NUTRIENTS, POLYPHENOLS, AND TOTAL ANTIOXIDANT ACTIVITIES IN MAMAKI, *PIPTURUS ALBIDUS*

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By

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We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Human Nutrition, Food, and Animal Science (Food Science).

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CHAPTER I

Introduction

Mamaki characteristics and discoverers

The Mamaki plant (*Pipturus albidus*) is poorly characterized in the scientific literature as a traditional medicinal plant. Hawaiian *Pipturus* falls under Mamakea (derived from the Hawaiian name Mamake). Mamaki is a simple flora of *Pipturus* with its glomerules arranged in the axillary heads (Skottsberg, 1934). Skottsberg described it as "A shrub or rarely small tree with somewhat hairy stems. The bark color ranges from grayish brown to reddish brown while its flowers are small and grouped in clusters. The fruits are unusual white masses with seeds on their surface like berries. The leaves are ovate in shape, light green on the upper side, but underneath sometimes almost white with very fine short hair. They possess three main veins which are often bright red in color." The leaf texture varies from dull papery to glossy leathery.

Mamaki are found not only in the Hawaiian Islands, but also in the Philippines, Polynesia, Micronesia, and Papua New Guinea. Hawaiian *Pipturus* are known for their varieties and are difficult to classify. What distinguishes Hawaiian *Pipturus* varieties from others are the shallow cleft stipules while others have deeper cleft stipules. They are also difficult to classify because the characteristics of one species tend to overlap another species (Nicharat, 1966).

The discovery of Hawaiian *Pipturus* for scientific classification goes back to the 19th century. The major discoverers include Hillerbrand (1888) with one species and one

variety; Rock (1913) with two species; and Skottsberg (1934) and Degener (1944) with thirteen species. Skottsberg also discovered one subspecies and two hybrids. The two hybrids proposed by Skottsberg were *P. oahuensis x albidus* and *P. gaudichaudianus x hawaiiensis*.

Habitat of Mamaki

The general habitat of Mamaki is limited to areas with at least forty inches of rainfalls in the altitude range from 100 feet to 4000 feet. Its habitat consists mostly of moist and well drained soils (Gehring, 1967). Mamaki is usually found along roadsides, erosion channels, streambeds, and edges of lava flow such as the road from Hilo to Kilauea (Gehring, 1967).

Use of Mamaki

Traditionally, Mamaki bark has been used for tapa cloth and its fruits are consumed as a laxative agent and to cure thrush (Skottsberg, 1934). The leaves are typically hand-harvested and dried indirectly from the sunlight. Mamaki is currently one of the only native Hawaiian plants suitable for use in herbal tea preparation. The traditional making of Mamaki tea is by placing the fresh leaves in a gourd calabash to which fresh spring water and red-hot stones are added (Krauss, 2001). The active ingredients are presumably leached out as soon as the water reaches its boiling point. It is also used as an energy drink to lower blood pressure, reduce cholesterol, and modulate diabetes. From a medical perspective, Chun (1994) reported that the "medicine" made from Mamaki is appropriate to drink for childbirth, to discharge blood, and is also an

appropriate agent to mix with other medicines. Locker (1995) found also the benefit of Mamaki as anti-microbial agents by inhibiting the growth of *Staphylococcus aureus* and *Streptococcus pyogenes*. He also found that Mamaki showed anti-viral activity against Herpes Simplex Virus-1 and 2 and Vesicular Stomatitis virus and anti-fungal activity to a lesser extent.

Research Objectives

There is only limited information available regarding Mamaki leaves, especially their nutrients, polyphenols, and antioxidant activities. Thus, this study is a compilation of two manuscripts which involved three parts, with each of which has its own objectives.

1. Part I (Chapter 2): Nutrients Composition in Dried Mamaki Leaves and Teas

   Objectives:

   - To determine differences in nutrients and mineral composition among five varieties of dried Mamaki leaves between two seasons.
   - To determine differences in mineral composition among five varieties of Mamaki teas between two seasons.

2. Part II (Chapter 3): Polyphenols Identification in Mamaki Leaves

   Objectives:

   - To determine three major polyphenols available in four varieties of Mamaki leaves.
   - To quantify the concentration of each of the three major polyphenols available in four varieties of the Mamaki leaves.

3. Part III (Chapter 3): Antioxidant Activities in Mamaki Teas

   Objectives:

   - To quantify the total antioxidant activity available in three varieties of Mamaki teas.
   - To quantify the total antioxidant activity available in purple Mamaki teas after storage.

CHAPTER II

Nutrients and Minerals Composition in Dried Mamaki Leaves and Teas

Abstract

Mamaki, *Pipturus albidus*, is currently one of the only native Hawaiian plants suitable for use in herbal tea preparation. This study includes determination of the nutritional values in dried Mamaki leaves and teas, mineral solubilities, and mineral comparison between those in Mamaki and other commercial tea products. Five varieties, "hybrid purple", "green", "purple sub-variety 1", "purple sub-variety 2", and "panaewa", of Mamaki were harvested in winter and summer. The nutrient composition of dried Mamaki leaves and teas were analyzed using AOAC methods. Statistical analysis for dried Mamaki showed that macronutrient values, such as protein, fat, neutral and acid detergent fibers, lignin and cellulose, in summer were significantly different from those in winter. The mineral values, such as phosphorus, magnesium, boron, iron, manganese, molybdenum, and zinc, were also significantly different between the two seasons. No significant nutrient differences among the varieties of Mamaki leaves were found. Statistical analysis for Mamaki teas showed that potassium, magnesium, boron, iron, and manganese level in summer were significantly different from those in winter. There were no significant nutrient differences among the varieties of Mamaki teas. Sodium (22.1%) was the most soluble mineral while iron and manganese (0.1%) were both the least soluble minerals in infusion of Mamaki leaves to teas. Mamaki leaves contained high amounts of potassium, calcium, magnesium, zinc, iron, and copper in

comparison to those in other commercial tea leaves. Mamaki teas contained high amounts of calcium, magnesium and sodium in comparison to those available in other commercial teas.

Keyword: Mamaki, teas, mineral composition, proximate analysis, *Pipturus albidus*

Introduction

The Mamaki plant (*Pipturus albidus*) has not been well characterized as a traditional medicinal plant. Skottsberg (1934) described it as "A shrub or rarely small tree with somewhat hairy stems. The leaves are ovate in shape, light green on the upper side, but underneath sometimes almost white with very fine short hair. They possess three main veins which are often bright red or purple in color." Hawaiian *Pipturus* (or Mamaki) are difficult to classify because the characteristics of one species tend to overlap another species (Nicharat, 1966). However, the general habitat of Mamaki is limited to areas with at least forty inches of rainfalls in the altitude range from 100 feet to 4000 feet. Its habitat consists mostly of moist and well drained soils (Gehring, 1967).

Traditionally, Mamaki leaves are typically hand-harvested and dried indirectly from the sunlight. Mamaki is currently one of the native Hawaiian plants suitable for use in herbal teas preparation. The traditional making of Mamaki teas is by placing the fresh leaves in a gourd calabash in which fresh spring water and red-hot stones are added (Krauss, 2001). Chun (1994) reported that the "medicine" made from Mamaki is appropriate to drink for childbirth, to discharge blood, and is also an appropriate agent to mix with other medicines. Folklore believed that consuming Mamaki teas help them in

reducing their cholesterol level, modulating blood glucose level, and lowering blood pressure. Locker (1995) recently found the benefit of Mamaki as anti-microbial agents by inhibiting the growth of *Staphylococcus aureus* and *Streptococcus pyogenes*. He also found that Mamaki showed anti-viral activity against Herpes Simplex Virus-1 and 2 and Vesicular Stomatitis virus and anti-fungal activity to a lesser extent.

However, research on Mamaki, especially the leaves and teas, is very limited. Thus, the purpose of this study was to determine differences of the mineral and nutritional contents of dried Mamaki leaves and teas. This study also included rate of mineral solubilities when they were extracted from dried Mamaki leaves into teas. Dried Mamaki leaves and teas were also compared to other commercial tea products available in the market.

