Distribution of Catechins, Epicatechins and Methylxanthines in Caffeinated and Decaffeinated Green Tea

by

Suvash Kafley

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Dr. Martin G. Ondrus, Research Advisor

Committee Members:

Dr. Cynthia Rohrer

Dr. Janice Coker

The Graduate School

University of Wisconsin-Stout

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The Graduate School
University of Wisconsin-Stout
Menomonie, WI

Author: **Kafley, Suvash**

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ABSTRACT

Catechins, epicatechins and methylxanthines were determined in commercially available caffeinated and decaffeinated green teas. The extraction procedure simulated consumer preparation of tea beverages at home. All analytes were measured using a reverse-phase, gradient HPLC separation with multi-wavelength photodiode-array detection. Red Rose® caffeinated green tea contained the largest amount of polyphenols among the brands investigated. The caffeinated green teas tended to have larger amounts of polyphenols than decaffeinated teas. Although most decaffeinated teas have lower polyphenol contents than their caffeinated counterparts, one brand had a greater total polyphenol content in the decaffeinated product. The decaffeinated products generally contained lower methylxanthines (4 to 16 mg/serving) compared to caffeinated green teas (23 to 49 mg/serving). None of the products analyzed was free of caffeine.

The Graduate School

University of Wisconsin Stout

Menomonie, WI

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Table of Contents

List of Tables

List of Figures

Chapter I: Introduction

Tea is the world's most consumed beverage after water (BBC, 1998). Green tea was first introduced in China and later carried to Japan (Green Tea PhD., 2003). Green tea was the first processed tea followed by other teas like black, oolong or white (Green Tea Lovers, 2005a). Green tea is the unfermented, rolled and dried fresh two leaves and the bud of the plant, *Camellia sinensis*.

Drinking tea is healthier than drinking water (BBC, 2006), in particular green tea, which is considered good for health because it contains large amounts of polyphenols mostly catechins and epicatechins, that are powerful antioxidants and disease fighters (Ho, Ferraro, Chen, Rosen & Huang, 1994). As found by Grassi et al., 2005, Rein et al., 2000, and Wang et al., 2000, (as cited in Kaspar, 2006), an abundance of antioxidants in the diet has been associated with a decreased risk of developing chronic diseases, such as cardiovascular disease and cancer (Bushman, 1998).

Green tea also contains methylxanthines, namely caffeine and theobromine. Methylxanthines are known for their physiological effects such as central nervous system stimulation, cardiac muscle stimulation, smooth muscle relaxation, and diuretic behavior (Winston, Hardwick, & Jaberi, 2005).

The concentrations of catechins, epicatechins and methylxanthines in green tea can be quantified by high performance liquid chromatography (HPLC). By analyzing the concentration of these compounds in various brands of green tea available in caffeinated and decaffeinated form, the health effects associated with their consumption can be reviewed in regards to the amount of each compound present per serving. Since the amount of each compound may vary

with brand and form (caffeinated and decaffeinated), it is important to quantify concentrations in a variety of green tea products in order to observe the degree of variation.

Statement of the problem

Catechins and epicatechins in different food items have aroused curiosity in many scientific studies relating to cancer and oxidative damages. To date there is limited research on the comparison and quantification of catechin (antioxidant) content in commercially available green tea; however, manufacturers claim high quantities in their products. Catechins, epicatechins and methylxanthines in caffeinated and decaffeinated green teas were quantified and compared by HPLC.

Objective of the study

The objectives of this research were to

1. Quantify and compare beneficial flavanols (catechin and epicatechin) and methylxanthines (caffeine and theobromine) in commercial green teas under the conditions that would simulate the tea as prepared by consumers.

2. Compare the level of flavanols (catechin and epicatechin) and methylxanthines between caffeinated green tea and its decaffeinated counterpart.

Definition of terms

Antioxidants: A broad group of compounds that destroy single oxygen free radical species, thereby protecting against oxidative damage.

Flavanols: Referring specifically to catechin, epicatechin, and their gallate derivatives.

Flavonoids: Produced as secondary plant metabolites and considered a very important category of polyphenols.

Methylxanthines: A group of compounds including caffeine, theobromine and theophylline.

Polyphenols: Referring to a compound comprised of two or more aromatic rings, with each ring containing one or more hydroxyl groups.

Caffeinated green tea: Referring to caffeinated product (green tea without caffeine removal). Does not mean caffeine fortified green tea.

Theaflavins: Compounds formed from catechins in tea leaves during the process of fermentation.

Assumptions of the Study

All the teas are made from *Camellia sinensis*. None of the green teas are supplemented with antioxidants or other plant materials components such as rose petals.

Chapter II: Literature Review

History of green tea

Very few herbs have as long or as impressive a history as green tea (Mitscher, Dolby and Toews, 1997). There is no clear documentation about when human beings began to consume tea or if people in ancient times ate tea leaves or drank brewed tea (Green Tea Lovers, 2005a). It is believed that the first recorded use of green tea dates back more than 5000 years in China. The earliest known reference to tea as a health aid dates back to 2737 B.C. when a legendary Chinese leader and medical expert discovered its extraordinary powers and acknowledged it by adding it to his medicinal herb list. Since the early reference to green tea as a health elixir, an impressive amount of evidence has accumulated on green tea for good health. Initially offered as a medicine, tea became most commonly used as a beverage during the western Han dynasty.

The development of tea processing started during the 8th century with the introduction of the steaming process (Green Tea Lovers, 2005a). The processed tea, "tea brick" was ground in powder, boiled or steeped in hot water and consumed as a hot beverage. The Japanese were unaware of tea until the 12th century; Japanese monks visiting China brought the tea culture home with them. There was the transition from brick to loose tea and the production style changed again. The method of roasting was introduced during the 13th century. Prior to that steaming was the primary process for tea processing. Japanese travelers brought 'steaming and roasting' processing methods from China. Drinking tea was made available to the masses towards the end of the 16th century, and it soon became the nation's most popular beverage. The development of tea processing methods started speeding up. Numerous advancements were made during the 17th century. A modern green tea processing method was introduced during the 18th century. Soen Nagatani developed Japanese sencha in which the tea leaves are steamed, pressed,

rolled, and dried as loose tea rather than being ground as in the traditional method. The mass production of teas started during the 19th century. Introduction of sensors and computer controls to machine automation during the 20th century contributed to improved quality control and reduced labor for tea production (Green Tea Lovers, 2005b). The combination of nature's gift and human technological advancements produce the most exceptional green tea sold on the market today.

The Portuguese were the first to export tea to the west followed by the Dutch and the English through their colonial holdings in the Far East (Green Tea Lovers, 2005c). The production of tea in several African countries (Kenya, Malawi and South Africa) started after World War II. Introduction of the tea culture to USA was from Europe (Origin of Green Tea, n.d.). It was as a part of global trade and through Chinese restaurants operated by immigrants from southern China. Americans became familiar with black and oolong tea but not green tea. Green tea in America has exhibited increasing popularity in recent years.

Tea classification

Tea can be classified according to types and processing method.

1) *Classification by type.* There are two types of tea based on form and shape of the final product: loose tea and brick tea.

2) *Classification by processing method.* White tea, green tea, oolong tea, and black tea are produced from the leaves of the same plant (Figure 1) *Camellia sinensis,* but processed differently to attain a different level of oxidation (Chu, 1997).

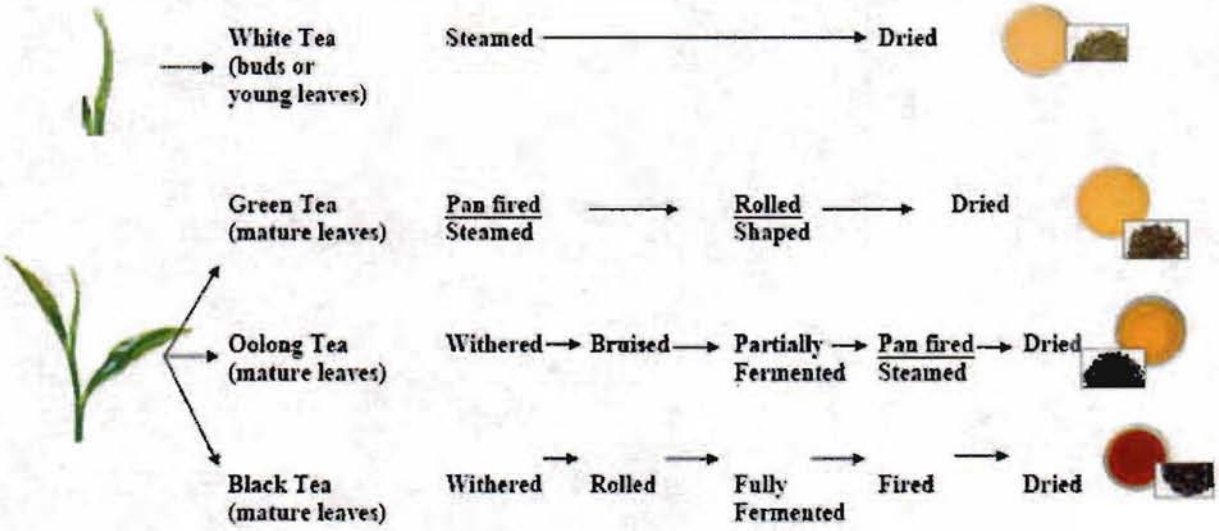

Figure 1. Generalized tea manufacturing process (Imperial tea garden, 2006).

Tea plant

Tea is made from the leaves and leaf buds of a shrub, *Camellia sinensis* (Chu, 1997). There are two major varieties of tea plant (Figure 2). The plant variety with small tea leaf known as *Camellia sinensis* (China variety), thrives in cool, high mountain regions of Central China and Japan. Greater volume comes out of the broad leaf variety, known as *Camellia assimica* (Assam variety) and grows best in moist, tropical climates found in northeast India and the Szechuan and Yunnan provinces of China.

Figure 2. Tea plant (*Camellia sinensis).* (World Science, 2007)

Cultivation of tea plant

Suitable climatic conditions for a tea plantation should have minimum rainfall of 114.3 - 127 cm (How Products are Made, 2006). Acidic soil (pH 5.8 – 5.4) favors growth as the plant will not grow in alkaline conditions. The tea plant can be cultivated up to 2,200 m above sea level and can grow between the equator and 45th latitude.

Green tea processing

Tea leaf harvesting. Tea leaves are harvested manually or mechanically. Generally tea shoots are plucked at 5 to 10 day intervals (How Products are Made, 2006). A bud and two leaves are picked from each plant to make green tea.

