In this issue

About TRI

2 Mission Statement and Vision
3 TRI News Updates
4 A Word from Program Manager, Lisa Bassani
5 The State of Tropical Resource Studies at Yale F&ES 2011, Director Michael R. Dove
9 Map of 2010 TRI and Compton Fellows in this issue

I. INSTITUTION BUILDING

10 The Myth of Sustainable Livelihoods: A Case Study of the Mnazi Bay Marine Park in Tanzania
   Geoffrey Mwanjela, MEM 2011
18 Gender and Community-Led Total Sanitation: A Case Study of Ekiti State, Nigeria
   Adenike Adeyeye, MEM 2011
28 A Regional Strategy for Water Resource Management in the Caribbean Region of Colombia
   Juan Pablo Vallejo, MEM 2011

II. HUMAN HEALTH

36 Characterizing the Urban Environment of Dengue Mosquitoes in Patillas, Puerto Rico
   Eliza Little, MESc 2011
43 Hawaiian Tea: The Relationship Between Chemical Concentrations, Leaf Age, and Levels of Shade
   Ran Song, MESc 2011, Dovi Kelman, Kimberly Johns, and Anthony D. Wright

III. ADAPTATION TO CLIMATE CHANGE

53 Household Level Adaptation in The Southern Region of the Yucatan Peninsula
   Ana Karla Perea Blázquez, MEM 2011
61 Farming the Fouta Djallon: The Effects of Climate on Agrobiodiversity and Household Economies
   Stephen Wood, MESc 2011
70 Distribution of Climate Change Impacts on Household Electricity Consumption in Brazil
   Guilherme DePaula, MESc 2011
76 Announcing the 2011 TRI, Compton, and Sabin Fellows

Please access the 2011 Bulletin at environment.yale.edu/tri in order to view maps, graphs, photographs, and figures in color.

All figures used in these articles are the authors’ own unless otherwise indicated.
Hawaiian Tea: The Relationship Between Chemical Concentrations, Leaf Age, and Levels of Shade

Ran Song ∆§ (MEM, 2011), Dovi Kelman©, Kimberly Johns©#, and Anthony D. Wright©*

©University of Hawaii at Hilo, College of Pharmacy, 34 Rainbow Drive, Hilo, Hawaii 96720, USA;
*Corresponding author. E-mail address: adwright@hawaii.edu; Telephone: +1-808-933-2866.
§Yale University, 195 Prospect Street, New Haven, Connecticut 06511, USA
#Sheffield Hallam University, City Campus, Howard Street, Sheffield S1 1WB, UK

Abstract

Using samples collected from a Hawaiian tea plantation, this investigation explored the relationship between tea leaf age and shade levels on the relative concentrations of three naturally occurring compounds—L-theanine, caffeine and epigallocatechin gallate (EGCG)—all of which are reported to have positive effects on human health. A High-Pressure Liquid Chromatography (HPLC) method was developed that utilized a reverse phase C-18 stationary phase and a mobile phase composed of water and acetonitrile to quantify the relative amounts of the three natural compounds. The analyses showed that the concentration of L-theanine and caffeine decreased as leaf age increased in moving from the bud to first and then second leaf. EGCG concentration increased in moving from the bud to first, and second leaves. Shade was found to have an influence only on concentrations of EGCG, exhibiting a positive correlation. Antioxidant activity, as determined using the Ferric Reducing Antioxidant Power (FRAP) assay system, was found to correlate positively with EGCG concentrations. This is the first investigation of its type using Hawaiian tea samples. The present findings show that certain chemical components of tea can potentially be used as markers for the age, quality and authenticity of various teas.

Introduction

In recent years, the consumption of green tea (Camellia sinensis) products, particularly as a drink, has become increasingly popular in western cultures because of its reported positive health effects (Dulloo et al. 1999, Venables et al. 2008, Yamamoto et al. 1997). In contrast to coffee’s long history of production in Hawaii, tea in Hawaii has been produced only in very limited quantities and has failed to enter the global market. However, recent interest has developed for producing high quality teas that can be grown and processed by smallholders and marketed in conjunction with tourism and regional cuisine (Gradisar et al. 2007). In order to achieve this end, it is essential to develop analytical methods to determine the effects of agricultural practices on the levels of desirable natural constituents that are biosynthesized by the plant.