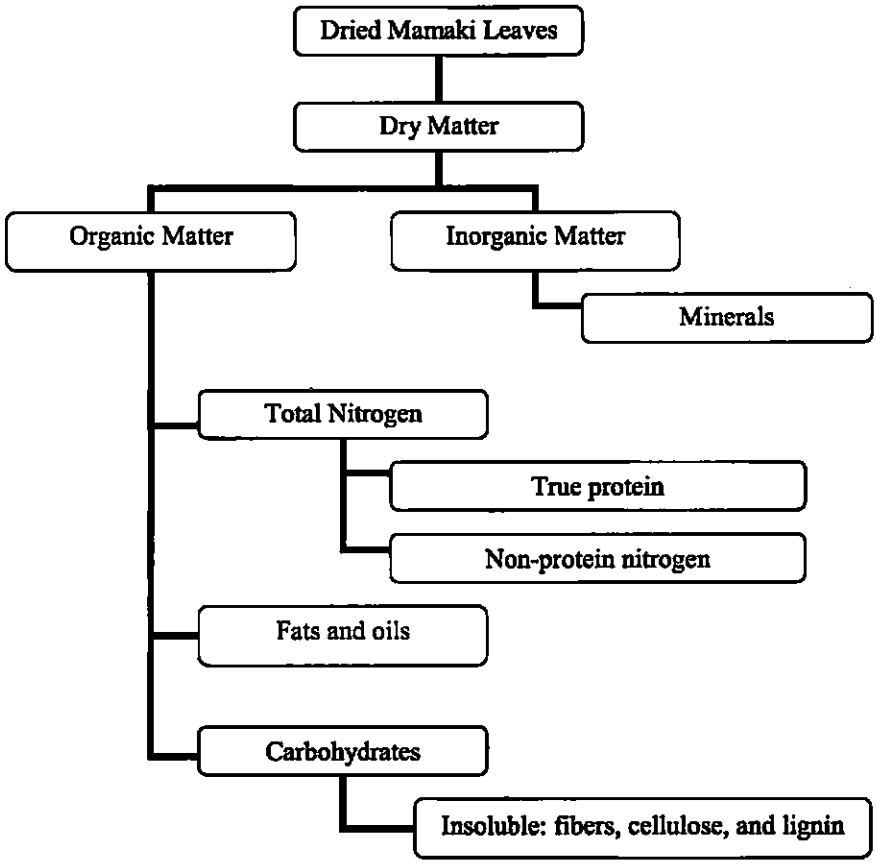
Materials and methods

Mamaki leaves were harvested from the Hawaiian Mamaki Teas Plantation in winter and summer. Five varieties (appendix) of Mamaki trees were differentiated by physical characteristics as 1) purple veined and purple leaves (sub varieties 1 and 2), 2) green veined and green leaves, 3) a hybrid plant with green leaves and purple veins, and 4) the so called "panaewa" by the farmer with green leaves and light pink veins. The main difference between purple sub-variety 1 and purple sub-variety 2 is the leaf size and color. The size of purple sub-variety 2 is about half the size of the purple sub-variety 1 and purple sub-variety 1 tends to have a deeper purple color than the purple sub-variety 2. Pictures of Mamaki leaf varieties are available on the Appendix section. The harvested leaves were weighed then dried under indirect sunlight over a two-day period. Dried

leaves were weighed again, sealed in moisture-proof containers and kept at room temperature (21°C) until further analysis.

For tea preparation of each variety, water extract was made by adding 150 ml boiling water to 1.5 g leaves and allowing the tea to stand for 30 min in a covered container. The extract was filtered with a strainer to remove all the leaf residues and bottled in a clean and sealed container. Each sample was prepared in duplicate for measurement of macronutrients and minerals. Dry matter (DM), ash, crude protein (CP), crude fat (CF), neutral detergent fibers (NDF), acid detergent fibers (ADF) lignin, and cellulose of dried leaves were determined using the standard AOAC method (Figure 1) (AOAC, 1990). The nitrogen content was estimated using micro-Kjeldhal techniques and the protein content was calculated as N x 6.25. For minerals, the content of phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), boron (B), copper (Cu), manganese (Mn), iron (Fe), molybdenum (Mo), and zinc (Zn) were determined from ash samples using inductively coupled plasma (ICP) spectroscopic method (Isaac and Johnson, 1985). The data for both Mamaki leaves and teas were then analyzed statistically with hierarchial ANOVA.

Figure 1. Nutrient and Mineral Analysis Flow Chart



Results and Discussion

1. Dried Mamaki Leaf Analysis

The nutrient compositions of five varieties of dried Mamaki leaves are presented in Table 1. Statistical analysis of the results showed that the macronutrients protein, fat, fibers (neutral and acid), lignin, and cellulose, in dried Mamaki leaves were significantly different between summer and winter. The levels of the minerals phosphorus, magnesium, boron, iron, manganese, molybdenum, and zinc in dried Mamaki leaves were also significantly different between the two seasons. The average calcium level in dried

Mamaki leaves was the highest (35 mg/g for summer and 38 mg/g for winter) while the average molybdenum level was the lowest (1 ppm for both seasons) among all of the minerals being analyzed. The average value of phosphorus, potassium, magnesium, and sodium in dried Mamaki leaves for summer were 1 mg/g, 10 mg/g, 3 mg/g and trace amounts respectively. The average value of phosphorus, potassium, magnesium, and sodium in dried Mamaki leaves for winter were 2 mg/g, 11 mg/g, 3 mg/g and trace amounts respectively. Other minerals found in the dried Mamaki leaves in trace amounts are boron (30 ppm - 36 ppm), copper (8 ppm – 12 ppm), iron (50 ppm – 54 ppm), manganese (69 ppm – 94 ppm), molybdenum (1 ppm) and zinc (12 ppm – 14 ppm). The levels of these minerals were not significantly different among the varieties.

Table 1. Macronutrients and mineral composition of dried Marnaki leaves for winter and summer seasons												
	Concentration in winter sample						Concentration in summer sample					
Nutrients & Minerals	Full green	Hybrid purple	Purple sub-var 1	Purple sub-var 2	Panaewa	Average ± SD	Full green	Hybrid purple	Purple sub-var 1	Purple sub-var 2	Panaewa	Average ± SD
Macro nutrients (mg/g)												
Dry Matter	866	862	865	864	865	864 + 1.4 [aa]	865.9	864.4	867.6	873.7	867.3	866 ± 3.5 [aa]
Ash	116	126	134	116	147	128 ± 12.9 [aa]	125.3	128.8	134.7	120.8	131.4	128 ± 5.4 [aa]
Crude Protein	135	158	137	140	136	141 ± 9.6 [ab]	80.9	68.4	82.7	75.4	69.4	108 ± 6.5 [ab]
Crude Fat	23	21	23	25	32	25 ± 4.4 [ab]	37.2	39.9	25.3	34.1	40.8	30 ± 6.3 [ab]
Neutral Detergent Fiber	309	347	324	277	316	315 ± 25.7 [ab]	227.7	243.9	275.1	263.1	213.5	280 ± 25.1 [ab]
Acid Detergent Fiber	163	197	193	174	187	183 ± 13.7 [ab]	194.3	224.3	216.1	223.8	212.5	198 ± 12.2 [ab]
Lignin	106	114	114	105	109	109 ± 4.4 [ab]	124.7	132.9	127.5	120.7	119.4	117 ± 5.4 [ab]
Cellulose	40	59	56	41	34	46 ± 10.9 [ac]	42.7	46.2	50.4	66	55.2	49 ± 9.1 [ac]
Macro-minerals (mg/g)												
P	1	2	2	2	1	2 ± 0.2 [ab]	1	0.8	1.1	0.9	0.7	1 ± 0.2 [ab]
K	9	13	14	12	10	11 ± 2.0 [aa]	10.2	7.7	8.7	7.6	7.9	10 ± 1.1 [aa]
Ca	43	36	37	39	37	38 ± 2.8 [aa]	33.8	39	35.3	31.3	35	37 ± 2.8 [aa]
Mg	3	3	3	2	5	3 ± 1.2 [ab]	2	2.1	1.8	1.6	1.4	3 ± 0.3 [ab]
Na	0	0	0	0	0	0 ± 0.0 [aa]	0.4	0.4	0.3	0.5	0.4	0 ± 0.1 [aa]
Micro-minerals (μg/g)												
B	28	23	30	31	40	30 ± 6.2 [ab]	37	43	42	41	42	36 ± 2.3 [ab]
Cu	22	11	10	8	7	12 ± 6.0 [aa]	5	5	5	5	5	8 ± 0.0 [aa]
Fe	57	59	54	48	50	54 ± 4.6 [ab]	42	43	45	49	49	50 ± 3.3 [ab]
Mn	98	105	110	80	78	94 ± 14.5 [ab]	44	42	41	37	50	69 ± 4.8 [ab]
Mo	0	1	1	1	1	1 ± 0.4 [aa]	0	1	1	1	1	1 ± 0.4 [aa]
Zn	14	13	11	14	16	14 ± 1.8 [ab]	9	9	13	9	9	12 ± 1.8 [ab]
[aa] indicates that the nutrient or mineral is not significantly different												
[ab] indicates that the nutrient or mineral is significantly different at p = 0.01												
[ac] indicates that the nutrient or mineral is significantly different at p = 0.05												

2. Mamaki Tea Analysis

The mineral composition of Mamaki tea is listed on Table 2. Among all of the minerals, the average level of potassium in the winter teas was the highest (128 μg/ml) while the average calcium was second highest (101 μg/ml) followed by the average sodium level (67 μg/ml). The average level of calcium in the summer teas was highest (103 μg/ml) followed by the average of potassium (98 μg/ml) and sodium (70 μg/ml). The average magnesium in the winter and summer teas were 35 μg/ml and 32 μg/ml, respectively. The average phosphorus in the teas from both seasons was the same (2 μg/ml). Other minerals present in the teas in very small quantities were boron (0.17 μg/ml – 0.2 μg/ml), copper (0.03 μg/ml – 0.08 μg/ml), iron (0.02 μg/ml – 0.04 μg/ml), manganese (0.07 μg/ml – 0.09 μg/ml), and zinc (0.11 μg/ml – 0.13 μg/ml). Molybdenum was not detected in the Mamaki teas.

Statistical analysis suggested that potassium, magnesium, boron, and manganese levels in the Mamaki tea were significantly different ($p<0.05$) while their iron levels were highly significantly different ($p<0.01$) between the two seasons. The phosphorus level was significantly different at actual p value of 0.095, while calcium, sodium, copper, and zinc were significantly different at actual p value of 0.216, 0.308, 0.075, and 0.767, respectively. No significant difference was found in the levels of the minerals among the varieties.