Enzyme inactivation. The enzyme present in the leaf should be inactivated before further processing to prevent fermentation and oxidation (How Products are Made, 2006). Two processes are used for inactivation, one being pan firing and the other steaming. In China, the leaves are commonly heat treated in large woks with continuous stirring to heat evenly. Another

type of process practiced in Japan uses steam to inactivate enzymes. The clean and sorted leaves are conveyed above the hot water or through a steam chamber. The amount of time the leaves are exposed to steam depends upon the product. Sencha is normally steamed for 30 to 90 seconds.

First rolling and drying. Rolling and shaping of green tea leaves are usually done by machines for mass production (How Products are Made, 2006). Rolling creates a distinctive look and also regulates release of tea components while brewing. The Japanese process uses number of rolling and drying steps. Special equipment is used for first rolling and drying simultaneously. The leaves are dried to improve their strength so they can be pressed during the next drying process. Moisture from the leaf surfaces and inside is removed during this process. This machine consists of a spindle with finger-shaped extensions that stir the leaves while heated air (34-36°C) is blown into the machine.

Second rolling and drying. The moisture in the leaves coming out of the first rolling process is not uniformly distributed so another process is used to uniformly distribute the remaining moisture present in leaves (How Products are Made, 2006). This process rolls the leaves by pressing under a rotating disk to bring the moisture from the center of the leaves to the surface.

Third rolling and drying. The leaves are transferred to another rolling/drying machine, which uses a spinning pedal inside of a revolving drum to convert the leaves into a round shape (How Products are Made, 2006). It is very important to take out the leaves at the same moisture level every time.

Final drying. After the final rolling in the Japanese tea processing, the processed tea is dried slowly to moisture content of 5% or less (How Products are Made, 2006). Then the tea is sorted, graded, and packed for marketing.

Decaffeination

Decaffeination of processed tea is commonly done by two methods: one uses the solvent ethyl acetate and the other uses carbon dioxide or water (Green Tea Lovers, 2005d). The process using the solvent ethyl acetate retains only 30% of the polyphenol. Water even though it is a good solvent for caffeine, is not preferred for decaffeination due to its low selectivity and ability to dissolve other compounds present in green tea (King and Bott, 1992). Today, supercritical carbon dioxide is always used for the decaffeination process because it is more selective towards caffeine and safer.

Plant phytochemicals

Tea, coffee, fruits and spices contain a diverse group of phenolic compounds which have structural requirements for scavenging free radicals (Fennema, 2008). These phenolic compounds include simple phenolics, phenolic acids, anthocyanins and hydroxycinnamic acid derivatives. Phenolics are primary antioxidants that react with peroxyl radicals producing stable products before the radicals can react with unsaturated lipids (Madhavi, Deshpande & Salunkhe, 1995).

The composition of tea polyphenol is dependent upon the variety of tea plant, climatic conditions, season, and the age of the leaf (Ho, Chen, Wanasundara, Shahidi, 1997). Overall, it has been reported that the polyphenol content in the extractable solids in fresh green tea leaves is approximately 36%. The predominant polyphenols in teas are catechins and epicatechins, with epigallocatechin gallate as the predominant polyphenol present in green leaves. The composition of green tea polyphenol is presented in Table 1.

Table 1

Composition of green tea polyphenol fractions

Compound	Percentage of polyphenol fraction (%)
Epigallocatechin gallate (EGCG)	48 – 55
Epigallocatechin (EGC)	9 – 12
Epicatechin gallate (ECG)	9 – 12
Caffeine (CAF)	7 – 9
Epicatechin (EC)	5 – 7
Catechin	0.3 – 0.6
Gallic acid	0.3 – 0.5

Huang et al. (1992) as cited in Ho et al., (1997).

Antioxidants

Antioxidants are chemical compounds capable of donating a hydrogen atom (Madhavi et al., 1995). Antioxidants scavenge free radicals and inhibit or slow down lipid oxidation by reacting faster with free radicals so that free radicals do not affect unsaturated fatty acids (Fennema, 2008).

Catechins and epicatechins are known for their antioxidant properties. The four major epicatechins (Figure 3) in green tea are: epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (EGCG) (Bushman, 1998). The three most potent green tea components against tumor cell lines are EGCG, GC, EGC, of which EGCG is the most potent of the green tea components (Valcic et al., 1996). In addition EGCG is the primary component, accounting for 40% of the total polyphenolic mixture.

Methylxanthines are plant alkaloids. Methylxanthines commonly found in teas are caffeine, theobromine and theophylline.

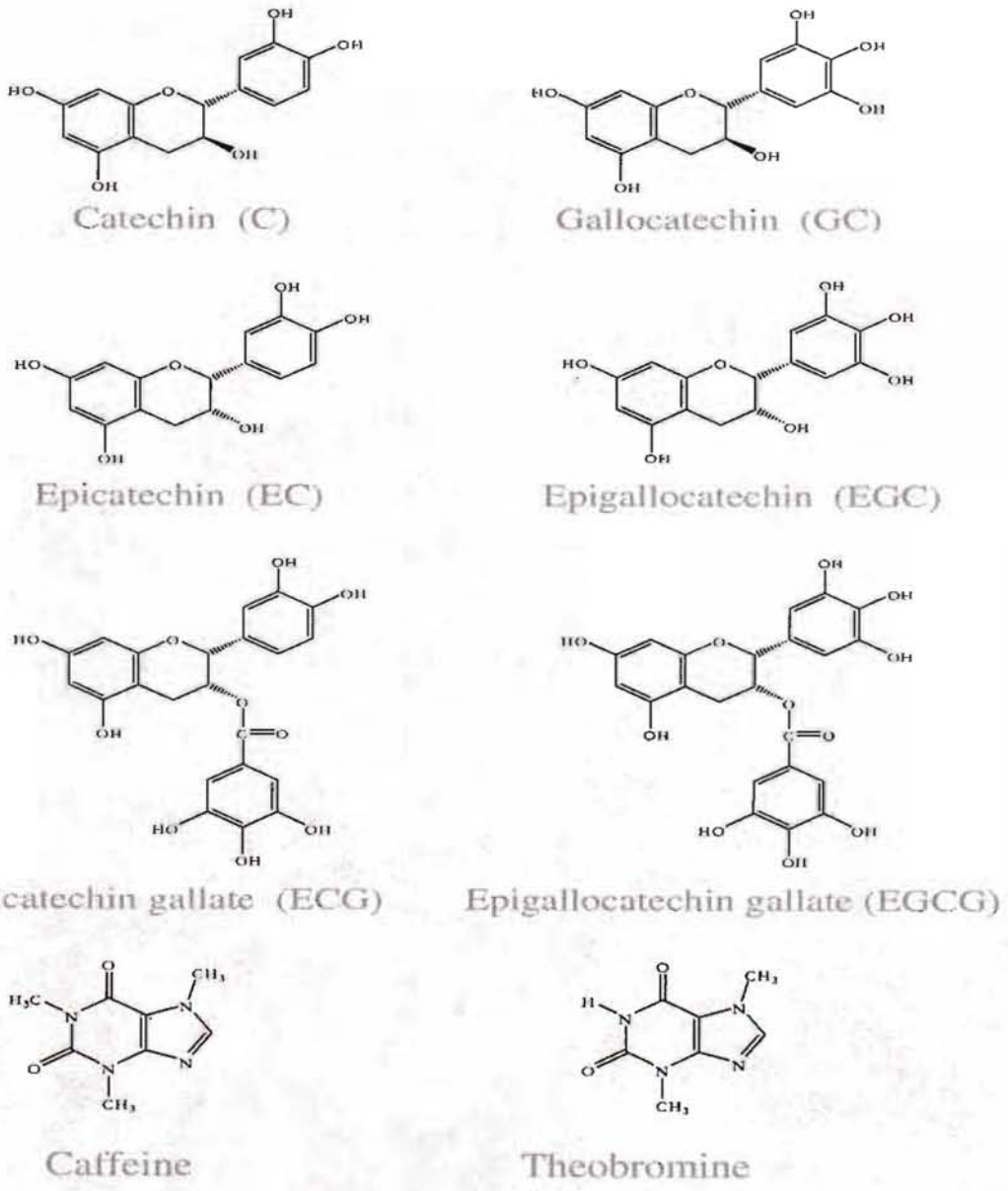

Figure 3. Structures of catechins, epicatechins and methylxanthines.

Biological lipid oxidation

Antioxidants function by disrupting the free radical chain reaction in lipid oxidation or by changing lipid peroxides into stable end products (Madhavi et al., 1995).

In a cellular system, lipid oxidation occurs mainly in bio-membranes where the content of unsaturated fatty acids and oxygen activating enzymes is relatively high (Madhavi et al., 1996). The oxygen activating enzymes are responsible for induction of oxidative stress that leads to formation of reactive oxygen species. Lipid peroxidation activates the process of cell death by degradation of cellular components and inactivates the cellular defense system. According to the oxygen radical and lipid peroxidation theory of aging, the major cause of oxidative of stress is damage to DNA and other macromolecules. Cancer and other degenerative diseases such as heart disease are known to be caused by this destructive process (Madhavi et al., 1996).

Green tea components and health benefits

Before green tea was consumed as a beverage it was renowned for its pharmacological properties (Green Tea Lovers, 2005). The aqueous infusion of green tea has reported antimutagenic, antibacterial, hypocholesterolemic, antioxidant, antitumor, antiaging, and cancer preventive activities (Valcic et al., 1996). Green tea contains 35-52% (measured in mass percent of extract of solids) catechins and flavanols combined (Bushman, 1998). Scientific studies have shown that the beneficial health properties of green tea are due to the presence of its antioxidants (Green Tea Lovers, 2005e). Antioxidants play an important part in reducing the risk of free radical-related oxidative damage associated with a number of clinical conditions and degenerative diseases (Dreosti, 1996). The major tea components are catechins, epicatechins and methylxanthines. Several forms of catechin occur in red wine, apple and grape skins, and the

bark of certain trees; however, their chemical profile is different from that of the green tea and their concentration in edible material is generally lower.

Green tea and cancer

Cancer is the second principal cause of death in the United States, heart disease being the first (Bushman, 1998). Antioxidants are among the dietary factors that may play a role in cancer protection. Table 2 shows that the cancer onset of patients who had consumed over 10 cups of green tea per day was 8.7 years later among females and 3.0 years later among males, compared with the patients who had consumed under three cups per day (Fujiki, Sugamuma, Okabe, Sueoka, Komori, Sueoka, Kozu, Tada, Suga, Imai and Nakachi, 1998).