Tea contains a group of polyphenol compounds known for their antioxidant
enzymatic antioxidants for this purpose, such as ascorbic acid (vitamin C), reduced glutathione (GSH), tocopherols, carotenoids, flavonoids, phenols, and aromatic amines (Halliwell 1999, Mallick and Mohn 2000). A variety of assays have been developed to measure the contribution of specific antioxidants, as well as the total antioxidant activity present within living cells. Numerous studies have been conducted to determine how levels of the catechin polyphenols in tea vary according to the tea varieties, agricultural conditions and processing. However, there have not been similar studies that evaluate changes in polyphenols, caffeine and L-theanine using a single analytical method. This current study aimed to develop a high-pressure liquid chromatography (HPLC) method to further investigate young tea leaves to compare the concentrations of one specific polyphenol, EGCG (1), as well as caffeine (2) and L-theanine (3) in extracts of the bud and the first and second leaves of selected tea plants. A second aim was to assess and confirm that EGCG levels in all investigated samples and pure compounds were correlated with antioxidant activity. It was hoped that the results would provide definitive evidence of whether compounds having positive effects on human health are found in the bud in higher concentrations than in the leaves underneath.

To counteract the deleterious effects of potentially harmful reactive oxygen species (ROS), aerobic cells have developed complex antioxidant systems based on low-molecular weight scavengers and specifically adapted enzymes. The major enzyme systems—superoxide dismutase (SOD), catalase, and peroxidase (Halliwell 1999, Halliwell and Gutteridge 1999)—are the primary intracellular antioxidant defenses in living organisms. However, cells also maintain a variety of non-enzymatic antioxidants for this purpose, such as ascorbic acid (vitamin C), reduced glutathione (GSH), tocopherols, carotenoids, flavonoids, phenols, and aromatic amines (Halliwell 1999, Mallick and Mohn 2000). A variety of assays have been developed to measure the contribution of specific antioxidants, as well as the total antioxidant activity present within living cells.

Recent studies of tea leaves have revealed a relationship between leaf age and concentrations of natural compounds. Young leaves are defined as the bud and the two leaves underneath the bud; all other leaves are considered to be old leaves (Lu et al. 2009, Chiu et al. 2005, Rios et al. 2001, Lin et al. 1996). Three of these studies clearly demonstrated that young leaves contain more antioxidants than older leaves (Chiu and Lin 2005, Lin et al. 1996, Rios et al. 2001).

To counteract the deleterious effects of potentially harmful reactive oxygen species (ROS), aerobic cells have developed complex antioxidant systems based on low-molecular weight scavengers and specifically adapted enzymes. The major enzyme systems—superoxide dismutase (SOD), catalase, and peroxidase (Halliwell 1999, Halliwell and Gutteridge 1999)—are the primary intracellular antioxidant defenses in living organisms. However, cells also maintain a variety of non-enzymatic antioxidants for this purpose, such as ascorbic acid (vitamin C), reduced glutathione (GSH), tocopherols, carotenoids, flavonoids, phenols, and aromatic amines (Halliwell 1999, Mallick and Mohn 2000). A variety of assays have been developed to measure the contribution of specific antioxidants, as well as the total antioxidant activity present within living cells.

Figure 1. Chemical structure of epigallocatechin gallate (1, EGCG), caffeine (2) and L-theanine (3).
of tea undertaken that has looked specifically at concentrations of the three natural products investigated as we report here, and certainly not for tea grown in Hawaii.

Some prior studies have also shown that higher shade levels seemed to increase the L-theanine and caffeine levels in tea leaves (Hirai et al. 2008, Ohta et al. 1996, Suzuki et al. 1985), while causing a decrease in the catechin (polyphenol) levels (Saijo 1980). To further investigate these findings for plants used in the current study, a specific selection of leaves was grouped and analyzed according to shade levels in order to determine whether or not a similar trend could be observed. Such findings could have ecological implications arising from the agricultural conditions used in tea production.

**Materials and methods**

**Study site selection**

On the Big Island of Hawaii, favorable geography and climate in certain regions have allowed many small tea farms, run by dedicated and enthusiastic growers, to produce fine, locally grown tea products. For this study it was essential to select a study site that was growing tea in an area that had varying shade levels and was also producing a green tea considered by local growers to be an above average product. Several farms were visited and those with too little or too much shade were ruled out. Discussions with local experts and growers led to the selection of the Mauna Kea Tea farm, whose owners granted us permission to use their plants in this investigation.

**Tea leaf collection**

Fresh leaves were collected from the Mauna Kea Tea farm on the Big Island of Hawaii. The farm is owned and operated by Takahiro and Kimberly Ino and is located at 46-3870 Old Mamalahoa Highway, Honokaa, Hawaii 96727, USA. The average elevation is 590 m and atmospheric temperature typically falls between 10 and 25°C. Annual rainfall ranges from 196 to 245 mm per year and falls mostly between December and April.

