Identification and Quantification of Flavanols and Methylxanthines in Chocolates with Different Percentages of Chocolate Liquor

By

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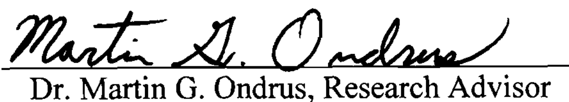
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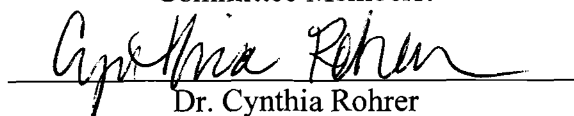
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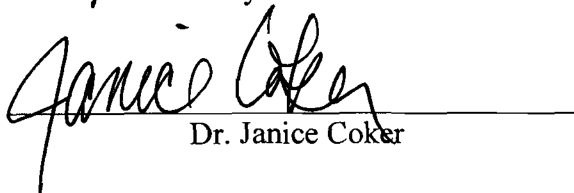
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ABSTRACT

Chocolate liquor is the source of antioxidant flavanols (catechin and epicatechin) and methylxanthines (caffeine and theobromine) found in dark chocolate. Factors that can influence the flavanol and methylxanthine concentration of dark chocolate investigated in this study include the amount of chocolate liquor added, alkalization, and cacao bean genus. The purpose of this study was to quantify flavanols and methylxanthines in different dark chocolates from Legacy Chocolates with different weight percentages of chocolate liquor and different cacao bean genera, Criollo and Forastero. Chocolate samples were analyzed by reverse-phase high performance liquid chromatography (HPLC). Results indicated that the greater the percentage of chocolate liquor added to the final product, the more flavanol antioxidants present. When comparing chocolates with similar weight percentages, the Forastero genus had a significantly greater ($p < 0.05$)

flavanol concentration than the Criollo genus. The Criollo genus resulted in a significantly greater ($p < 0.05$) caffeine content in dark chocolate when compared to a product prepared with similar weight percentages of chocolate liquor from the Forastero genus. Conversely, the Forastero genus produced a chocolate that was significantly greater ($p < 0.05$) in theobromine when compared to a Criollo product with similar weight percentages of chocolate liquor. Alkalization processing did not appear to affect catechin, epicatechin, caffeine, or theobromine concentrations in chocolates with similar weight percentages of chocolate liquor. Commercial brand chocolates were analyzed for comparison.

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Chapter I: Introduction

Dark chocolate, also referred to as sweet chocolate, differs from other types of chocolate based upon the level of chocolate liquor added by weight to the final product. Chocolate liquor is the product attained by grinding the solid contents of the cacao (cocoa) bean, including the cocoa butter. The amount of chocolate liquor in a chocolate product is often referred to as the percentage of cocoa or cacao-derived ingredients per weight.

In the United States, the Food and Drug Administration (FDA) has regulated the level of chocolate liquor to be at least 15% by weight for any chocolate product to be labeled as "dark or sweet chocolate" (Food and Drug Administration, 2003). Increasingly, many commercially sold dark chocolates are packaged with a label stating the percentage of cocoa-derived ingredients by weight.

Chocolate liquor is the source of antioxidant polyphenols that are present in dark chocolate, cocoa, and other chocolate products prepared from chocolate liquor (Natsume et al., 2000). Since chocolate liquor contains polyphenols, it would be expected that the polyphenol content of chocolate would depend on the percent of chocolate liquor added to the finished product. However, a greater percentage of chocolate liquor may not always represent an increased polyphenol content in the finished chocolate product. Factors that affect the final polyphenol content of a finished chocolate product include the cacao bean variety, amount of chocolate liquor, amount of milk solids, alkalization processing, and cacao bean fermentation (Adamson et al., 1999; Natsume et al., 2000).

Polyphenols are a group of antioxidants most universally found in the human diet (Scalbert, Johnson, & Saltmarsh, 2005). They are widely distributed throughout the plant

kingdom and typical dietary sources include fruit, vegetables, tea, coffee, red wine, grains, and chocolate. The polyphenols present in chocolate liquor, cocoa, and dark chocolate are classified as flavonoids. Flavonoids, such as catechin and epicatechin, are classified as flavon-3-ol monomer units (flavanols); their oligomers are classified as procyanidins (Lazarus, Hammerstone, Adamson, & Schmitz, 2001). An abundance of polyphenol antioxidants in the diet has been associated with a decreased risk of developing chronic diseases, such as cardiovascular disease (Grassi et al., 2005; Rein et al., 2000a, 2000b; Wang et al., 2000) and cancer (Arteel et al., 2000; Keeny et al., 2004; Yamagishi et al., 2002a). Studies have demonstrated that cocoa has more polyphenols and flavonoids than green tea and red wine, therefore, resulting in a higher antioxidant activity once ingested (Lee, Kim, Lee, & Lee, 2003).