Table 2

The average age at cancer onset and the amount of daily green tea consumed

No. of patients	Daily consumption of green tea (cups per day)		
	≤ 3	4 – 9	≥ 10
Male (220)	65.3 ± 1.5 (54)	67.6 ± 1.0 (102)	68.3 ± 1.2 (64)
Female (164)	65.7 ± 1.7 (49)	66.8 ± 1.2 (94)	74.4 ± 2.5 (21)

(Fujiki et al., 1998).

Negative health effects of green tea

No negative health effects associated with green tea have been found to date (About, 2006). The only side effect due to excessive drinking of tea can be insomnia due to the caffeine content. The caffeine content of green tea however is 4 to 6 times lower than that present in instant coffee (Wilstar, 2006).

Previous studies on polyphenols and methylxanthines of green tea

Various extraction conditions and a wide variety of methods have been utilized to measure the concentration of tea components. Most of the studies conducted involved the use of the products or samples from other countries besides the USA so information on these teas could not be used for the current study. Table 3 displays the summary of the distribution study of catechins, epicatechins and methylxanthines carried out by Friedman, Kim, Lee, Han, Han, Lee, and Kozukue (2005). In the study, Friedman et al. (2005) used acetonitrile and KH_2PO_4 for the mobile phase and the Hitachi HPLC used was equipped with 250mm x 4.0mm internal diameter column.

Table 3

Distribution of catechins, epicatechins and methylxanthines from a study carried out by Freidman et al., (2005).

Sample	EGC	C	EC	ECGC	ECG	TBR	CAF	T.M.	T.P.
C.S. 'C'	n.d.	n.d.	0.7	13.8	4.0	0.7	6.0	6.7	18.5
Lipton® 'C'	n.d.	n.d.	1.3	18.4	8.3	0.3	8.2	8.5	28.0
Stash® 'D'	4.8	4.4	0.8	13.5	6.0	0.6	3.8	4.4	29.5
Stash® 'C'	13.6	2.0	0.9	38.4	15.9	0.8	15.1	15.9	70.8

Values in mg/g; n.d. = non detected; C.S. = Celestial Seasoning®; 'C' = caffeinated; 'D' = decaffeinated; CAF = caffeine; TBR = theobromine; T.M. = total methylxanthines; T.P. = Total polyphenol.

HPLC and different combinations of mobile phase have been used by other researchers to analyze tea components. As cited in Friedman et al. (2005), Leen and Ong (2000) water extracted catechins and theaflavins from tea using HPLC and electrophoresis. The mobile phase

consisted of acetonitrile/trifluoroacetic acid, compared to Lin and others (2003), who used HPLC with mobile phase consisting of ethanol/H_2O/formic acid to determine 5 catechins in 31 Taiwanese tea leaves and tea flowers, as cited in Friedman et al. (2005). Different extraction conditions leads to wide range of flavonoids and alkaloids concentrations (Freidman, Levin, Choi, Kozukue, and Kozukue, 2006). As cited in Friedman et al. (2005), the extraction conditions used by different researchers are presented in Table 4. While many solvent systems have been investigated, hot or boiling water has been used most frequently.

Table 4

Extraction methods used by researchers

Extraction methods	Researchers
Boiling water for 3 min	Del Rio and others 2004
Boiling water for 5 min	Khokhar and Magnusdottir (2002)
	Bonoli and others (2003)
Boiling water for 10 min	Aucamp and others (2000)
Water at 80°C for 30 min	Sakakibara and others (2003)
Water at 90°C for 30 min	Lee and Ong (2000)
Acetonitrile/water for 1 h	Fernandez and others (2002)
Ethanol at room temperature	Lin and others (2003)
80% methanol for 3h, followed by 80% methanol containing 0.15% HCL for 3h	Cabrera and others (2003)
80% acetone for 2 wk	Menet and others (2004)
Pressurized liquids	Pineiro and others (2004)

Conclusion

Many studies have reviewed green tea as a potential source of antioxidants. These studies discussed previously, have demonstrated that polyphenols from green tea may provide numerous health benefits. These studies offer sufficient evidence to conclude that polyphenols from green tea have probable health benefits directly related to their consumption; therefore, the presented literature review validates the basis of this research to investigate commercially available green tea for catechins, epicatechins and methylxanthines content.

Chapter III: Methodology

This chapter includes a discussion on the methods of sample preparation and analysis used by different researchers. This chapter concludes with the process of raw material acquisition, instrumentation, data collection procedure, data analysis, and limitations.

Polyphenols such as catechin, epicatechin and methylxanthines, such as caffeine and theobromine, are unique because these compounds have similar polarities and ultraviolet absorption spectra; therefore, they are able to be simultaneously extracted and analyzed via high performance liquid chromatography (HPLC). According to Bloor (2000), HPLC is the preferred technique because this system offers the spectra of multiple wavelengths and the ability to record them (cited in Kaspar, 2006).

HPLC can be classified based on the polarity of the column stationary bed; normal phase and reverse-phase (Types of HPLC, n.d.). In normal phase, the stationary bed is polar and the mobile phase nonpolar. The stationary bed in reverse-phase HPLC is nonpolar or hydrophobic in nature and the mobile phase is polar. Nonpolar analytes bond to reverse-phase HPLC columns in the presence of aqueous mobile phase and are eluted from the columns with aqueous/organic mobile phase. Reverse-phase gradient HPLC with multi-wavelength photodiode-array detection was used to quantify levels of catechin, epicatechin, caffeine and theobromine in fourteen caffeinated and decaffeinated green teas.

Sample selection

Caffeinated and decaffeinated green teas (of different brands) were obtained from local (Menomonie and Eau Claire, WI) stores. The caffeinated and decaffeinated teas were analyzed, and any flavored and herbal teas were excluded from the study.

Standard solutions preparation

The standard solutions of polyphenolic compounds, catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, epigallocatechin and methylxanthines compound (caffeine and theobromine) were prepared. Catechin (C), caffeine (CAF), and theobromine (TBR) standards were used from Sigma (St. Louis, MO). The epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), epigallocatechin (EGC) standard was obtained from Aldrich (Milwaukee, WI). Stock solutions of caffeine (1000 mg/L) and theobromine (800 mg/L) were prepared by dissolving the measured amount of compounds in approximately 700 mL of a 50:50 (V/V) methanol-Milli-Q® water solution heated to 60°C. After the caffeine and theobromine were dissolved, they were allowed to cool to room temperature and diluted to volume up to one liter. Standard solutions of polyphenols were prepared at different concentrations by weighing exact amounts of each substance on a microbalance and diluted to mark with methanol in a 25 mL standard volumetric flask. The concentrations of the standard mixtures are listed in Table 5.

Table 5

Standard mixture solution concentrations

Standard mixture	Concentration (mg/L)					
	I	II	III	IV	V	VI
Polyphenols:						
Catechin	10	20	30	40	50	60
Epicatechin	10	20	30	40	50	60
Epicatechin gallate	10	20	30	40	50	60
Epigallocatechin	10	20	30	40	50	60
Epigallocatechin gallate	20	40	60	80	100	120
Methylxanthines:						
Caffeine	25	50	75	100	125	150
Theobromine	160	240	320	400	480	560

Tea sample preparation

Samples were kept in ambient conditions similar to those found in stores and homes. Each sample (tea bag) was weighed without tearing the tea bag. Two hundred fifty milliliters of Milli-Q® water was used to make the sample following the directions given on the box or individual wrapper of the tea bag for the temperature of the water and the steeping time. Prior to HPLC quantification about 2 mL of each sample was transferred to a sample vial with a syringe equipped with a Whatman® 0.45 um polypropylene membrane filter.

Instrumentation

A Waters high performance liquid chromatography (HPLC) system with Empower® 2 software was used for qualitative and quantitative analysis of catechin, epicatechin, epicatechin gallate, epigallocatechin gallate, epigallocatechin, caffeine and theobromine from modified a HPLC method. The column used in the analysis was a Waters Radial Compression™ 10 cm x 8 mm ID Novapak™ C_{18} column with a NovaPak™ GuardPak in a RCM-100 radial compression module. Two aqueous solutions were used to generate a solvent gradient. Gradient conditions are described in Table 6. Solvent A consisted of 99.5% of Milli-Q® water and 0.5% of glacial acetic acid (V/V) and solvent B consisted of 0.5% glacial acetic acid, 40% acetonitrile and 59.5% Milli-Q® water. Solvent composition was changed from 100% A to 100% B over a 30-minute time period. The gradient was slightly concave (nonlinear), corresponding to curve 8 on the Waters solvent programmer (Table 6). Acetic acid was necessary to prevent ionization of the polyphenol and resulting peak broadening. At the end of the 30 minute gradient, the solvent was returned to 100% A (1 minute) and the system equilibrated for 5 minutes. The total run time was 36 minutes. The flow rate was set at 2 mL/minute. The injection volume of each sample was 25 µL. The HPLC instrument system consisted of a Waters 717 Plus autosampler, Waters 1525

Binary HPLC Pump, and a Waters Photodiode Array Detector. The system was controlled with a PC using Windows® NT operating system and Waters Empower® 2 Software.

Table 6

Mobile phase gradient separation conditions

	Time (min)	Flow (mL/min)	%A	%B	Curve
1	---	2.00	100.00	0.00	---
2	30.00	2.00	0.00	100.00	7
3	31.00	2.00	0.00	100.00	6
4	32.00	2.00	100.00	0.00	6
5	36.00	2.00	100.00	0.00	6

The UV-Vis spectra of the all analytes were determined by measuring with an Aligent® UV-Vis spectrophotometer with UV-Vis Chem Station Software. Kaspar (2006) determined that at the concentration of 10 mg/L, the UV-absorption maxima of catechin (Figure 4) and epicatechin (Figure 5) were 280 nm; caffeine (Figure 6) and theobromine (Figure 7) were at 272 nm.