The selected study area on the plantation had an approximate area of 300 m² and a northeast-facing slope. Young seedlings, generated from cuttings in order to maintain varietal characteristics (variety: Mauna Kea #1, MK1), were transplanted from nursery into this field, and had never been harvested. The field was mostly open to the sky, except on the northwest and southwest sides where ohia lehua (*Metrosideros polymorpha*) trees form a canopy over some tea plants.

Samples were collected on July 12 and July 25, 2010. The weather on both days was cloudy. Three sets of replicate samples were collected on July 12 and nine on July 25. Samples were grouped according to prevailing shade conditions of the parent plant. Shade level (canopy closure) was determined with a

![Figure 2. Diagram showing positions of tea plants relative to shade trees and progression of sun from morning to afternoon.](image)
70-75% of their weight, assumed to be loss of water. After leaf stems had been removed, dried samples were powdered and resultant material was accurately weighed. Weighed powders were extracted with a mixture of methanol and water (70:30 v/v, 10 mL) for 30 minutes at room temperature. At the end of this period samples were filtered employing 8µm 2V grade Whatman prepleated qualitative filters (Fisher Scientific Co.) prior to HPLC analysis.

**Chemicals and reagents**

References standards of L-theanine (>99%), epigallocatechin gallate (EGCG, >95%), and caffeine (>99%) came from the Sigma Chemical Company (St. Louis, MO). Ultrapure water was prepared using a Millipore Direct-Q purification system and used in HPLC analyses as mobile phase A. HPLC-grade acetonitrile was purchased from ACROS Organics (New Jersey) and used in HPLC analyses as mobile phase B.

**HPLC-PDA analysis**

A Shimadzu HPLC system consisting of a degasser, a liquid chromatograph, auto-sampler, a photodiode array detector, column oven, and LC Solution software was used for all HPLC analyses. A 150 × 4.6 mm, 5 µm Ultra II C-18 column (Restek, Bellefonte, PA) was used for all HPLC separations. The elution program used for all analyses was: 0-2 min: 2% acetonitrile, 98% ultrapure water; 2-20 min: percentage of acetonitrile increased linearly from 2% to 20%; 20-25 min: percentage of acetonitrile increased linearly from 20% to 21%; 25-35 min: 50% acetonitrile, 50% ultrapure water; 35-40 min: percentage of acetonitrile decreased linearly from 50% to 2%; 40-45 min: 2% acetonitrile, 98% ultrapure water.

For all analyses the injection volume was 10 µL/min, with a flow rate of 1 mL/min and oven temperature of 25°C. The PDA detection wavelength range was set between 190 and 290 nm.
**HPLC method validation**

Individual calibration curves were created for each of the standards, which were L-theanine, caffeine and EGCG, dissolved in a mixture of methanol and water (70:30 v/v). For each standard, a range of stock solutions was prepared.

**FRAP antioxidant assay**

The FRAP assay employed was modified from a previous protocol (Benzie and Strain 1996, 1999). The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution, and 20 mM FeCl₃·6H₂O in a 10:1:1 ratio and heated to 37°C prior to use. The 300 mM acetate buffer was prepared by mixing 3.1 g of sodium acetate trihydrate (NaOAc·3H₂O) with 16 mL glacial acetic acid and made to 1 L with doubly distilled (dd) H₂O. The TPTZ solution was prepared by mixing equal volumes of 10 mM TPTZ with 40 mM HCl.

For the actual assays 150 µL of FRAP reagent was added to each well of a 96-well microtiter plate. A blank reading was then taken at 595 nm using a Bio-Rad microtiter plate reader. To each well 20 µL of sample (tests were done in triplicate) was then added, incubated for 8 min at room temperature and read at 595 nm. Triplicate standards of known FeII concentrations were run simultaneously using concentrations between 50 to 1000 µM of FeSO₄·7H₂O. A standard curve was plotted and the FRAP values, in µM, for each sample were determined. Since results may vary between plates, a new standard curve was prepared for each plate.

**HPLC method development**

For the investigation an analytical HPLC method was required that would enable the unequivocal determination of the amounts of L-theanine, caffeine and EGCG in the aqueous methanol extract made from dried and powdered tea leaves of various ages. With the equipment available the methods of choice were to employ a PDA detector, a stationary phase of C-18 reversed phase material, and a gradient elution employing a mobile phase consisting of water and either methanol or acetonitrile. After several trials a system was selected that generated good peak shape, produced excellent baseline separation of the three compounds of interest, had a recycle time of approximately 45 minutes, and used water and acetonitrile as the mobile phase components.