Other compounds present in chocolate, caffeine and theobromine, are a class of methylxanthines that have been widely quantified in the literature. Methylxanthines have known physiological effects in the body such as central nervous system stimulation, cardiac muscle stimulation, relaxation of smooth muscle (especially bronchial muscle), and diuretic effects (Apgar & Tarka, 1999; Gilbert, 2004).

Like polyphenols, chocolate liquor is also the source of methylxanthines that are present in dark chocolate. In addition to the percentage of chocolate liquor added to the finished product, the caffeine and theobromine content of dark chocolate depend on a variety of factors, including cacao bean genus, cacao bean maturity, and cacao bean fermentation conditions (Apgar & Tarka, 1999). Therefore, the associated health effects due to methylxanthine consumption from chocolate may differ by chocolate brand or type.

The concentration of catechin, epicatechin, caffeine, and theobromine present in dark chocolate can be quantified by high performance liquid chromatography (HPLC). By analyzing the concentration of these compounds in different chocolate products, the health effects associated with their consumption can be reviewed in regards to the amount of each compound present per serving size. Since the amount of each compound may differ by brand, it is important to quantify the concentrations in a variety of dark chocolate products to observe the degree of variance.

Statement of the Problem

Flavanol monomers, such as catechin and epicatechin, and methylxanthines such as caffeine and theobromine have been extensively quantified in different types of chocolates. However, the comparison and quantification of flavanol monomers and methylxanthines from different sources of dark chocolate (Dove®, Ghirardelli, Hershey's®, Legacy Chocolates, and Lindt) have not previously been conducted.

Legacy Chocolates sells two main types of finished dark chocolate samples, medallions and truffles. Five types of raw chocolate with different weight percentages of chocolate liquor (41%, 58.5%, 60%, 73.5%, and 100%) are used to produce Legacy Chocolates' medallions and the truffle shell. These raw chocolate products were tested using reverse-phase HPLC to quantify levels of catechin, epicatechin, caffeine, and theobromine.

For comparison, commercial brand solid dark chocolate products (Dove®, Ghirardelli, Hershey's®, and Lindt) were also analyzed. HPLC testing was conducted at the UW-Stout Chemistry Department, 3rd floor Jarvis Hall Science Wing during the spring 2006 semester.

Purpose of the Study

The purpose of this study was to quantify flavanols (catechin and epicatechin) and methylxanthines (caffeine and theobromine) in different dark chocolate samples from Legacy Chocolates by HPLC. The specific objectives were to:

1) Develop an HPLC method for detecting monomer flavanols (catechin and epicatechin) and methylxanthines (caffeine and theobromine) simultaneously from solid dark chocolate samples;
2) Quantify the concentration of catechin, epicatechin, caffeine, and theobromine in Legacy Chocolates dark chocolate samples containing five different weight percentages of chocolate liquor: 41%, 58.5%, 60%, 73.5%, and 100% (chocolate liquor);
3) Determine the pure medallion and truffle shell monomer flavanol/methylxanthine concentration in chocolates containing 41%, 58.5%, 60%, 73.5%, and 100% weight percentages of chocolate liquor available from Legacy Chocolates.
4) Quantify and compare the monomer flavanol and methylxanthine content of Legacy Chocolates with four commercial dark chocolates (Dove®, Ghirardelli, Hershey's®, and Lindt).

Assumptions

Flavanols and methylxanthines were assumed to be present in all varieties of dark chocolate samples. In addition to the analytes of interest being present in all of the dark chocolates, the samples with the greater percentages of chocolate liquor by weight were assumed to contain a greater concentration of flavanols/methylxanthines than the dark chocolates containing a lower amount of chocolate liquor by weight.

Definition of Terms

The following terms will be commonly used throughout this research paper and are defined as follows.

Antioxidant. Any substance that when present at low concentrations compared with those of an oxidizable substrate significantly delays or prevents oxidation of that substrate (Halliwell & Gutterridge, cited in Halliwell, 2001).