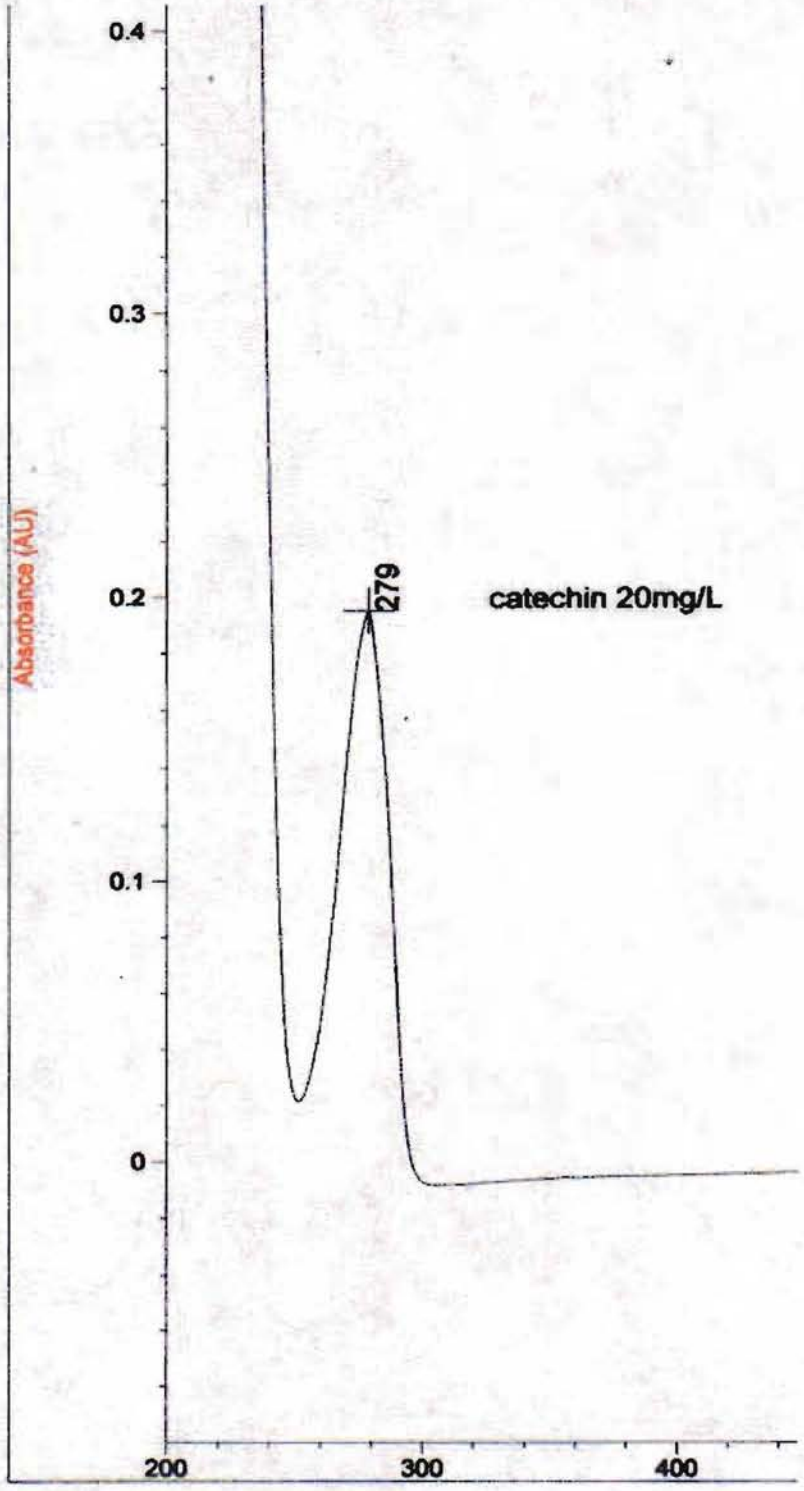

Figure 4. Catechin UV-spectra.

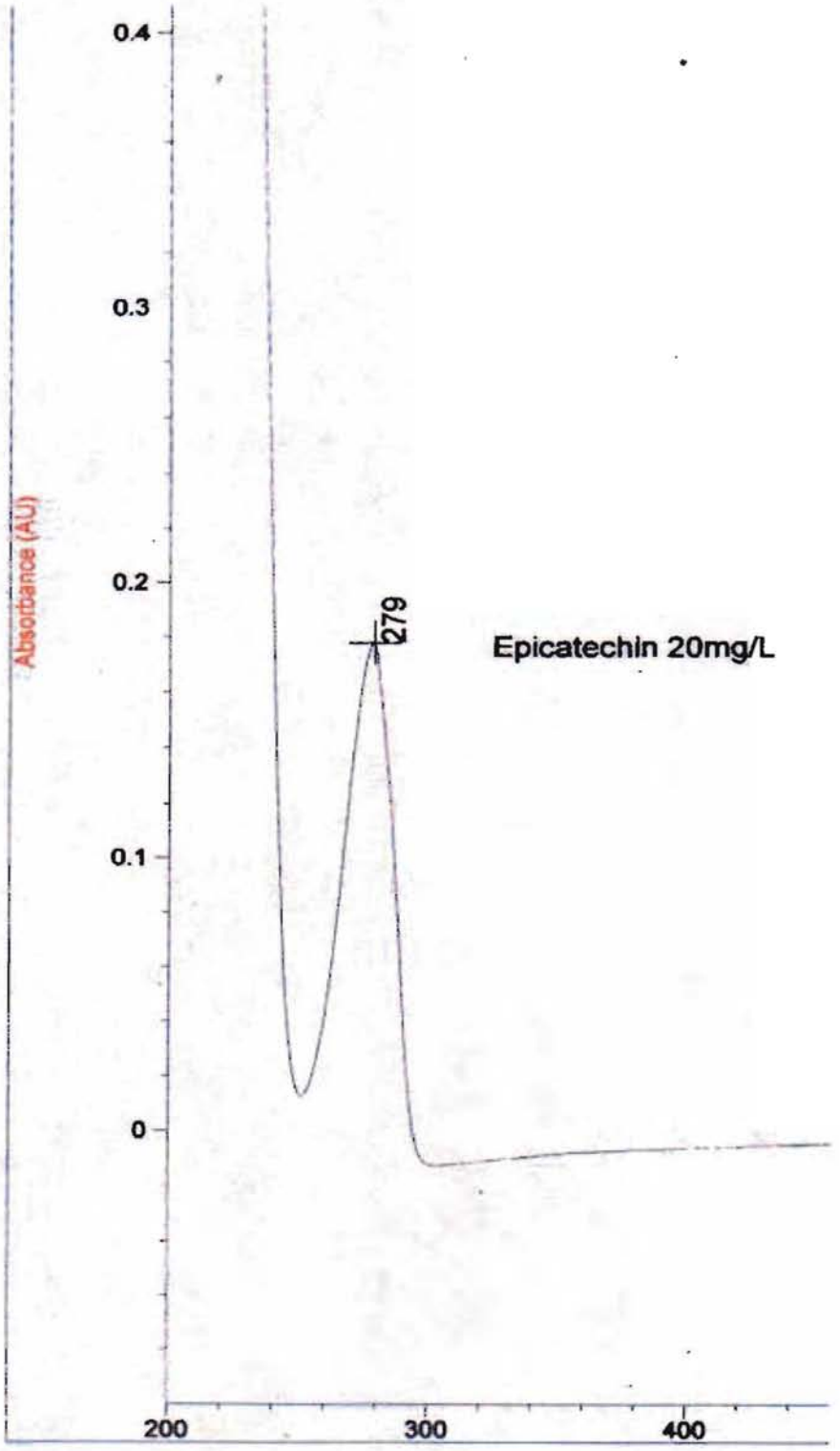

Figure 5. Epicatechin UV-spectra.

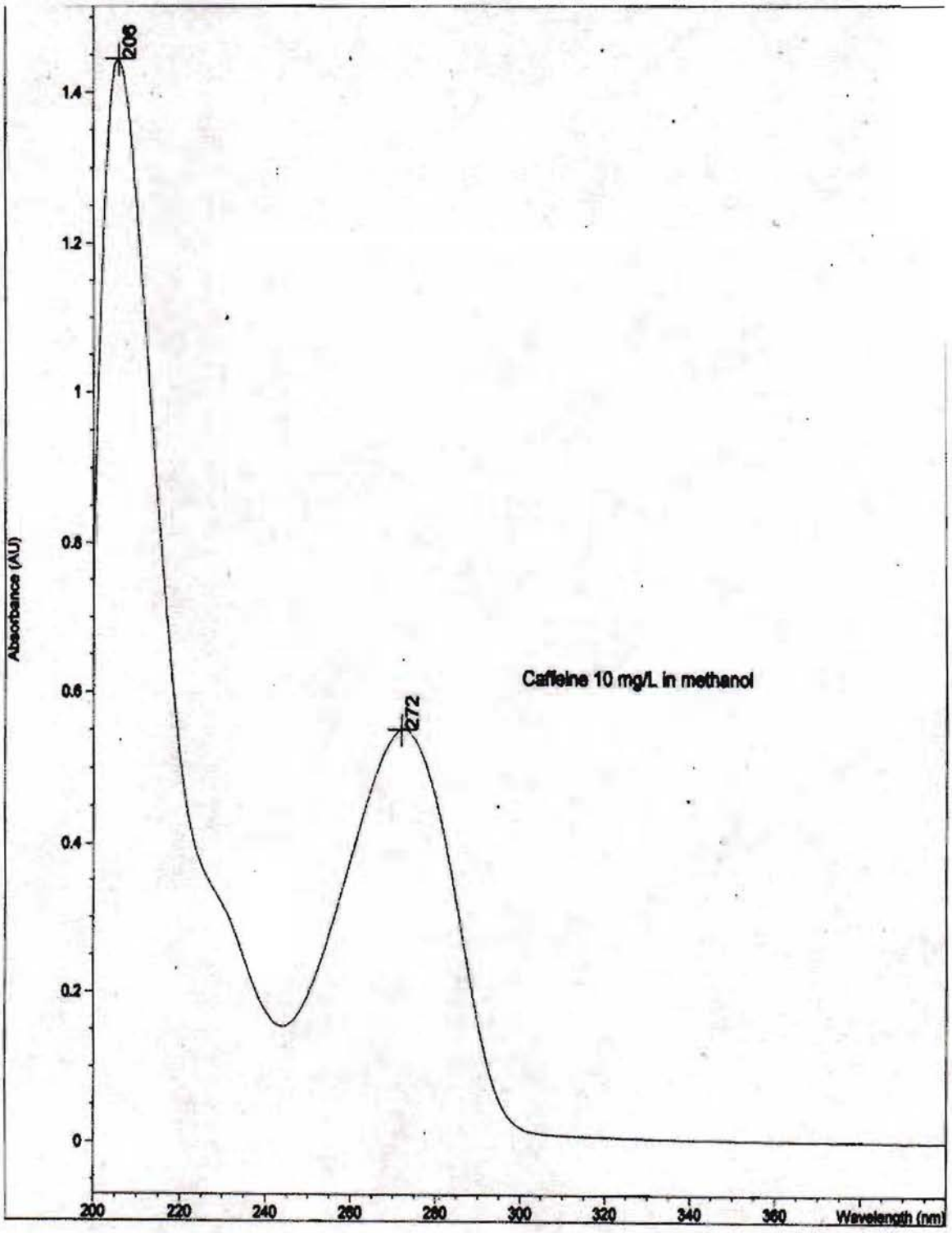

Figure 6. Caffeine UV-spectra.