**Identification and quantification of L-theanine, caffeine and EGCG in tea leaves**

Peak, and hence compound, identification in tea leaf extracts was achieved by comparing HPLC retention times, and peak shapes in chromatograms of standards with those found in the chromatograms of prepared extracts. Quantification was done through calibration of external standards by HPLC. Calibration curves of EGCG and caffeine were obtained at a detection wavelength of 280 nm, while for L-theanine the wavelength employed was 200 nm. All calibration curves were linear over the range of concentrations examined. The relative standard deviation percent (RSD%) values were below 5% in the test of inter-day repeatability, and below 5.09% for intra-day repeatability. Thus, it was concluded that the employed method was reliable and reproducible. Here, it should be noted that it is likely that any catechin polyphenol that has the same overall HPLC characteristics as EGCG will probably contribute to all of the measurements related to EGCG.

**Results and Discussion**

**Relationship of natural component levels, leaf age and shade**

Using the developed HPLC method, concentrations of L-theanine, caffeine and EGCG were determined in the aqueous methanol extracts made from the buds, first,
and second leaves of different tea samples. The results of these analyses are shown in Table 1 and Figures 3-4. The mean percentage of each compound reflects the weight of each compound extracted from dried and powdered leaves.

From the data presented in Table 1 the differences in % L-theanine extracted from dry green tea leaves of various ages and shade levels were found to be statistically significant, as were the levels of caffeine (Table 1, Figures 4A and 4B). These findings clearly show that the concentrations of both L-theanine and caffeine decrease in going from the bud to the second leaf of the plant and that there is no obvious correlation with shade level.

From the information in Table 1, Table 2, and Figure 4C, the differences in % EGCG extracted from dry green tea leaves of various ages and shade levels were found to be statistically significant. These results show that the levels of EGCG increase with leaf age when correlated with shade level. When shade is not considered as a variable there appears to be no statistically significant correlation between leaf age and EGCG levels in the leaves examined.

Many tea experts worldwide suggest that green teas of the finest quality and flavor are made from selecting the bud and the next two leaves, or simply the bud alone, from actively growing plants. Our results suggest that the levels of L-theanine, caffeine and EGCG are, due to their concentrations, playing a significant role in helping determine this quality. Indeed, there is some suggestion that of all the amino acids founds in tea, L-theanine is the one responsible for the exotic flavor of green tea (Lin et al. 1996). At this stage optimal levels of the substances examined in this study for producing fine quality tea are not known. A next step would be to compare these levels among teas.

**Table 1.** Mean level of theanine, caffeine, and EGCG as % of dry weight (± SD) of green tea leaves of various ages.

<table>
<thead>
<tr>
<th></th>
<th>Bud</th>
<th>1st leaf</th>
<th>2nd leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean % Theanine of dry tea leaf weight</td>
<td>0.71 ± 0.19</td>
<td>0.51 ± 0.13</td>
<td>0.40 ± 0.11</td>
</tr>
<tr>
<td>Mean % Caffeine of dry tea leaf weight</td>
<td>2.86 ± 0.52</td>
<td>2.58 ± 0.53</td>
<td>1.92 ± 0.39</td>
</tr>
<tr>
<td>Mean % EGCG of dry tea leaf weight</td>
<td>5.98 ± 1.32</td>
<td>7.15 ± 1.90</td>
<td>7.18 ± 1.82</td>
</tr>
</tbody>
</table>

EGCG - Epigallocatechin gallate  
SD - Standard deviation
that are agreed upon by a group of professional tasters to be of low, medium and high quality to assess if these three substances are in some way determinant.

Also associated with the quality of tea and tea products are their reputed antioxidant properties. The ferric reducing antioxidant power (FRAP) assay is a direct test that measures the total antioxidant levels in a given sample (Benzie and Strain 1999). The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method. In this method the reduction of FeIII, as part of the ferric tripyridy1-s-triazine (FeIII-TPTZ) complex, to FeII is measured colorometrically. The assay was selected because it is considered a robust and direct method of assessing the “total” antioxidant activity of a given system, as no activity-changing interactions between antioxidants occurs in the system (Benzie and Strain 1999). The antioxidant activities of L–theanine, caffeine, EGCG and ascorbic acid are given in Table 2 and Figure 4D, and show that, among the compounds examined in

<table>
<thead>
<tr>
<th>Mean FRAP value (µM µg⁻¹ dry tea leaf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud</td>
</tr>
<tr>
<td>1st leaf</td>
</tr>
<tr>
<td>2nd leaf</td>
</tr>
<tr>
<td>Theanine</td>
</tr>
<tr>
<td>Caffeine</td>
</tr>
<tr>
<td>EGCG</td>
</tr>
<tr>
<td>Ascorbic acid</td>
</tr>
</tbody>
</table>