Table 2. Mineral composition of Mamaki teas in winter and summer seasons

Minerals	Concentration in winter samples						Concentration in summer samples					
	Full green	Hybrid purple	Purple sub-var 1	Purple sub-var 2	Panaewa	Average ± SD	Full green	Hybrid purple	Purple sub-var 1	Purple sub-var 2	Panaewa	Average ± SD
Macro-minerals (μg/ml)												
P	1.46	2.40	1.45	2.27	2.26	2 ± 0.5 [aa]	1.53	1.44	1.74	1.87	1.20	2 ± 0.3 [aa]
K	111.30	171.25	129.90	128.65	97.37	128 ± 27.8 [ac]	108.50	98.89	104.76	85.15	93.29	98 ± 9.3 [ac]
Ca	114.45	101.34	89.30	98.05	103.12	101 ± 9.1 [aa]	102.34	132.65	102.86	84.88	90.31	103 ± 18.5 [aa]
Mg	31.18	37.53	33.53	30.66	42.94	35 ± 5.1 [ac]	32.11	36.54	29.67	28.13	31.58	32 ± 3.2 [ac]
Na	62.56	68.07	67.22	68.92	69.54	67 ± 2.8 [aa]	71.76	80.19	66.66	62.30	67.70	70 ± 6.8 [aa]
Micro-minerals (μg/ml)												
B	0.14	0.21	0.11	0.18	0.21	0.17 ± 0.04 [ac]	0.27	0.33	0.19	0.20	0.20	0.2 ± 0.0 [ac]
Cu	0.04	0.02	0.05	0.03	0.02	0.03 ± 0.01 [aa]	0.08	0.06	0.09	0.12	0.04	0.08 ± 0.03 [aa]
Fe	0.03	0.02	0.02	0.03	0.02	0.02 ± 0.01 [ab]	0.04	0.04	0.04	0.04	0.03	0.04 ± 0.00 [ab]
Mn	0.07	0.12	0.09	0.11	0.07	0.09 ± 0.02 [ac]	0.08	0.07	0.06	0.08	0.07	0.07 ± 0.01 [ac]
Zn	0.12	0.11	0.15	0.16	0.09	0.13 ± 0.03 [aa]	0.11	0.12	0.12	0.14	0.08	0.11 ± 0.02 [aa]

[aa] indicates that the mineral is not significantly different

[ab] indicates that the mineral is significantly different at p = 0.01

[ac] indicates that the mineral is significantly different at p = 0.05

3. Extraction rate of minerals from the dried Mamaki leaves to teas

Table 3 shows the percentage of the minerals that were extracted after brewing the dried Mamaki leaves into teas for 30 min. In general, all Mamaki varieties are leaching out different minerals at about the same quantity. However, each mineral has a different solubility rate of leaching out from the leaves to water (Natesan and Ranganathan, 1990). In this case, sodium (22.1%) is the most soluble mineral among all minerals being analyzed. Magnesium (1.3%), potassium (1.1%), and zinc (1.0%) solubility rates were about the same and they were the second most soluble minerals after sodium. Even though magnesium, potassium, phosphorus, and calcium were abundant in the dried Mamaki leaves, they were not very soluble in water. Micro-minerals such as iron, manganese and zinc were not easily soluble in water as well. The least soluble minerals in the Mamaki teas were the iron and manganese, where the percent of extraction was only 0.1%. .

Several factors may play roles in mineral extractions from leaves to teas. Increased efficiency in the brewing method could increase the amount of minerals leaching out from the dried Mamaki leaves to teas. Minerals will be leached out at a higher quantity if leaves were steeped for a longer time and were extracted multiple times (Ravichandran and Parthiban, 1998). It should be noted that different mineral contents (background) of the water (tap, mineral, spring) used for the tea making will also affect the amount of minerals available in teas before the tea brewing. Processing the Mamaki leaves such as rolling, bruising, cutting, oxidation, etc as is done in conventional tea, can also affect mineral extraction rates.

Table 3. Percent (%) minerals extracted from dried Mamaki leaves to teas	
Minerals	**Percent (%) extracted**
Macro-minerals	
Phosphorus (P)	**0.1**
Potassium (K)	**1.1**
Calcium (Ca)	**0.3**
Magnesium (Mg)	**1.3**
Sodium (Na)	**22.1**
Micro-minerals	
Boron (B)	**0.6**
Copper (Cu)	**0.7**
Iron (Fe)	**0.1**
Manganese (Mn)	**0.1**
Zinc (Zn)	**1.0**
[a]Each value is calculated by average value from both seasons in table 2 (in μg/g) divided by average value from both seasons in table 1(in μg/ml) * 100%	

4. Minerals comparison between Mamaki leaves/teas to other commercial tea leaves/teas

In order to know where the mineral compositions of Mamaki leaves and teas stand, some of the minerals in the Mamaki leaves and teas are compared to other commercial tea leaves products available in the market. Data for these commercial tea products were derived from the published literature. Figures 2A and 2B show the macro-mineral and micro-mineral comparisons between dried Mamaki leaves and other commercial tea leaves. The concentration of potassium, calcium, magnesium, sodium, copper, iron, and zinc in Mamaki leaf are compared to those in black tea leaf, green tea leaf, oolong tea leaf, and Lipton tea leaf (Ferrara, et. al., 2001). Figure 2A shows that the concentrations of potassium, calcium, and magnesium in dried Mamaki leaves are much higher than those in other commercial tea leaves while the sodium level in dried Mamaki leaves is about the same as other tea leaves. Figure 2B shows that the concentration of iron and zinc in dried Mamaki leaves are also much higher than those in

other commercial tea leaves. The concentration of copper in dried Mamaki leaves is relatively higher than other commercial tea leaves.

Figure 3A and 3B shows the macro-mineral and micro-mineral comparisons between Mamaki teas and other herbal drinks. Mineral concentrations in Mamaki teas are compared to those in Echinacea tea, peppermint tea, Chinese green tea, and raspberry tea (Gallaher, et. al., 2006). Figure 3A shows that the amounts of calcium and sodium in Mamaki tea are much higher than those in other herbal drinks. We speculate that the high amount of sodium in Mamaki tea might be related to the Hawai'i's geographical location. Hawai'i is surrounded by the ocean and with its trade winds; salt spray can be blown far inland and mix with rainfall. The minerals in rain might be absorbed on the plant, or left as residues on the leaves, since the leaves are not washed as part of the processing method. As sodium is known to be the most soluble mineral in water, it is likely that the high amount of sodium in or on the leaves is easily leached out in the tea. Thus, the Mamaki tea shows higher amounts of sodium than other herbal drinks.

Figure 3B shows that the amounts of micro-minerals in Mamaki tea are about the same as other herbal teas, with the exception of the Chinese green tea. The amount of manganese in Chinese green tea, in this case, is much higher than the Mamaki tea. It should be noted that the tea extraction methods, nutrients analyses, and the statistical analyses used for other commercial tea leaves/teas are unknown, and hence may not be directly comparable to Mamaki leaf or teas.

Figure 2A. Comparison of Macro-mineral in Dried Tea Leaves

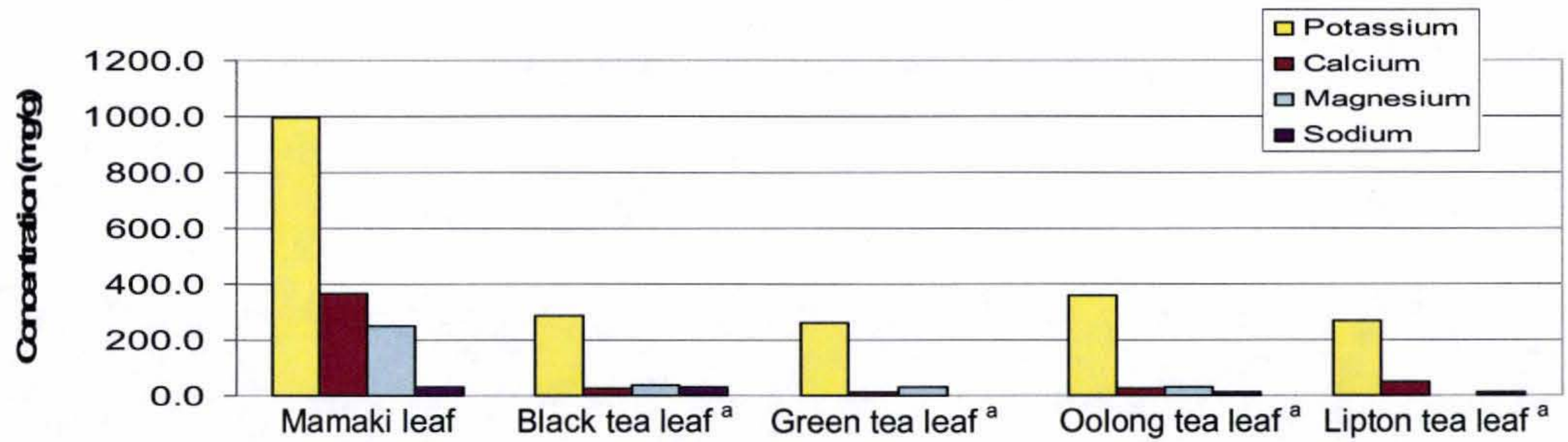
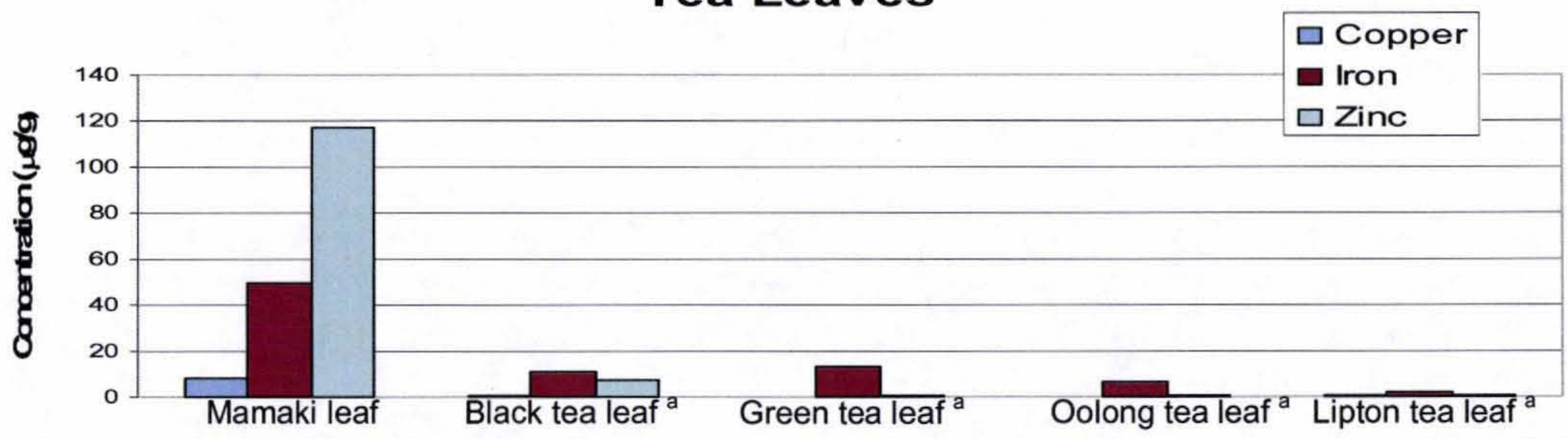