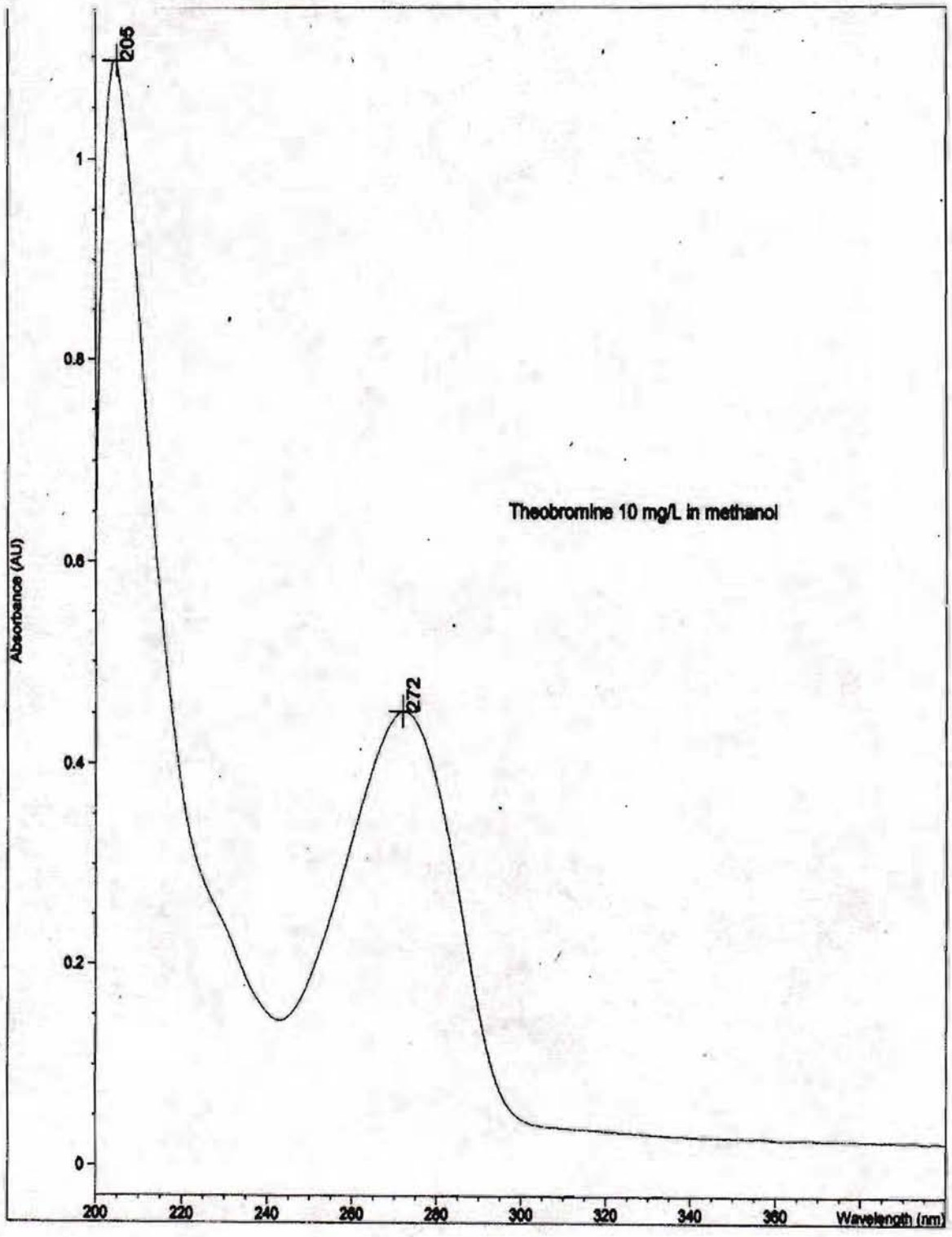

Figure 7. Theobromine UV-spectra.

Peak detection and verification

Four wavelengths (260 nm, 270 nm, 280 nm and 290 nm) were used for detection and peak verification, but only the results from the 270 nm chromatograms were used for quantification. The 270 nm and 280 nm results were used to calculate peak area ratios.

Data collection procedures

The samples were injected in 2-ml sample vials via micro-filter. Vials were placed in the auto-sampler tray of the HPLC setup and analyzed using above discussed instrumentation. The print out of the results for the analytes area were obtained and used for data analysis.

Data analysis

Statically differences between the mean concentrations (mg/serving) of flavanols and methylxanthines for each green tea variety were examined by ANOVA. The statistical analysis was performed using the computer statistical package SPSS version 14.0.

Limitations

The two limitations of the study are:

1. The quantification of flavanols and methylxanthines concentrations is dependent on the extraction process described in Table 4.
2. Analyte identification is limited by the peak resolution produced by the column and mobile phase.
3. Peak quantification is limited by the diode-array detector sensitivity.

Chapter IV: Results and Discussions

This chapter includes a discussion on the results obtained and compares those findings to those of other researchers. This chapter concludes with the interpretation of data obtained.

A reverse-phase HPLC method developed previously (Kaspar, 2006) in order to quantify polyphenols (catechin and epicatechin) and methylxanthines (caffeine, theobromine and theophylline) in chocolate, was utilized in this study to determine the polyphenols and methylxanthines content in green teas. Seven brands of caffeinated and decaffeinated forms of green tea that were locally available from grocery stores were used in this study.

Replicate injections of individual standard solutions were used to determine the retention time of each analyte. Multiple wavelength detection (260 nm, 270 nm, 280 nm and 290 nm) was used because of the variation in the wavelength of maximum absorption between these compounds. Figure 8 displays the chromatogram of a standard mixture containing Catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG), epigallocatechin (EGC), caffeine (CAF), theobromine (TB) and theophylline (TP).

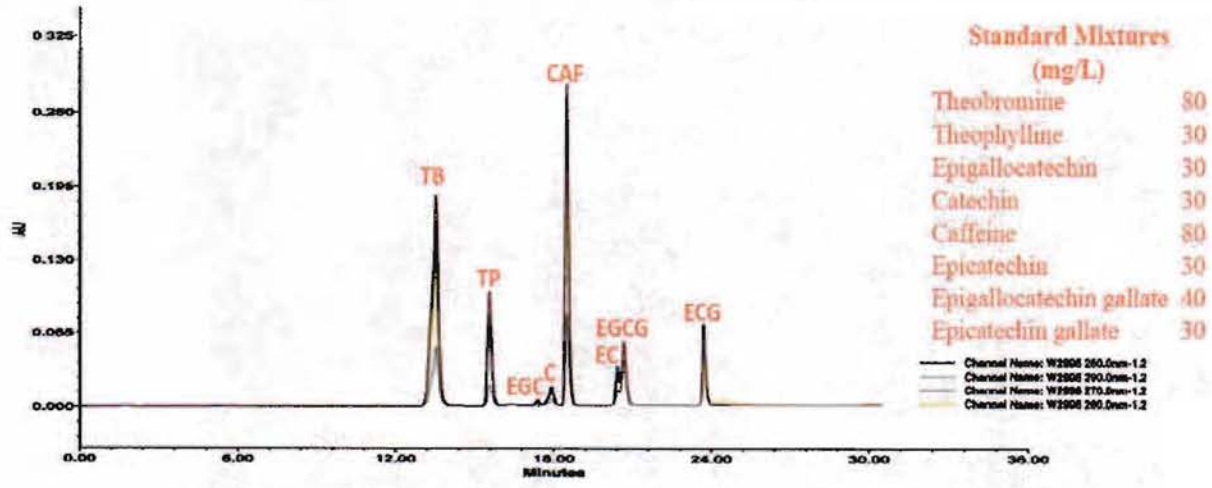

Figure 8. Chromatogram of standards.

The chromatograms of caffeinated and decaffeinated green teas produced by the same manufacturer are shown in Figures 9 and 10. These chromatograms were obtained from caffeinated and decaffeinated products and differ in level of polyphenols to some extent but exhibit a large difference in the level of caffeine. Figure 9 shows the chromatograms of caffeinated and decaffeinated Red Rose® green tea. Red Rose® is the brand with the largest amount of total polyphenols among the brands investigated. Figure 10 shows the chromatograms of caffeinated and decaffeinated Bigelow® green tea samples. Bigelow® is the only brand having a larger amount of total polyphenols in its decaffeinated form than in its caffeinated form.

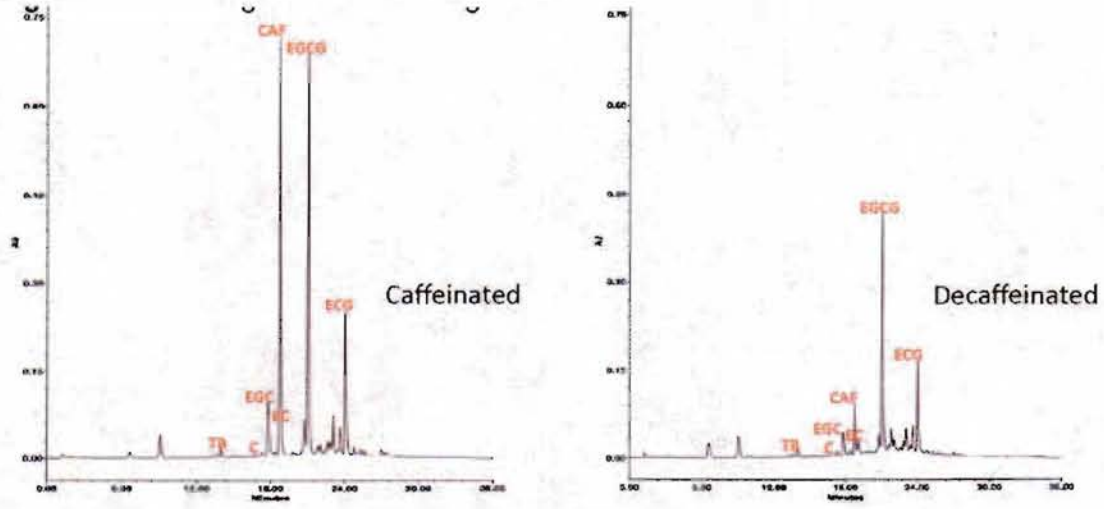

Figure 9. Chromatograms of Red Rose® green tea.

