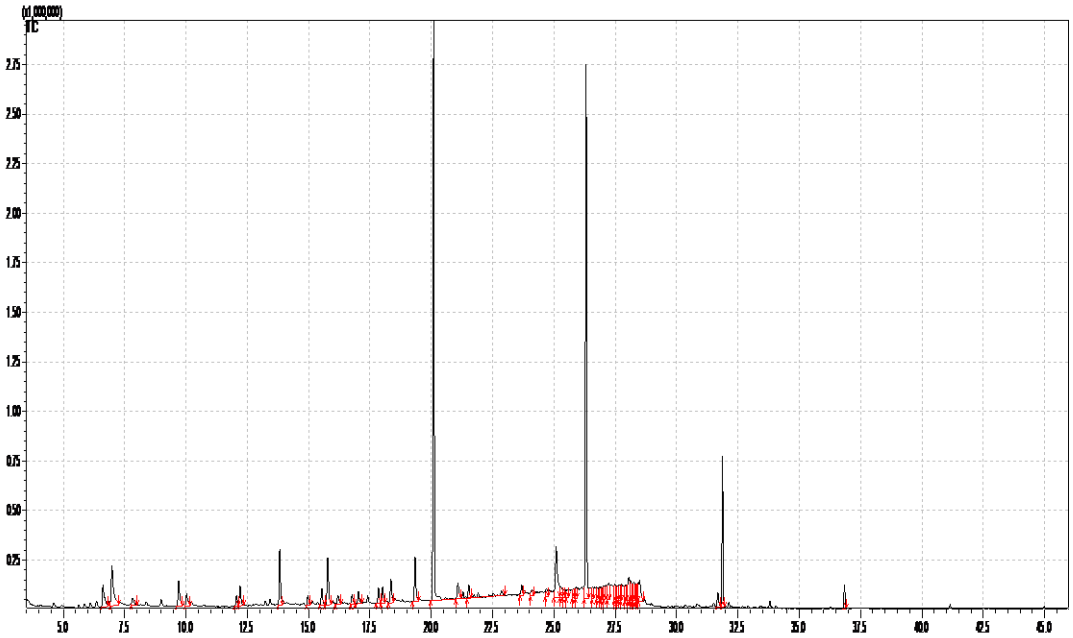

Figure A.9 GC-MS Chromatogram of panned Jin Xuan oolong tea from SPME extraction

APPENDIX B

IRB Approval Letter

VirginiaTech

Office of Research Compliance
Institutational Review Board
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
540/231-4606 Fax 540/231-0959
email irb@vt.edu
website http://www.irb.vt.edu

MEMORANDUM

DATE: June 21, 2013

TO: Sean O'Keefe, Ershad Sheibani

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)

PROTOCOL TITLE: Flavor analysis of Oolong tea

IRB NUMBER: **13-580**

Effective June 20, 2013, the Virginia Tech Institution Review Board (IRB) Chair, David M Moore, approved the New Application request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

http://www.irb.vt.edu/pages/responsibilities.htm

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As:	**Exempt, under 45 CFR 46.110 category(ies) 6**
Protocol Approval Date:	**June 20, 2013**
Protocol Expiration Date:	**N/A**
Continuing Review Due Date*:	**N/A**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

Date*	OSP Number	Sponsor	Grant Comparison Conducted?

* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

APPENDIX C

Table of Effects of Panning on 12 Major Volatiles

Table C.1 Effects of panning on peak percentage of 12 major volatiles in the oolong tea isolated from SDE and SPME techniques by GC-MS

Compounds	Odor Description	Panning Effects	*P* Value
trans-Nerolidol	Waxy, floral	Decrease	0.0015
2-Hexenal	Apple, green	Decrease	<.0001
Geraniol	Rose, geranium	Decrease	0.0007
Indole	Pungent, floral, burnt	Decrease	0.0120
Linalool	Flower, citrus	n.d. *	0.0834
Linalool Oxide	Flower, wood	Increase	0.0099
Cis-Jasmone	Woody, herbal and floral	Increase	0.0276
Methyl salicylate	Peppermint	Increase	0.0010
Jasmine lactone	Fruity, floral	n.d. *	0.8634
Benzyl Alcohol	Sweet, flower	n.d. *	0.4622
Benzaldehyde	Almond, burnt sugar	Decrease	<.0001
Benzeneacetaldehyde	Honey, floral, rose	Decrease	0.0225

*= No Significant Difference (P>0.05)

APPENDIX D

List of Chemicals for GC-O Training

Table D.1 List of aroma compounds used in GC-O training

#	Compounds	Aroma Description*
1	2-Methoxypyrazine	Sweet, nutty, chocolate
2	2,5- Dimethylpyrazine	Nutty, earthy, roasted cocoa
3	Ethylpyrazine	Nutty, coffee
4	2-Methylpyrazine	Nutty, musty
5	4,5- Dimethylthiazole	Fishy, rancid
6	2,3- Diethylpyrazine	Nutty, musty
7	2,3,5- Trimethylpyrazine	Nutty, musty, cocoa
8	2-Ethyl—3-methylpyrazine	Nutty, musty
9	2-Acetylpyridine	Popcorn,musty
10	2-Acethylthiazole	Nutty, popcorn
11	Thiazole	Meaty, fishy
12	4-methlyacetophenone	Cherry, vanilla
13	Hexanal	Green
14	Octanal	Wax, floral
15	Nonanal	Lemon peel

16	Benzaldehyde	Fruity, cherry
17	Ethlynonanoate	Apple, banana,waxy
18	p-ethylphenole	Smoky
19	Thymol	Herbal, spicy
20	Vanillin	Vanilla
21	Linalyl acetate	Citrus, floral, green
22	Linalool	Almond, sweet, woody
23	Citral	Citrus
24	Furfural	Bready, woody
25	Citronellal	Floral, citrus
26	Myrcene	Spicy, celery
27	L–Carvone	Minty
28	2-Nonanone	Soapy,fruity, cheese
29	2,3- Pentadione	Buttery, nutty
30	(+) Limonene	Citrus
31	2,3-Dimethylpyrazine	Musty, nutty
32	Diacetyl	Buttery

33	2-Undecanone	Pineapple, fatty, waxy

*= Aroma descriptions are from Flavornet, Pherobase and thegoodscentscompany database