Figure 2B. Comparison of Micro-mineral in Dried Tea Leaves



[a]Reference: Ferrara, et. al. (2001)

Figure 3A. Comparison of Macro-mineral in Teas

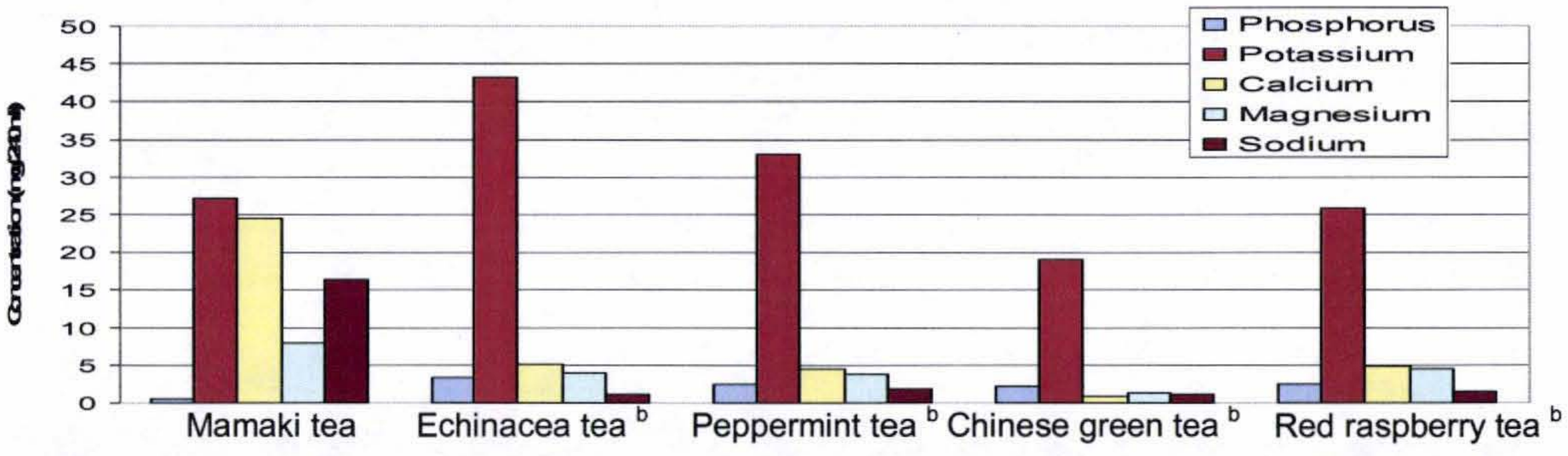
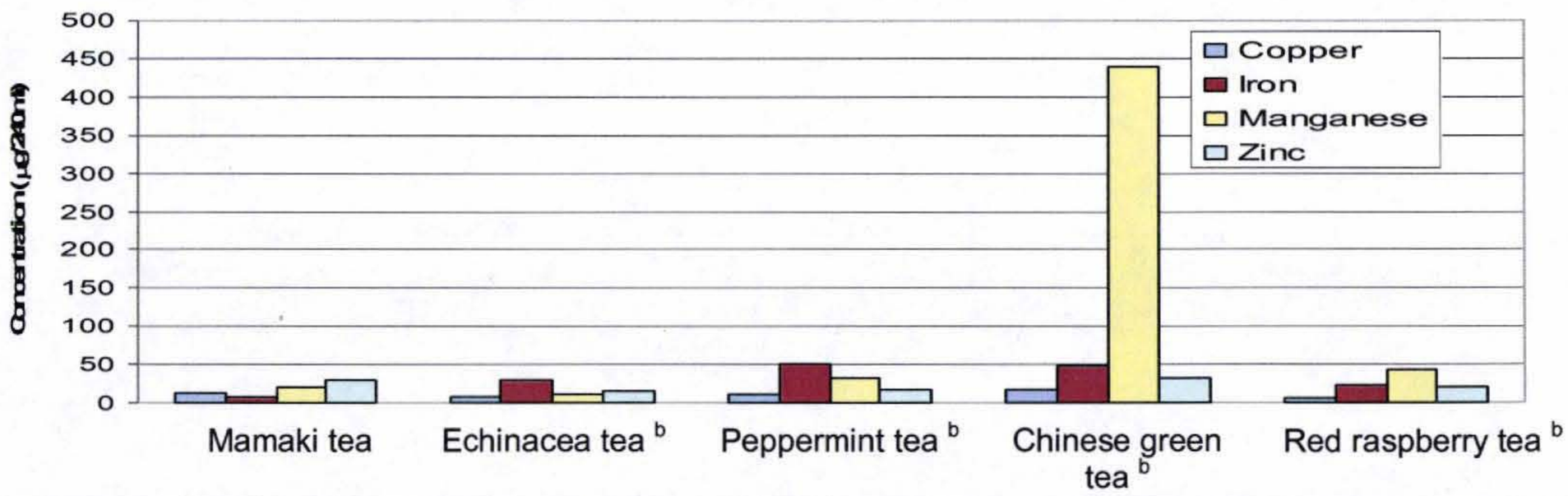


Figure 3B. Comparison of Micro-mineral in Teas



[b]Reference: Gallaher, et. al. (2006)

Conclusions

In conclusion, the levels of certain macronutrients and minerals in the dried Mamaki leaves and teas were significantly different between two seasons. Protein, fat, fibers, lignin, and cellulose of Mamaki leaves harvested in winter season were significantly different from those harvested in the summer season. The levels of phosphorus, magnesium, boron, iron, and manganese in Mamaki leaves harvested between the two seasons were significantly different as well. For Mamaki teas, the levels of iron, potassium, magnesium, boron, and manganese were significantly different between the two seasons. The solubility rates of minerals tend to vary when the dried Mamaki leaves were brewed into teas. The most soluble mineral in Mamaki teas was sodium with a percent of extraction of 22.1% and the least soluble minerals in the Mamaki teas were iron and manganese with a rate of extraction of 0.1%. Mamaki leaf contained high amount of potassium, calcium, magnesium, iron, zinc, and copper in comparison to those in other commercial tea leaves. Mamaki tea contained high amounts of calcium, magnesium, and sodium in comparison to those in other commercial teas. The high amount of sodium in Mamaki teas maybe related to the Hawai'i's geographic location.

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CHAPTER III

Phenolic acids and total antioxidant activity in Mamaki, *Pipturus albidus*

Abstract

Three phenolic acids in Mamaki leaves were identified and quantified using the LCMS method. (+)Catechins, chlorogenic acid, and rutin were identified at t_r 20.7 min, 23.0 min, and 55.6 min respectively. Concentrations of (+) catechins, chlorogenic acid, and rutin in a gram of Mamaki extract vary from 1.1 mg to 5.0 mg. The concentration of (+) catechins in Mamaki leaves was higher than those in Gyokuro green tea leaf, Chinese oolong tea leaf, and Kenya black tea leaf. The concentration of rutin in Mamaki leaves was about the same as those in Kenya black tea leaves but much higher than those in Gyokuro green tea leaves and Chinese oolong tea leaves

This study also quantified total antioxidants using the photochemiluminescence (PCL) method and was measured in equivalents to ascorbic acid (AA). Mamaki teas brewed for thirty minutes contained total antioxidant activity (TAA) ranges from 238 mg AA/gram of tea to 259 mg AA/gram of tea. Mamaki teas brewed for one hour and stored at 4 hours, 1 day, 3 days, and 6 days at 4°C had available TAA 293, 271, 172, 163, and 262 mg AA/g of tea, respectively. Mamaki teas contained relatively low amounts of TAA compared to green teas and Lipton teas.