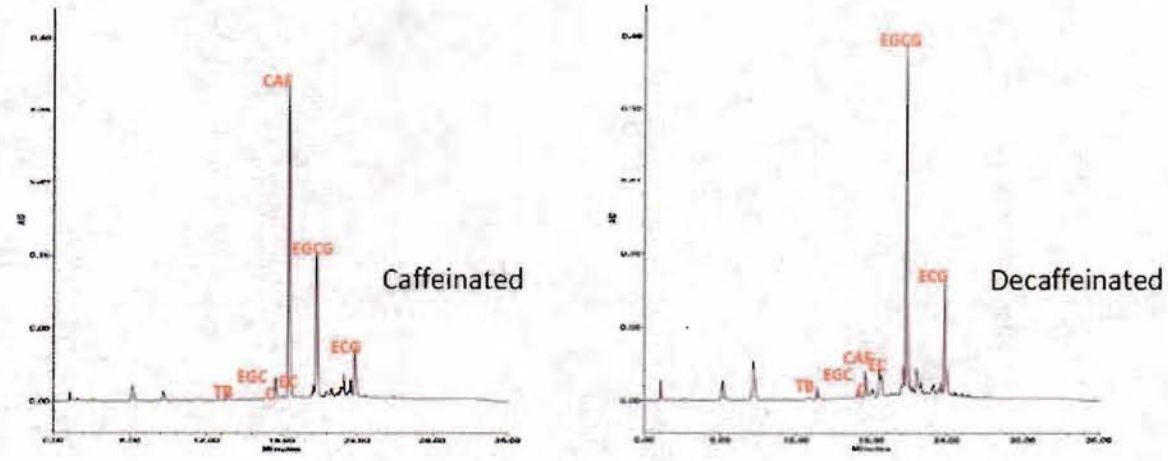

Figure 10. Chromatograms of Bigelow® green tea.

Figure 11 shows the standard curve obtained from the concentrations of standards and the peak area at 270 nm. The standard curve was used to calculate the amount of each component of polyphenols present in the product analyzed.

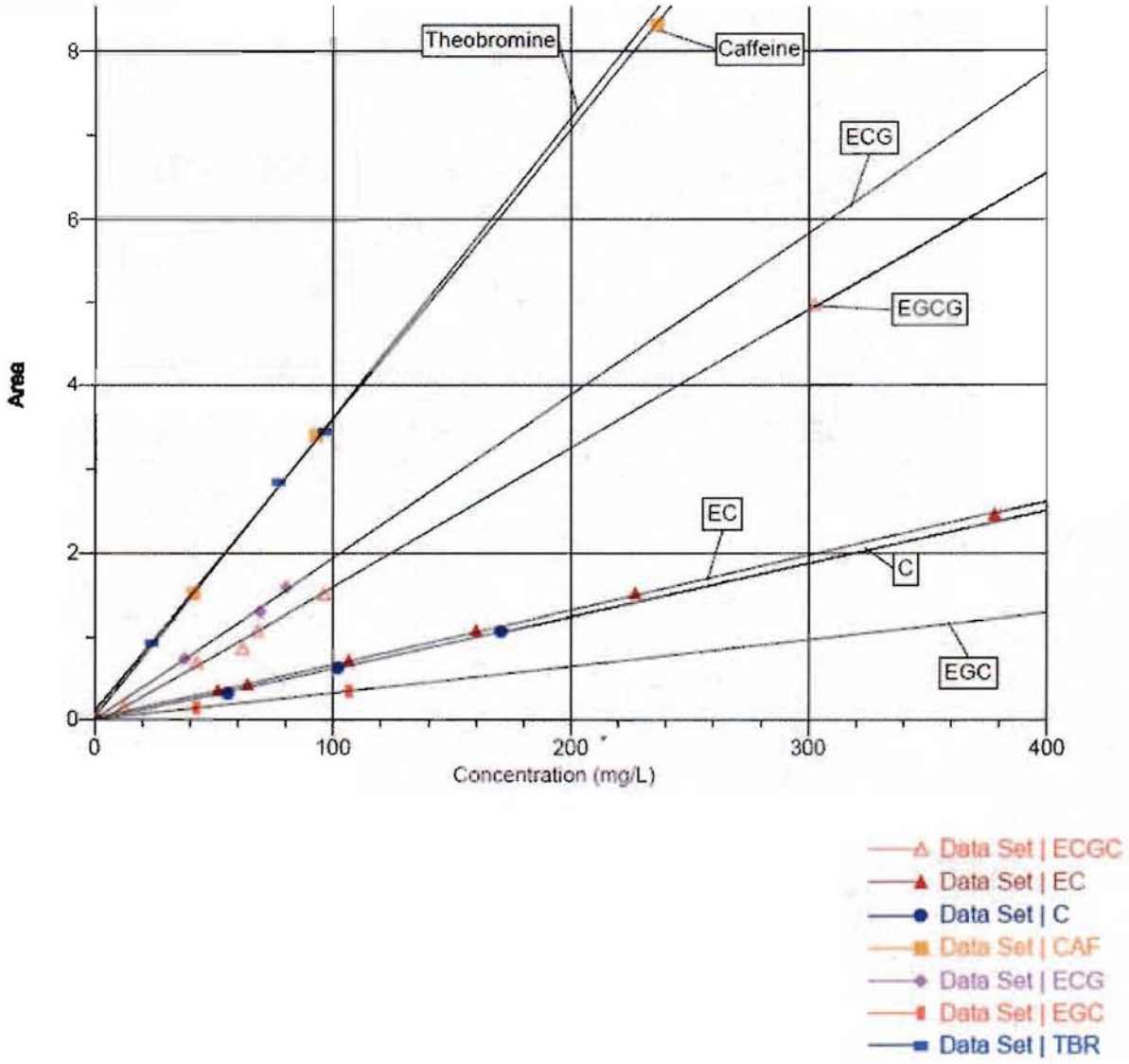

Figure 11. Standard curve of the analytes obtained using laboratory grade pure standards.

To verify analyte peaks, the 280 nm/270 nm peak area ratios of the peak in each tea chromatogram were compared to the corresponding peak area ratios of known compounds with similar retention times. Table 7 shows the retention time and peak area ratio of each standard component at 280 nm and 270 nm. The concentration of each component in the sample was determined using the standard curve of each component.

Table 7

Compound retention time and peak area ratio at 280 nm and 270 nm[1]

Compound	Retention time (minutes)	Peak area ratio (280 nm/270 nm)
Caffeine	18.8	0.89 ± 0.00
Theobromine	13.9	0.88 ± 0.00
Theophylline	15.9	0.81 ± 0.00
Catechin	18.4	1.37 ± 0.01
Epicatechin	20.9	1.35 ± 0.02
Epigallocatechin	17.9	0.64 ± 0.01
Epicatechin gallate	24.3	1.13 ± 0.01
Epigallocatechin gallate	21.2	0.99 ± 0.01

[1]mean of samples ± standard deviation

Average individual analytes concentration (mg/L) and its standard deviation, in green tea aqueous infusion are presented in Tables 8 and 9. Table 8 displays the individual component concentration in caffeinated green tea. Table 9 displays the concentration of the individual components in decaffeinated green tea. In both the caffeinated and decaffeinated green tea, EGCG was the largest component among all polyphenols. Caffeine was largest component among the methylxanthines present in caffeinated and decaffeinated green tea. Stash® caffeinated

green tea contained the largest amount of EGCG (399 mg/L) and caffeine (191 mg/L). Similar findings were found by Friedman et al. (2005). Among the caffeinated brands investigated by Friedman et al. (2005), Stash® caffeinated green tea contained the largest amount of EGCG and caffeine.

Table 8

Average distribution of polyphenols and methylxanthines in caffeinated green tea samples (mg/L)[1]

Sample	Compounds analyzed[2]								
	Caf	TBR	C	EC	ECG	EGC	EGCG	MET	PPH
Bigelow®	88.4 ±4.6	1.59 ±0.24	5.27 ±4.0	22.7 ±4.4	26.1 ±4.7	62.1 ±8.9	95.9 ±8.8	90.0 ±4.7	209 ± 7.1
Celestial Seasoning®	126 ±27	1.81±0.28	10.4 ±7.6	44.1 ±7.4	55.7 ±11	196 ±52	224 ±41	128 ±27	528 ±114
Lipton®	127 ±13	3.75±0.42	13.5 ±2.7	68.9 ±5.6	81.4 ±9.7	287 ±43	268 ±29	131 ±13	718 ±80
Our Family®	118 ±8.7	2.11 ±0.19	11.9 ±3.0	52.1 ±8.1	69.1 ±10	199 ±25	224 ±33	120 ±8.9	556 ±77
Red Rose®	171 ±2.4	4.36 ±0.18	23.3 ±2.9	84.2 ±8.3	127 ±1.9	359 ±21	397 ±7.0	176 ±2.5	990 ±20
Salada®	172 ±8.1	3.57 ±0.29	18.6 ±9.1	70.2 ±8.3	93.6 ±7.9	268 ±44	362 ±15	175 ±8.3	809 ±74
Stash®	191 ±7.5	6.25 ±0.30	16.2 ±5.6	91.2 ±8.8	103 ±1.6	299 ±24	399 ±18	197 ±7.7	908 ±30

[1]mean and standard deviation of the individual brands.

[2]Caf = Caffeine, TBR = Theobromine, C = Catechin, EC = Epicatechin, ECG = Epigallocatechin, EGC = Epigallocatechin, EGCG = Epigallocatechin gallate. MET = Methylxanthines = Caf + TBR, and PPH = Polyphenols = C + EC + ECG + EGC + EGCG.

Table 9

Average distribution of polyphenols and methylxanthines in decaffeinated green tea samples (mg/L)[1]

Sample	Compounds analyzed[2]								
	Caf	TBR	C	EC	ECG	EGC	EGCG	MET	PPH
Bigelow®	12.3 ±2.5	3.62 ±0.36	16.8 ±9.2	34.2 ±2.2	58.7 ±7.6	113 ±14	224 ±12	15.9 ±2.2	447 ±16
Celestial Seasoning®	12.0 ±3.3	2.68 ±0.35	12.8 ±9.9	27.2 ±2.2	41.0 ±6.1	98.5 ±18	171 ±27	14.7 ±9.9	351 ±58
Lipton®	11.4 ±1.6	4.43 ±0.32	9.81 ±6.5	48.0 ±11.9	77.3 ±8.1	169 ±26	268 ±25	15.8 ±1.9	571 ±67
Our Family®	38.3 ±4.7	6.94 ±0.65	13.8 ±4.5	43.1 ±5.4	62.0 ±8.8	144 ±22	199 ±13	45.3 ±5.1	462 ±29
Red Rose®	22.6 ±1.5	3.43 ±0.98	24.5 ±1.9	54.8 ±9.3	88.0 ±6.2	128 ±13	233 ±13	26.0 ±2.4	528 ±35
Salada®	17.6 ±4.1	3.61 ±0.69	5.55 ±6.35	34.4 ±4.5	55.8 ±13.6	121 ±34	205 ±40	21.2 ±4.7	421 ±96
Stash®	61.3 ±7.4	3.27 ±0.34	18.0 ±11.0	54.3 ±12.0	71.5 ±7.8	150 ±28	268 ±20	64.6 ±7.7	562 ±73

[1]mean and standard deviation of the individual brands.