*EGCG - Epigallocatechin gallate
*SD - Standard deviation
In this study, only EGCG can be considered an antioxidant. In this respect, the amount of EGCG present in the extracts when compared to the levels of L-theanine and caffeine may also be significant. Clearly, levels of EGCG increase with leaf age but this is only significant when correlated with shade. Not surprisingly, the antioxidant activity of extracts examined using the FRAP assay correlates well with the levels of EGCG found. That is, the differences in antioxidant activity of extracts made from green tea leaves of various ages and shade levels (Table 2 and Figure 4D) were statistically significant. This finding shows that antioxidant activity, like EGCG concentrations, increases with leaf age when associated with shade.

Effect of shade

Another interesting observation from this study is that natural shade has a direct positive correlation with the concentration of only one of the three natural chemicals investigated. Natural shade not only influences the amount of sunlight received by tea leaves, but also affects other environmental conditions such as air temperature, humidity, and soil chemistry. These factors are also important for the actively growing tea plant and could influence leaf chemistry in different ways. It has been suggested that shade levels increase the L-theanine and caffeine concentration in tea leaves (Hirai et al. 2008, Ohta et al. 1996, Suzuki et al. 1985), while at the same time cause a decrease in the catechin levels (Saijo 1980). The results of the current study neither support nor refute the deductions concerning L-theanine and caffeine but seem to be contrary to the findings concerning the catechin EGCG. Future studies will further investigate these variables for other leaf selections and at other locations.

Ecological implications

In order to survive and reproduce, plants must protect themselves—especially their most vulnerable sections and actively growing parts—from predators and disease. The three natural products investigated in this study, particularly caffeine (Nathanson J.A. 1984) and L-theanine, may be playing a role in defending young leaves and the growing tips from predation. The concentrations of these two compounds decrease as leaf age increases, suggesting that their role is less important in more mature leaves than in younger ones.

Future Investigations

This study was primarily undertaken to provide a basis for quality evaluation of Hawaiian tea samples and to determine if there was a relationship between tea leaf age, chemical content (caffeine, L-theanine and EGCG), shade levels, and antioxidant activity.

The ultimate aim is to use these data and subsequent methods to compare this and other varieties of tea grown in other parts of Hawaii under different conditions. The results of this study are a starting point for more detailed investigations of tea grown in Hawaii. Future studies should be attempted at different locations with more precise control of shade in order to see if these observed trends are more widespread. We also plan to examine volatile constituents of tea as well as other chemical components that can help inform professional assessments of tea quality.

The developed HPLC method was shown to be a reliable and reproducible way for the determination of the chemical components L-theanine, caffeine, and EGCG found in relatively high concentrations in young tea leaf extracts. From this determination it was concluded that leaf age has an obvious effect on the concentrations of the three compounds present in the bud and first two leaves of the plants investigated. L-theanine concentrations decrease when going from the bud, to the first, to the second leaf of the plant. In contrast, caffeine concentrations do not decrease
significantly in going from the bud to the first and second leaves. The reasons underlying this variation will need to be studied further. In the absence of shade as a variable, EGCG levels do not vary significantly with leaf age. However, when shade is considered as a variable, concentrations of EGCG clearly increase with leaf age. The significance of these findings is in showing that certain chemical components of tea can potentially be used as markers for the age, quality and authenticity of various teas, and may possibly be manipulated by the agricultural conditions of the tea plant.

Acknowledgements

We are grateful to M. Ashton and G. Benoit from Yale University; F. Zee, USDA/ARS Pacific Basin Agricultural Research Center, Hilo, Hawaii; C. Hu and J. B. Friday University of Hawaii, Manoa; H.J. Noh, National Academy of Agricultural Science, Republic of Korea; Mr. Fang, E. Lee and D. Disco from the Hawaii Tea Society who were very generous with provision of information, advice, laboratory assistance and equipment. We thank H. Ernstberger, Yale University, for assisting with HPLC experimental trials and for shipping the L-theanine and EGCG standards used in this study. We are also indebted to Takahiro and Kimberly Ino, proprietors of Mauna Kea Tea, who provided us with access to their farm to enable this research to be undertaken. Finally we thank Yale University's School of Forestry and Environmental Studies, including the Tropical Resources Institute, Carpenter Sperry Fund and F&ES Summer Globalization Internships and Research Fund for financial support.

References


Lin Y. L.; Juan I. M.; Chen Y. L.; Liang Y. C.; Lin