Keywords: polyphenols identification, LCMS, Mamaki, total antioxidant activity, storage study

Introduction

Phenolic compounds are commonly found in all plants as secondary metabolites. They are derived from phenylalanine and tyrosine during the shikimic acid pathway (Hermann, 1995). They are produced in response to environmental stress such as microbial infections, UV radiation, and chemical stressors, rather than involvement in plant growth and development (Dixon and Paiva, 1995). Polyphenols in plants includes simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins (Dixon and Paiva, 1995). These phenolic compounds contributed to color, bitter and astringent taste, flavor, odor, and antioxidant properties.

Recently, there is a growing interest in the study of nutraceuticals in plants due to their antioxidative, mild estrogenic, and hypolipidemic activity. Researches indicated that phytophenolics are potent antioxidants in scavenging free radicals and inhibiting lipid peroxidation in human tissues (Rice-Evans et al., 1997; Marja et al.,1999; Sugihara et al.,1999) They play roles as reducing agents, metal chelators, singlet oxygen quenchers, and hydrogen donors (Rice-Evans et al., 1997; Marja et al.,1999; Kuo et. al., 1998; Yoshino & Murakami, 1998; Sugihara et al.,1999). Furthermore, studies show that these phenolic compounds are linked with lower occurrence and lower mortality rates of various human diseases (Block et al., 1992; Ames et al., 1993; Vinson et al., 1995; Geleinjese et al.,2002).

Traditionally, Mamaki or *Pipturus albidus*, were used by native Hawaiian to ease childbirth, to discharge blood, and to alleviate listlessness (Chun, 1994). Previous studies also found that parts of Mamaki plants have anti-microbial, anti-viral, and anti fungal properties (Locker, 1995). Recently, Mamaki tea leaves became well-known in therapeutic

usage for alleviating various pre-existing diseases and according to folklore Mamaki leaves are a potential natural therapeutic medicine in regulating blood sugar levels, blood pressure and cholesterol levels.

However, due to limited available information on Mamaki and especially the leaves, more extensive research on its chemical and antioxidant properties are necessary. Thus, the purpose of this study is to identify and quantify three major polyphenols available in Mamaki leaves. Since Mamaki leaves are commonly prepared as herbal teas, this study also quantifies the total antioxidants available in Mamaki teas after being brewed for 30 minutes and 1 hour. Total antioxidants were also quantified after Mamaki teas were stored for 4 hours, 1 day, 3 days, and 6 days in the refrigerator.

Materials and methods

1. Identification and Quantification of Mamaki leaves

1a. Chemicals

Optima grade solvents including methanol, acetic acid, and formic acid were obtained from Fisher Scientific (Pittsburg, PA). (+) catechins, chlorogenic acid, caffeine, and rutin were purchased from Sigma-Aldrich (St. Louis, MO). Water was purified and filtered at all times. The water was passed through a Milli-Q water purification system (Millipore Corp., Bedford MA) set at 18 MΩ-cm resistance. HPLC solvents were filtered through Nylon 66, 47-mm i.d., 0.45-μm pore size (Millipore Corp., Bedford MA).

(+) Catechins, chlorogenic acid, and rutin were each dissolved in methanol and sonicated until the powder dissolved into solutions. (+) Catechins was prepared at 200, 400,

and 800 μg/ml. Concentration of chlorogenic acid was 100, 500, and 1000 μg/ml. Rutin was prepared with concentration of 80, 400, and 800 μg/ml.

1b. Mamaki sample preparation

Four varieties of fresh Mamaki leaves were hand harvested, placed in labeled zip lock bags and chilled in a cooler immediately. They were distinguished by leave physical characteristics as: 1) purple veined and purple leaves, 2) green veined and green leaves, 3) a hybrid plant with green leaves and purple veins, and 4) the so called "panaewa" by the farmer with green leaves and light pink veins. For each variety, Mamaki leaves were ground in a clean vegetable chopper with dry ice until it gave a uniform ground. Ground Mamaki leaves were then homogenated and lyophilized at 0.2 Pa for more than 48 hours and stored in the refrigerator before use.

1c. Extraction of polyphenols

0.5 gram of Mamaki samples were extracted with 12 ml of 90% methanol containing 0.5% acetic acid. The solution was sonicated for 3 minutes followed by centrifugation at 3000 rpm for 15 minutes. After three times of extraction, the supernatants were dried with vacufuge concentrator (Model 5301). The residues were dissolved in methanol and filtered through a PTFE 0.25-μm membrane filter (Millipore Co., Bedford, MA). The solution was concentrated to a final volume of 1.5 ml by drying under a nitrogen gas flow.

1d. LCMS conditions

The LC-MS system is an Agilent Series 1100 LC/MSD instrument (Agilent Technologies, Wilmington, DE) consisted of an Agilent 1100 series liquid chromatograph with a diode array detector and a single quadrupole mass spectrometer equipped with an electro spray (ESI) source. Mass spectra were detected in negative ion mode in a mass range of m/z 110-1000. The compounds were monitored at 250, 280, 320, 370, and 510 nm. The drying gas was nitrogen at a flow rate of 10 L/min. The carrier gas was also nitrogen set at 25 psi and 350°C.

Two variables, the fragmentor voltage and the capillary voltage, were modified in order to optimize the LCMS method. Fragmentor voltage is the most frequent element for method optimization because it has the greatest impact on sensitivity and fragmentation (HP Manual, 1998). The optimized fragmentor voltage will provide a stronger molecular ion and good relative abundance of fragment ions (HP Manual, 1998). The optimized fragmentor voltage was 120 V. Capillary voltage (Vcap) is a less frequent element for method modification. Normally, it is set between 2500 V and 5000 V to obtain broad optimum for most compounds. Generally, smaller compounds are optimized at lower Vcap and larger compounds are optimized at higher Vcap (HP Manual, 1998). Since it is expected that there will be plenty of large molecules in the Mamaki leaves, the capillary voltage chosen for the study is 4000 V.

For the analysis, LC separations were performed with a Phenomenex Luna, C-18 column (4.6 x 250 mm, 5-μm i.d.) joined with a guard column (4 x 3 mm i.d.) at 35°C. The gradient elution was set up according to Sakakibara et al. (2003) method with some modification. Gradient elution was carried out with solvent A, composed of water and 0.1%

formic acid, and solvent B, comprising 100% methanol, delivered at a constant rate of 1ml/min as follow: initially 100% of solution A, for the next 15 minutes, 70% of solution A, for another 30 minutes, 65% A, for another 20 min, 60% A, for another 5 min, 50% A, and finally for the last 25 min, 0% A. The injection volume for the extract was 10 μl.

2. Antioxidant activity of Mamaki tea

2a. Mamaki tea preparation

Three varieties of dried *Mamaki* leaves; 1) purple veined and purple leaves, 2) green veined and green leaves, and 3) a hybrid plant with green leaves and purple veins were used for the antioxidant analysis. For tea preparation of each variety, a water extract was made by adding 150 ml boiling tap water to 1.5 g leaves and allowing the tea to stand for 30 min in a covered container. The extract was filtered with a strainer to remove all the leaf residues and bottled in a clean and sealed container. The Mamaki teas were prepared in triplicates and analyzed for their water-soluble antioxidant activities.

For the storage study, teas from the purple variety were prepared by steeping 1.5 gram of leaves in 150 ml of boiling water for 60 minutes. These Mamaki teas were then stored for 4 hours, 1 day, 3 days, and 6 days in the refrigerator. The Mamaki teas were prepared in triplicates and each replicate had three sub-samples to ensure accuracy in measurement. Tea samples were then diluted with water before antioxidant analysis as follows: 1 ml of tea was measured directly for its antioxidant activities. Then, after 4 hours of storage, the tea was mixed with 125 ml water and analyzed for its antioxidant activities. For 1 day, 3 days, and 6 days of storage, the tea was mixed with 175 ml of water and analyzed for its antioxidant activities.

2b. Antioxidant Study

Instrumentation used for the antioxidant analysis was a Photochem® system (Analytik Jena AG, The Woodlands, TX). The system enables quantification of antioxidant capacity of water-soluble substances based on photochemiluminescence (PCL). This includes photochemical excitation to generate free radicals (superoxide anion radicals), followed by luminescence detection (luminol). The free radicals generated by the optical excitation of the photosensitizer substance are partly eliminated by the reaction of antioxidants in the sample to be analyzed. In a measurement cell, the luminescence of the detector substance (luminol) generated by the remaining radicals is measured and thus the quantity of antioxidants present in the sample is determined in equivalent to ascorbic acid.

The standardized kits used for the analysis were obtained from Analytik Jena AG (The Woodlands, TX). They were used for the measurement of integral antioxidative capacity of substance mixtures which consisted of four reagents: ACW-Diluent (reagent 1), reaction buffer (reagent 2), photo-sensitizer and luminol (reagent 3), and ascorbic acid standard (reagent 4).

2c. Antioxidant Reagents and Mamaki Tea Samples Preparation

The ascorbic acid standard (reagent 4) stock solution was prepared by mixing concentrated ascorbic acid standard with 490 μl of ACW-Diluent (reagent 1) and 10 μl H_2S0_4. The calibration standards were prepared daily with the ratio according to the following table:

Calibration standard	R1 (μl)	R2 (μl)	R3 (μl)	R4 (μl)
1	1500	1000	25	0
2	1490	1000	25	10
3	1485	1000	25	15
4	1480	1000	25	20
5	1475	1000	25	25

For the sample, an aliquot of 20 μl of Mamaki tea samples was mixed with 1480 μl of R1, 1000 μl of R2, and 25 μl of R3

Results and Discussion

1. Identification of three main phenolic acids in Mamaki extracts.

Figure 4 represents a typical HPLC profile of Mamaki leaves. The phenolics were observed at UV_{max} of 250, 280, 320, 370, and 510 nm. The main chromatogram difference among the four varieties of Mamaki leaves is that the "green" variety does not show a peak at t_r ~21min with UV_{max} of 510 nm. "Purple", "hybrid", and "panaewa" varieties show the peak at UV_{max} of 510 nm with strongest intensity in the chromatogram of the "purple" variety and the weakest intensity in the chromatogram of the "panaewa" variety.