[2]Caf = Caffeine, TBR = Theobromine, C = Catechin, EC = Epicatechin, ECG = Epigallocatechin, EGC = Epigallocatechin, EGCG = Epigallocatechin gallate, MET = Methylxanthines = Caf + TBR, and PPH = Polyphenols = C + EC + ECG + EGC + EGCG.

Comparison of total polyphenol content

The calculated total polyphenol content (PPH) was considered to be the sum of catechin, epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate. Table 10 shows the distribution of polyphenols per serving. The overall total polyphenols content for all brands ranged between 52.4 mg/serving to 239.0 mg/serving. In the caffeinated green tea analyzed, Red Rose® (239 mg/serving) has the largest amount of polyphenols followed by Stash® (227 mg/serving) and Salada® (202 mg/serving). Among the decaffeinated teas, Lipton® decaffeinated (143 mg/serving) had the largest amount of polyphenols followed by Stash® decaffeinated (140 mg/serving) and Red Rose® decaffeinated (132 mg/serving). The caffeinated tea containing the lowest level of polyphenols was Bigelow® caffeinated (52.4 mg/serving) and the decaffeinated green tea containing the lowest level of polyphenols was Celestial Seasoning® (88.0 mg/serving).

Table 10

Average polyphenol content (mg/ per serving)[1] per tea bag

Sample	Caffeinated	Decaffeinated
Bigelow®	52.4 ± 1.8	112 ± 4.0
Celestial Seasoning®	143 ± 15	88.0 ± 15
Lipton®	180 ± 20	143 ± 17
Our Family®	139 ± 19	116 ± 7.0
Red Rose®	239 ± 20	132 ± 9.0
Salada®	202 ± 18	105 ± 21
Stash®	227 ± 13	140 ± 18

[1]mean ± standard deviation

Freidman et al. (2005) also analyzed comparable green tea samples and similar findings were observed as in the current study. The green tea beverages analyzed were Celestial Seasoning® caffeinated, Lipton® caffeinated, Stash® caffeinated and Stash® decaffeinated. Freidman et al. (2005) found that Stash® contained the largest amount of polyphenols among caffeinated and decaffeinated green tea samples analyzed. Our findings also indicate that Stash® products contained a larger amount of polyphenols compared to Lipton® and Celestial Seasoning® in caffeinated green tea. The Lipton® decaffeinated and Stash® decaffeinated contained almost equal amounts of polyphenols. Differences in reported results are noted because Friedman et al. (2005) expressed their results in mg/g tea and the current study is presented in mg/serving. Also during total polyphenols calculation, Friedman et al. (2005) added additional types of polyphenols that the current study did not investigate.

The distribution of total polyphenol content in individual teas analyzed was compared to the overall mean of 144 mg/serving (Figure 12) to show the deviation from the average. It was observed that Red Rose® caffeinated green tea (239 mg/serving) was a better source of healthy beneficial antioxidants among all the brands investigated, and Lipton® decaffeinated green tea (143 mg/serving) was a better source of antioxidants for consumers of decaffeinated tea.

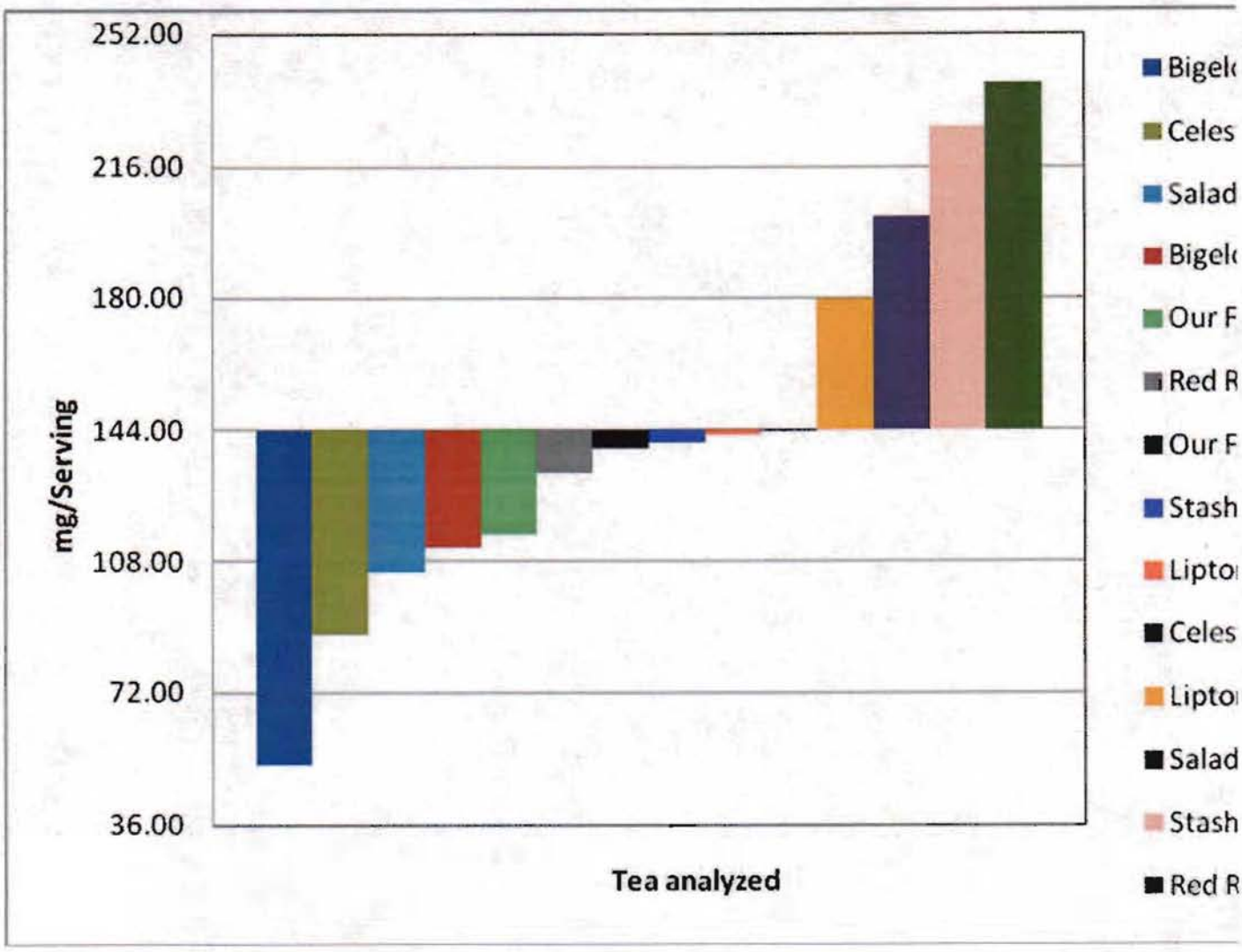

Figure 12. Comparison of total polyphenol content in teas to the mean value (144 mg/serving).

The difference between the polyphenol content of caffeinated and decaffeinated green tea within the same brand was analyzed (Figure 13). Table 11 shows the percentage of polyphenols in decaffeinated green tea compared to its caffeinated counterpart of the same brand. It was determined that the decaffeinated products generally contained from 53 to 89% of the total polyphenols found in their respective caffeinated counterparts. The Stash® caffeinated contained 227 mg/serving compared to Stash® decaffeinated at 140 mg/serving which is 62% of total polyphenols present in caffeinated green tea of the same brand. Bigelow® is the only brand with the exception of having a higher percentage (213%) of polyphenol in decaffeinated green tea (112 mg/serving) compared to caffeinated green tea (52.4 mg/serving). Bigelow® caffeinated contains the least amount of polyphenols (52.4 mg/serving) which is lower than any of the other caffeinated or decaffeinated brands analyzed.

Table 11

Percentage of polyphenol content in decaffeinated green tea compared to its caffeinated form

Brand	% polyphenol content in decaffeinated compared to its caffeinated form.
Bigelow®	213
Celestial Seasoning®	61.2
Lipton®	79.4
Our Family®	83.2
Red Rose®	55.2
Salada®	51.8
Stash®	61.8

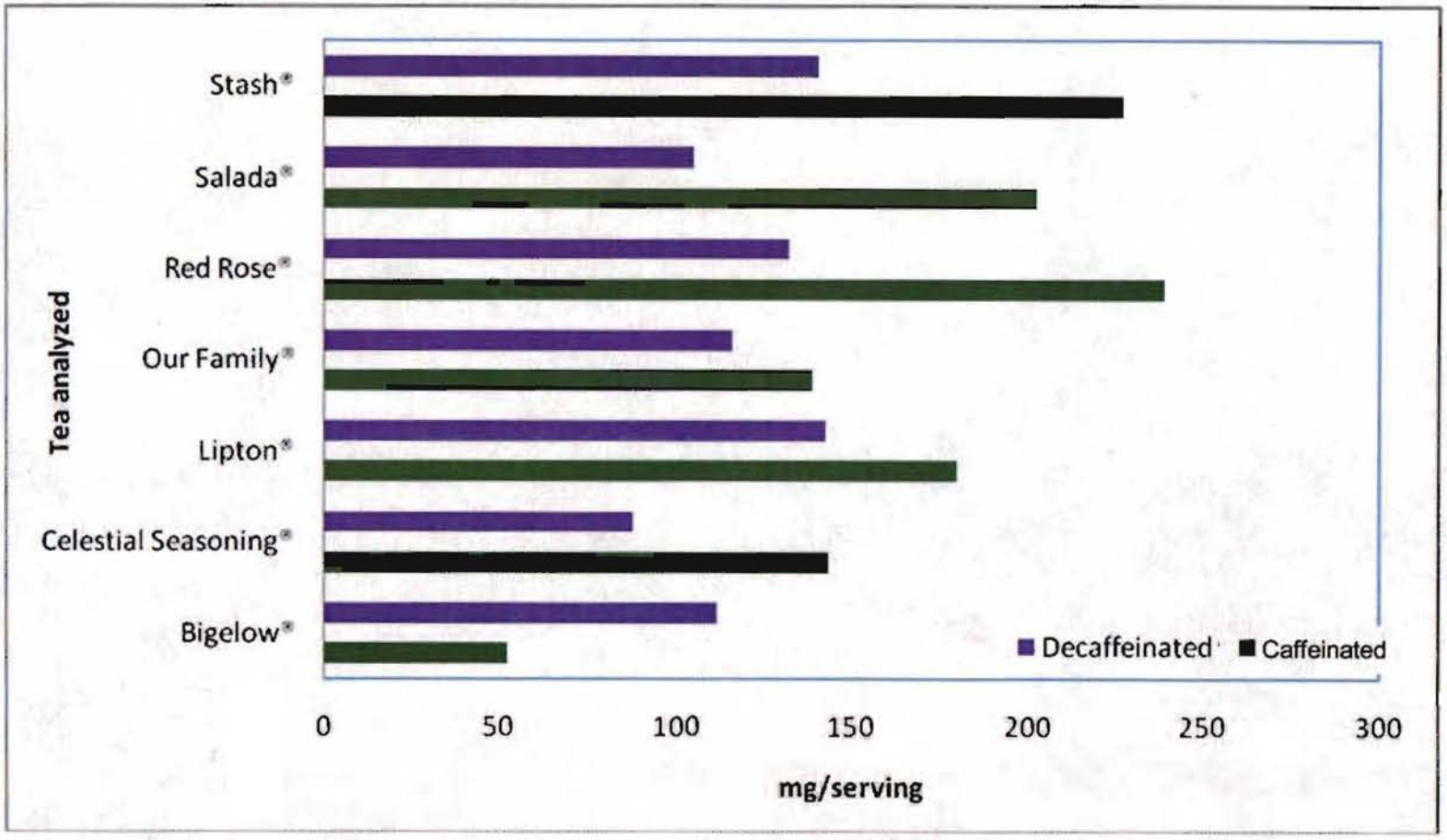

Figure 13. Comparison of total polyphenols (mg/serving) in caffeinated and decaffeinated green tea.

Comparison of total methylxanthines content

Comparisons were made for total methylxanthines (caffeine + theobromine) content among all brands of tea for both caffeinated and decaffeinated forms. Figure 14 shows the distribution of methylxanthines in the caffeinated and decaffeinated tea analyzed. Results indicated that decaffeinated products generally contained about 11% to 38% of the methylxanthine found in the corresponding caffeinated counterparts. Average methylxanthines reduction in decaffeinated green tea was found to be 19.7% $\pm$ 11%. This suggests that there is a large difference in the caffeine content of decaffeinated products. None of the decaffeinated products analyzed were completely caffeine free.

Average methylxanthines content in green tea was found to be 36.6 $\pm$ 9.4 mg/serving for caffeinated and 7.30 $\pm$ 4.7 mg/serving for decaffeinated forms of the same brand. Stash® caffeinated had the largest amount of methylxanthines (49.4 mg/serving) followed by Salada® caffeinated (43.8 mg/serving) and Red Rose® (43.6 mg/serving). Celestial Seasoning® decaffeinated green tea had the lowest amount methylxanthines (3.66 mg/serving) among the brands analyzed. The other two products containing lower amounts of methylxanthines were Lipton® (3.96 mg/serving) and Bigelow® (3.97 mg/serving).

On average, 90% of the total methylxanthines content is caffeine. The average caffeine content of caffeinated green tea was found to be 35.4 $\pm$ 9.8 mg/serving and 6.3 $\pm$ 4.7 mg/serving in decaffeinated green tea. The products with the largest caffeine content were Stash® caffeinated (49.8 mg/serving), Salada® caffeinated (42.9 mg/serving) and Red Rose® caffeinated (42.6 mg/serving). The lower caffeine-containing products were Lipton® decaffeinated (2.9 mg/serving), Celestial Seasoning® decaffeinated (3.0 mg/serving) and Bigelow® decaffeinated (3.1 mg/serving).

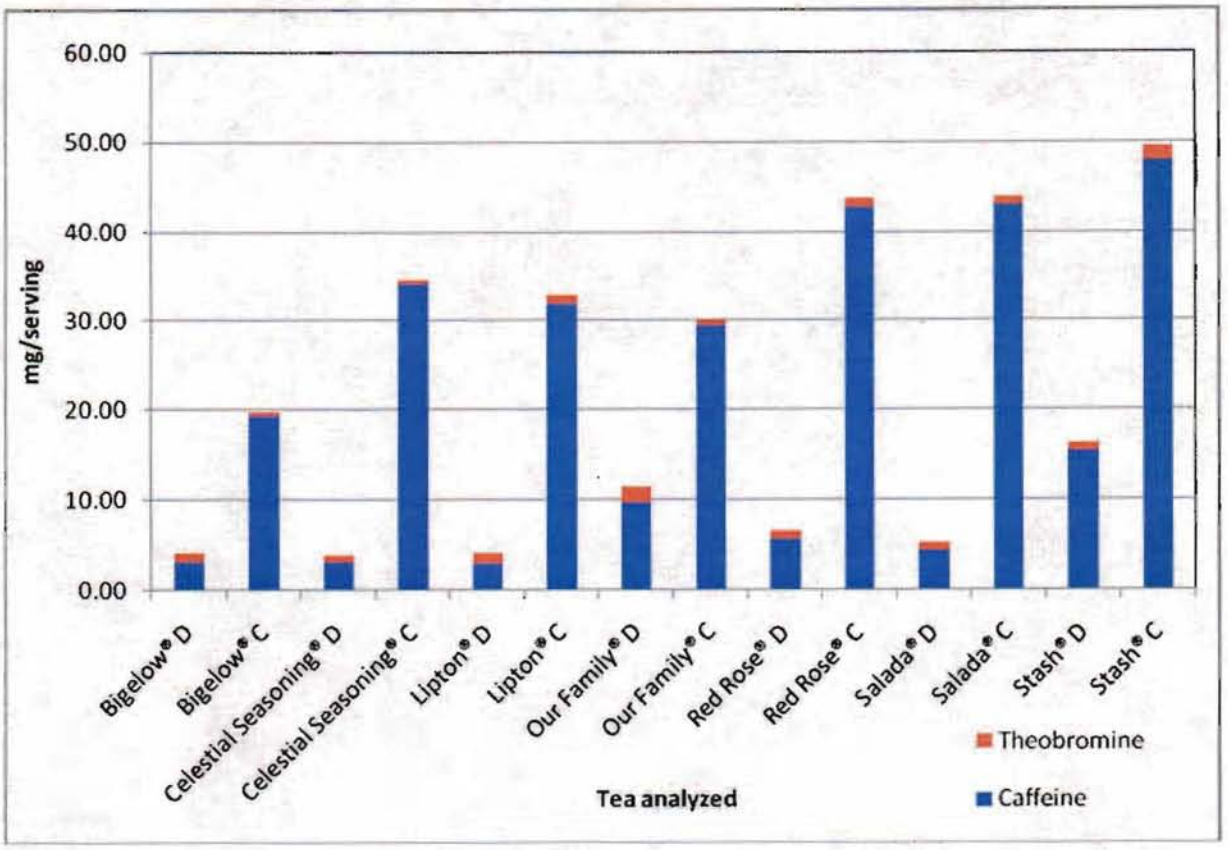

Figure 14. Distribution of methylxanthines (mg/serving) in green teas.

Chapter V: Conclusion

The purpose of this study was to analyze and compare polyphenols and methylxanthines present in commercially available green tea bags. The samples were obtained from local grocery stores (Menomonie, WI and Eau Claire, WI). The samples were prepared to imitate the consumer's green tea brewing process, i.e., following the manufacturer's direction on the label. The samples were analyzed using an HPLC technique. Data obtained were compared with the standard curve of individual components to quantify them. These results were compared according to types and brands of green tea.

The specific blends of tea used by the manufacturers were not known to the investigator. No information was known concerning the cultivar or places of cultivation and the production process. Assumptions made were that all products were derived from *Camellia sinensis* and none of these products were supplemented or fortified with antioxidants.

In general, caffeinated tea contained a greater percentage of polyphenols and methylxanthines compared to decaffeinated forms. Among the caffeinated green tea investigated, Red Rose® (239 ± 20 mg/serving) contained the greatest amount of total polyphenols, followed by Stash® (227 ± 13 mg/serving) and Salada® (202 ± 18 mg/serving). Among the decaffeinated green teas, Lipton® (143 ± 17 mg/serving) contained a larger polyphenol content followed by Stash® (140 ± 18 mg/serving) and Red Rose® (132 ± 9.0 mg/serving). The only brand of green tea having a larger amount of polyphenols in the decaffeinated form was Bigelow® (caffeinated = 112 mg/serving and decaffeinated = 52.4 mg/serving).

The decaffeinated teas contained 11% to 33% of the methylxanthines content found in their caffeinated counterparts. The percentage of caffeine ranged from 72% to 99% of total methylxanthines present. None of the decaffeinated tea was found to be caffeine free. The three

products containing the greater levels of methylxanthines were Stash® (49.4 mg/serving), Salada® (43.8 mg/serving) and Red Rose® (43.6 mg/serving). The lowest amounts of methylxanthines were found in decaffeinated green teas of the brands Celestial Seasoning® (3.7 mg/serving), Lipton® (4.0 mg/serving) and Bigelow® (4.0 mg/serving). The caffeine content in Lipton® decaffeinated (2.9 mg/serving) green tea was marginally lower compared to Celestial Seasoning® (3.0 mg/serving).

The findings of this research showed a similar trend as observed by Friedman et al. (2005). The Friedman et al. (2005) study was the only other published research (Journal of Food Science) found on quantification of polyphenols and methylxanthines in commercially available green teas in US markets.

This study demonstrated that, among the commercially available green tea investigated, Red Rose® caffeinated contained the greatest amount of polyphenols followed by Stash® and Salada®. The green tea containing the lowest level of caffeine with the greatest level of polyphenols was Lipton® decaffeinated.

This study concludes that, among the green tea investigated:

1) Red Rose® caffeinated green tea had the greatest amount of polyphenols, and
2) The caffeinated green tea contained greatest amount of polyphenols and methylxanthines compared to their decaffeinated counterparts.

Recommendations

The following studies are suggested to further explore the subject:

1) Investigate the effect of brewing water temperature on the extraction of polyphenols and methylxanthines content of green tea.
2) Compare the levels of polyphenols in different kinds of tea: white, oolong and black.
3) Investigate the effect of the length of brewing time on polyphenols and methylxanthines content of teas.
4) Investigate and quantify other types of polyphenols or vitamins present in tea.
5) Compare the levels of polyphenols and methylxanthines in bottled green tea.
6) Develop a method to detect other polyphenols present in fruits and vegetables using HPLC.
7) Investigate the effects of added ingredients such as citrus extract, ascorbic acid, and berry juices on polyphenol stability.

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