A phenolic compound at UV_{max} 510 nm is very rare and usually belongs to the anthocyanins group. This indication does relate to the physical feature of the Mamaki leaves. All of the Mamaki varieties, with the exception of the green one, have a purplish or reddish color either on the leaves, the vein, or both. With the m/z ion of 465, it is possible that this molecule is cyanidin glucoside. Cyanidin has a molecular weight of 286 and glucose has molecular weight of 180. Binding these two compounds together cause the unknown to elute early (t_r ~21min). However, further confirmation with the standard is necessary to identify the mystery compound.

Figure 4. A typical chromatogram of Mamaki Leaf

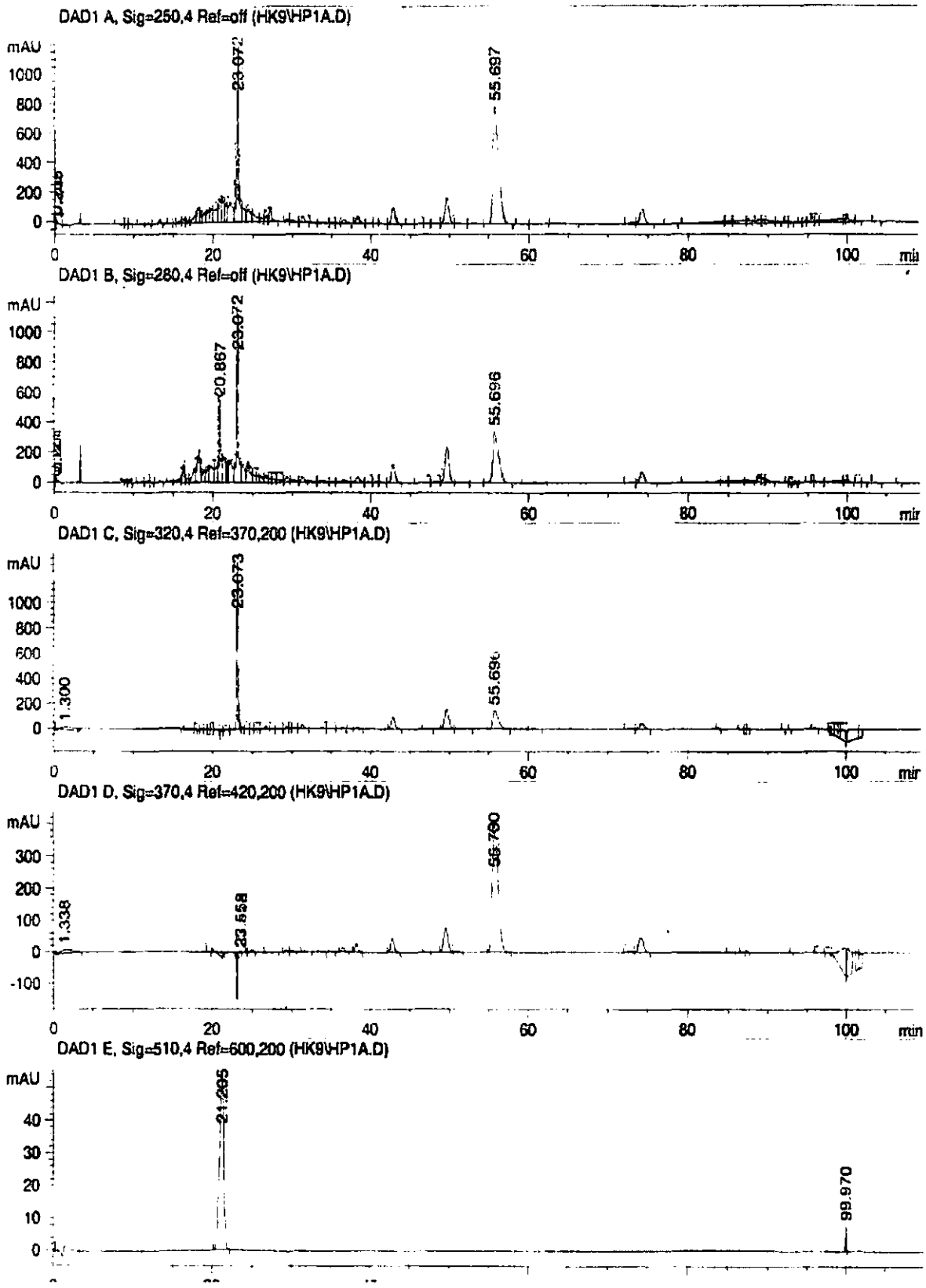
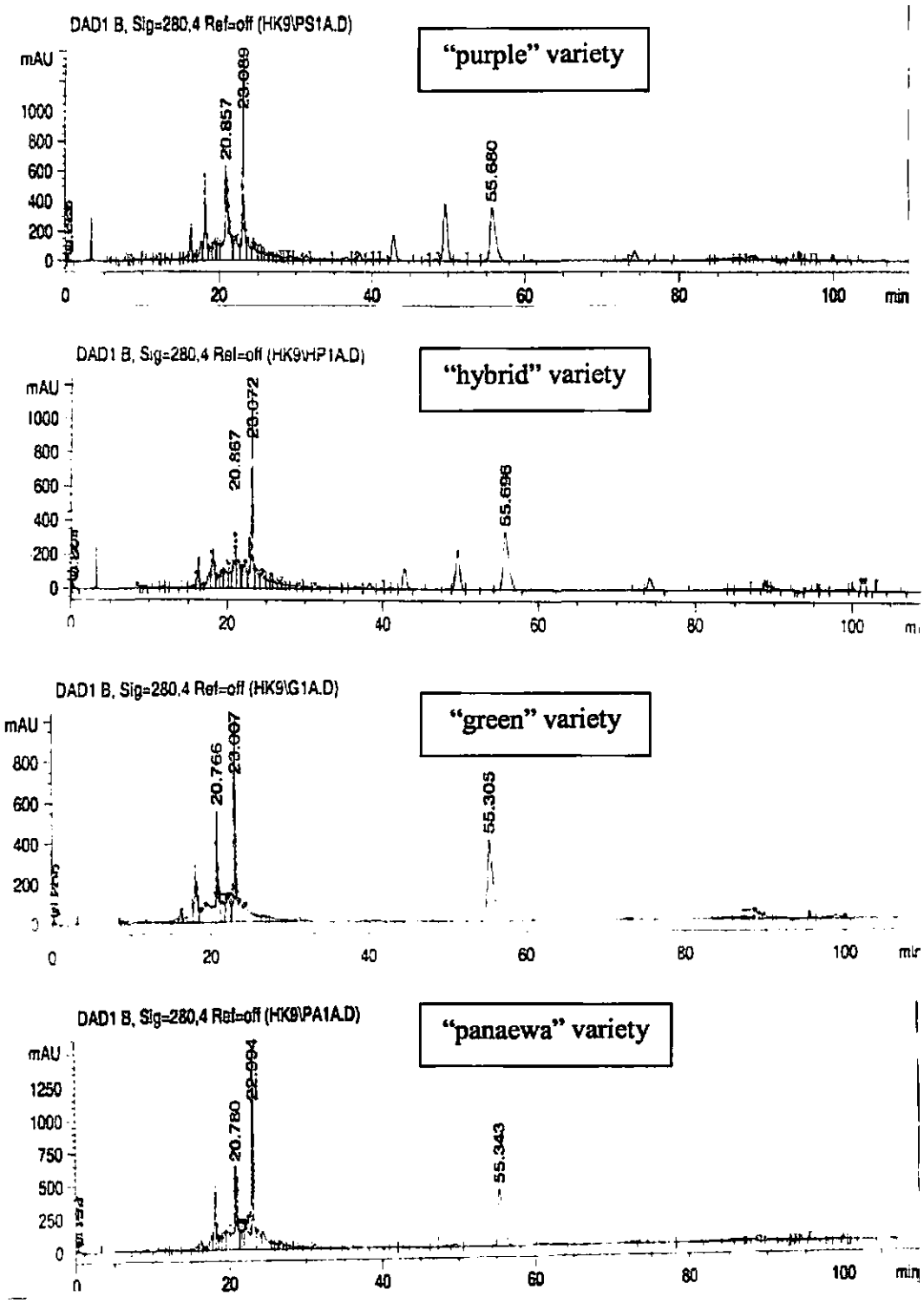


Figure 5 represents the chromatograms of the four varieties of Mamaki leaves at UV_{max} of 280 nm. The three strongest and well-defined peaks of the phenolics were then selected for identification. These three phenolics were at retention times of 20, 23, and 55 min and were identified as chlorogenic acid, (+) catechins, and rutin, respectively. Their retention time and molecular ions matched those of the standards.

These phenolics could also form dimmer compounds or conjugate compounds that are commonly formed in complex plant extracts. Because of the binding of the different types of sugar molecules, the phenolics tend to elute earlier than their aglycone alone (Hong, 1990). In a reverse-phase HPLC column, molecules that bind to galactose tend to elute faster than glucose and arabinose due to the degree of glycosilation and the nature of the sugar moieties (Hong, 1990). This theory was proven with the phenolic profile from Sakakibara (2003) which shows that the retention of rutin is 40.6 minutes while the retention time of quercetin is 75.5 minutes. In the Mamaki case, the sugar molecule of rutinose binds to the quercetin molecule and caused rutin (quercetin-3-O-rutinoside) to elute at a retention time of ~55 min. It is likely that quercetin will elute at a longer retention time than 55 min. However, such hypothesis has to be confirmed by testing the quercetin standard.

Figure 5. Chromatograms of Four Varieties of Mamaki Leaves



2. Quantification of three main phenolic acids in Mamaki extracts.

After the method was optimized, the standards were injected at three different concentrations. The calibration standards were analyzed using regression methods and showed R^2 equal or near to one. The concentrations of the phenolics available in Mamaki extracts are shown in Table 4. (+) Catechins were the most abundant phenolic among the three identified phenolics. Among the four Mamaki varieties, the purple variety contained the highest amount of (+) catechins and the panaewa variety contained the lowest amount. The concentrations of (+) catechins were quantified at UV_{max} 280 nm and they ranged from 2.1 mg/g to 5 mg/g. The concentrations of chlorogenic acid were quantified at UV_{max} 320 nm and they ranged from 1.1 mg/g to 1.7 mg/g. The concentrations of rutin were quantified at UV_{max} 370 nm and they ranged from 1.1 mg/g to 1.8 mg/g.

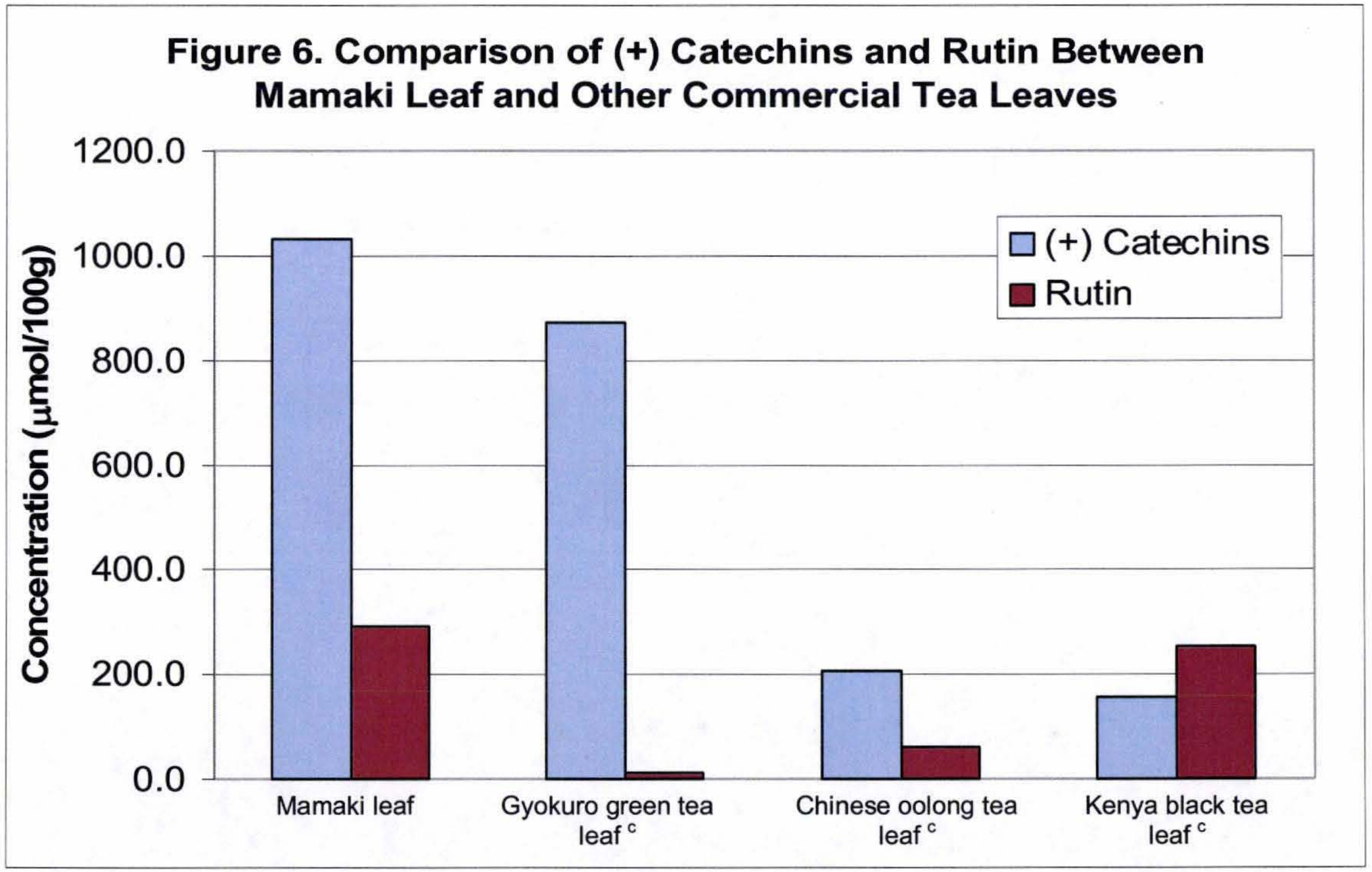
The benefits of each of these phenolic acids have been established in the literature. For example, (+) catechins is commonly found in green tea and plays a role as an antioxidant against cancer, obesity, diabetes, cardiovascular disease, aging, and neurodegenerative diseases. (Zaveri et. al., 2006). Chlorogenic acid is commonly found in root vegetables such as carrot, radish, turnip, and burdock (Sakakibara, 2003). Chlorogenic acid in hawthorn fruit is found to be beneficial as an antioxidant against LDL oxidation. (Zhang et. al., 2001; Wang et. al., 2006). Chlorogenic acid is also found in blueberries and play roles in browning reaction and polymerization (Kader et. al, 1997) Rutin is commonly found in red wine, buckwheat, citrus, and tomato skin (Heims et al., 2002). An animal study showed that rutin plays a role as an antioxidant and was effective in controlling animal body weight (Gao et. al., 2003)

Table 4. Concentration of (+) catechins, chlorogenic acid, and rutin in Mamaki leaves			
	Concentration of phenolic acid (mg/g)		
Mamaki variety	(+) Catechins	Chlorogenic acid	Rutin
Green	2.4 ± 0.0	1.1 ± 0.1	1.1 ± 0.2
Hybrid purple	2.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.0
Purple	5.0 ± 0.8	1.7 ± 0.1	1.8 ± 0.1
Panaewa	2.1 ± 1.4	1.7 ± 0.1	1.8 ± 0.1

3. Comparison of polyphenols in Mamaki leaves with other commercial tea leaves

Figure 6 shows a comparison of the amount of (+) catechins and rutin in Mamaki leaves and other commercial tea leaves. The commercial tea leaves used for the comparison are Gyokuro green tea leaf, Chinese oolong tea leaf, and Kenya black tea leaf (Sakakibara, 2003). Chlorogenic acid is commonly found in root vegetables such as radish, carrot, turnip, and burdock (Sakakibara, 2003). Because it is different part of the plant, there is no comparison for chlorogenic acid. The concentrations of (+) catechins available in Mamaki leaf is higher than in other commercial tea leaves, especially the Chinese oolong tea leaf and Kenya black tea leaf. The concentrations of rutin in Mamaki leaf are also higher than other tea leaves, especially the Gyokuro green tea leaf. It is noted that different instruments, solvent compositions, and methods were used in the quantification of the (+) catechins and rutin between in the Mamaki leaves and those in other commercial tea leaves.

Figure 6. Comparison of (+) Catechins and Rutin Between Mamaki Leaf and Other Commercial Tea Leaves



[c]Reference: Sakakibara, et. al. (2003)

4. Quantification of antioxidants in Mamaki teas.

Three varieties of Mamaki leaves were chosen for total antioxidant activity (TAA) in teas and were quantified in equivalents to ascorbic acid (AA). The three Mamaki leaves selected for the study were the purple, hybrid, and green varieties. Purple Mamaki tea was also selected for the storage study.

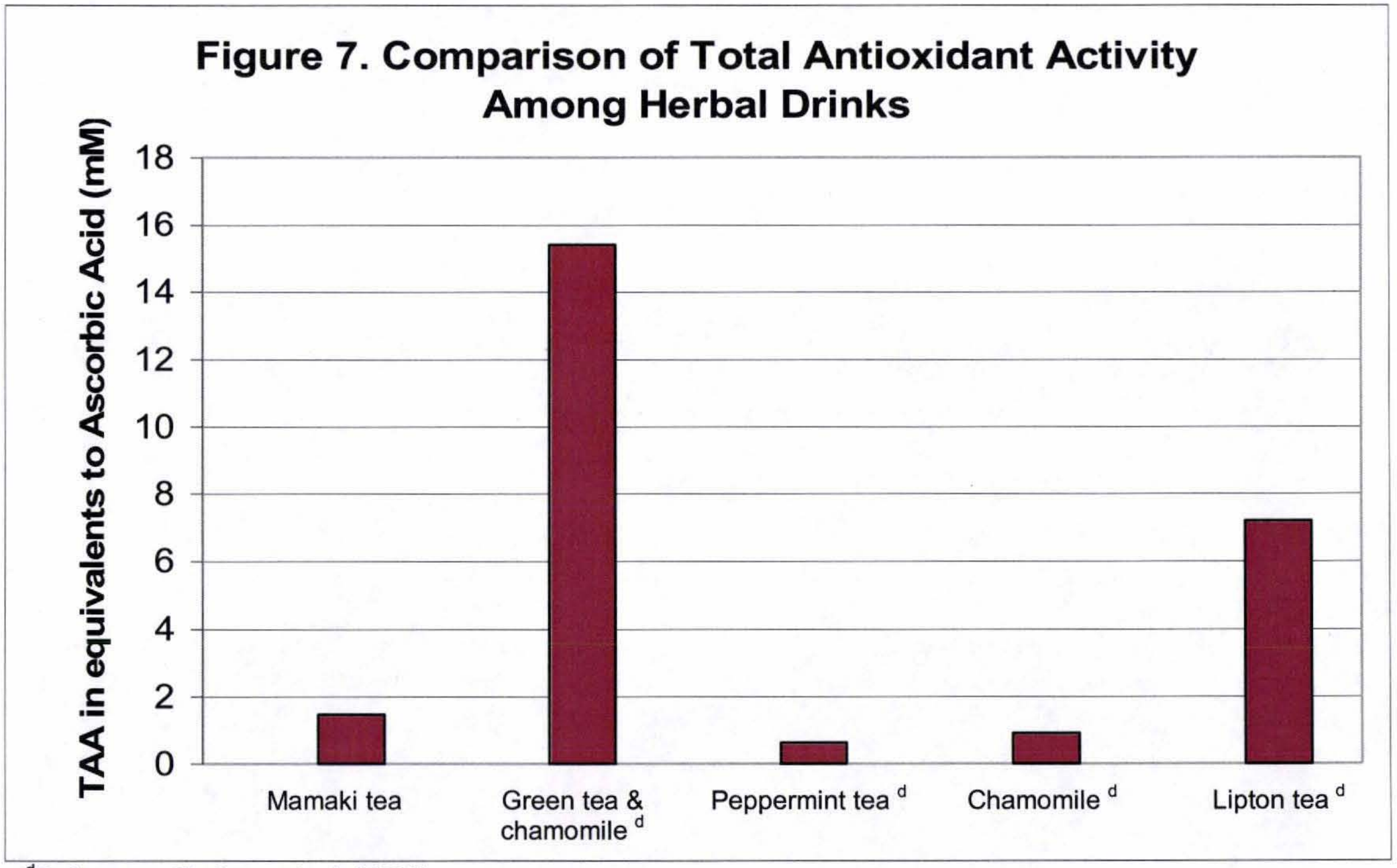
Table 5 shows that all Mamaki varieties contain about the same amount (mg) of TAA in a gram of brewed tea. The average amount of TAA available in the purple, hybrid, and green Mamaki teas are 238 mg, 2434 mg, and 259 mg respectively.

Table 5. Total antioxidant activity in Mamaki teas (mg AA/g)

Mamaki tea	Winter	Summer	Average $\pm$ SD
Purple	253.9	221.6	237.7 $\pm$ 22.8
Hybrid	236.3	250.9	243.6 $\pm$ 10.4
Green	306.7	211.3	259.0 $\pm$ 67.4

The amount of TAA available in Mamaki teas is also compared to those in other commercial teas (Figure 7). Other commercial teas used for the comparison are peppermint tea, chamomile tea, Lipton tea, and a mixture of green tea and chamomile tea (Shpigun, et. al., 2006). In this case, the TAA available in Mamaki tea is much lower than those in Lipton tea and the mixture of green tea and chamomile tea. However, different methods of antioxidant quantification and tea preparation may cause such large differences. Further investigation is necessary to confirm these results.

Figure 7. Comparison of Total Antioxidant Activity Among Herbal Drinks



[d]Reference: Shpigun, et. al. (2006)

The purple variety of Mamaki is selected for the storage study since color often indicates the abundance of antioxidants. Freshly brewed purple Mamaki teas were prepared for the storage study. The highest TAA (293 mg AA/g tea) is immediately after Mamaki tea was brewed for an hour while the lowest TAA (163 mg AA/g tea) is for a three-day stored Mamaki tea (Table 6). More research is needed to explain the apparent increase after six days of storage. Total antioxidant activity stabilized over time, as indicated by the decreasing standard deviations as storage time increases.

Table 6. Total antioxidant activity in purple Mamaki teas after brewed for 1 hour and stored for 4 hours, 1 day, 3 days, and 6 days at 4°C (mg AA/g tea)

Length of storage (hours)	Average ± SD
0	293 ± 145
4	271 ± 134
24	172 ± 65
72	163 ± 18
144	263 ± 10

Conclusions

In conclusion, three identified polyphenols in Mamaki leaves are (+) catechins, chlorogenic acid, and rutin with concentrations that varies from 1.1 mg/g to 5.0 mg/g of Mamaki leaves. The concentrations of (+) catechins and rutin in Mamaki leaves are higher than those in other commercial tea leaves. The amount of TAA available in Mamaki teas varies from 237.7 mg to 259 mg of ascorbic acid equivalents. The amount of TAA in Mamaki teas is relatively very low in comparison to other commercial teas. The amount of TAA available in the purple Mamaki tea is highest immediately after an hour of brewing and lowest after day 3. However, the amount of TAA stabilizes with the increasing storage time.

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CHAPTER IV

Summary and Future Research

This study represents the first investigation of nutrients, minerals, polyphenols, and total antioxidant activity in Mamaki, *Pipturus albidus*.

Nutrient levels of protein, fat, neutral and acid detergent fibers, lignin, and cellulose in five varieties of dried Mamaki leaves were significantly different between the summer and winter seasons. The minerals phosphorus, magnesium, boron, iron, manganese, molybdenum, and zinc in dried Mamaki leaves were also significantly different between the two seasons. No significant nutrient or mineral differences among the varieties of dried Mamaki leaves were found. Potassium, calcium, magnesium, iron, zinc, and copper levels in Mamaki leaves are much higher than those in black tea leaf, green tea leaf, oolong tea leaf, or Lipton tea leaf.

The levels of potassium, magnesium, boron, iron, and manganese in Mamaki teas were significantly different between summer and winter seasons. No significant mineral differences among the varieties of Mamaki teas were found. The amount of calcium and sodium in Mamaki tea is much higher than in Echinacea tea, peppermint tea, Chinese green tea, or raspberry tea. The high concentration of sodium in Mamaki tea maybe related to unique Hawai'i's unique geographical location. Sodium is the most soluble mineral while iron and manganese are the least soluble minerals when brewing Mamaki tea.

Three identified polyphenols in four varieties of Mamaki leaves were (+) catechins, chlorogenic acid, and rutin. The concentration of (+) catechins varies from 2.1 mg/g leaf to 5

mg/g leaf. The concentration of chlorogenic acid varies from 1.1 mg/g leaf to 1.7 mg/g leaf and the concentration of rutin varies from 1.1 mg/g leaf to 1.8 mg/g leaf. The concentrations of (+) catechins and rutin in Mamaki leaves are compared to Gyokuro green tea leaves, Chinese oolong tea leaves, and Kenya black tea leaves. The amount of (+) catechins in Mamaki leaves is much higher than for Chinese oolong tea leaves and Kenya black tea leaves. The amount of rutin in Mamaki leaves is higher than in Gyokuro green tea leaves.

Total antioxidant activity (TAA) in three varieties of Mamaki teas varied from 238 mg of ascorbic acid equivalents per gram of tea to 259 mg of ascorbic acid equivalents per gram of tea. The amount of TAA in Mamaki tea was compared to the peppermint tea, chamomile tea, Lipton tea, and a mixture of green tea and chamomile tea. TAA in Mamaki tea is much lower than in Lipton tea and the mixture of green tea and chamomile tea.

TAA in stored purple Mamaki tea was also quantified. TAA in stored purple Mamaki tea was highest immediately after one hour of brewing (293 mg ascorbic acid/ gram tea) and was lowest after three days of storage (163 mg ascorbic acid/ gram tea). TAA levels stabilize with increasing storage intervals.

Future possible research in nutrient and mineral analysis of Mamaki leaves and teas includes a more intensive study on comparison of nutrients and minerals among the Mamaki leaf or tea varieties, seasons, and interaction between seasons and varieties. It means that more research should be done by increasing the number of Mamaki trees, the location of Mamaki plantations, and the number of seasons for collecting the Mamaki leaves. Another possible area of research includes comparison between Mamaki leaf or tea with other commercial teas using identical method of preparation and analysis.

Future research in phenolics identification of the Mamaki leaves includes identification and quantification of the remaining unknown peaks, especially the unknown peak at retention time of 21 min with UV_{max} 510 nm. Other possible research for the phenolic identification in Mamaki include identification and quantification of phenolics in the Mamaki teas, comparison between phenolics concentrations in Mamaki leaves and teas, and method development to optimize phenolics concentrations available in the Mamaki leaves as well as the Mamaki teas. Comparison of phenolic concentrations in Mamaki leaves with other commercial tea leaves using the same method of preparation and analysis is also necessary.

Future research in total antioxidant activity of the Mamaki teas includes comparison of the total antioxidant activity in Mamaki tea with other commercial teas using the same method of preparation and analysis. A longer storage study on Mamaki tea is also necessary to determine factors that influence fluctuation of the total antioxidant activity in Mamaki tea.

APPENDIX

"purple" variety

"hybrid" variety

"green" variety

"panaewa" variety