Chocolate liquor/Cocoa/Dark Chocolate Flavonoids. Referring specifically to catechin, epicatechin, and procyanidins.

Cocoa Liquor. Cocoa nib which is finely ground (Beckett, 2000).

Cocoa Mass. Another name for cocoa liquor (Beckett, 2000).

Cocoa Nibs. Cocoa beans with the shell removed (Beckett, 2000).

Dark chocolate. Any chocolate that has 15 % or greater chocolate liquor.

Flavonoids. A category of polyphenols that are considered to be very important because they are the most commonly occurring and widely distributed throughout the plant kingdom, produced as secondary plant metabolites (Bloor, 2001; Bravo, 1998).

Ganache. Referring to the inside content of truffles.

Methylxanthines. Referring specifically to caffeine and theobromine.

Monomer Flavanols. Referring specifically to catechin and epicatechin.

Polyphenols. Referring to a compound comprised of two or more aromatic rings, with each ring containing one or more hydroxyl groups (Lazarus et al., 2001).

Chapter II: Literature Review

History of Chocolate

Chocolate is produced from cocoa beans of the cacao tree, *Theobroma cacao*. *Theobroma cacao* is native to the Amazon rainforests of South and Central America and is believed to have naturally spread northward to Mexico and Guyana (Minifie, 1989). There are over twenty species of *Theobroma* trees, but only *T. cacao* is used to produce commercial cocoa/chocolate products. Two distinct subspecies of *T. cacao* are believed to have developed from the spread of the cacao tree, Criollo and Forastero. A third subspecies, Trinitario, is a hybrid of the Criollo and Forastero.

Commercial cacao trees are grown in tropical regions 20° north and 20° south of the equator, where suitable growing conditions such as high average temperatures, humidity, and rainfall favor tree growth (Beckett, 2000). Currently, there are three major cocoa growing regions: West Africa, South-East Asia, and South America.

The first known cocoa plantations were developed by the ancient Mayan culture in the south Yucatan of present day Mexico, around 600 AD, where cocoa beans were roasted and milled (Beckett, 2000). Cocoa beans were highly regarded in the Central and South American cultures of the Mayans, Aztecs, and Incans. Historical illustrations depict cups of "chocolatl"—a mixture of roasted and crushed cocoa beans, water, maize and spices—being consumed at wedding ceremonies and in the court of the Aztec emperor, Montezuma (Minifie, 1989). The chocolate drink was very fatty due to the high levels of cocoa butter present and had a very bitter taste.

Christopher Columbus first introduced cocoa beans to Europe, but when the Spaniards conquered Mexico, Don Cortez introduced the chocolatl drink to Spain in the

1520s (Beckett, 2000). The concoction was primarily unknown in Europe until the early to mid1600s, when it first came to Italy, then spread to France and England. The chocolate drink was very expensive; therefore, it could only be afforded by European royalty and aristocracy. By 1657, chocolate drinking houses were present in London.

The development of chocolatl to chocolate as it is known today was produced by over 200 years of cocoa bean processing developments. The most important development occurred in 1828, when Van Houten developed the cocoa press (Beckett, 2000). The cocoa press was a machine that involved treating the cocoa beans during the roasting process with an alkali liquid, pressing the cocoa beans to remove about half of the fat content, and milling the product into a powder.

The modern chocolate bar of today can be attributed to the development of the cocoa press machine. Since half of the fat from the cocoa bean was removed using this machine process, cocoa powder producers were left with a considerable amount of excess fat, called cocoa butter (Beckett, 2000). Confectioners discovered that a solid, uniform eating chocolate could be produced by combining milled cocoa, sugar, and cocoa butter, therefore, providing a market for the previously unwanted cocoa butter.

Cacao Bean Genus and Chocolate Attributes

Three genera of *T. cacao,* Criollo, Forastero, and Trinitario, produce cacao beans with different taste characteristics (Lass, 1999). Forastero beans are the most widely produced, accounting for 93.5% of world production. The Forastero beans are described as having a more astringent taste because they have more polyphenol tannins than the other cacao tree genera (Lopez, 2002). The Criollo beans have a milder, nutty flavor than the Forastero, and only make up 1.5% of world production (Lass, 1999). Because of their

delicate flavor, the Criollo genus is used to make fine or premium chocolates. The Trinitario is a hybrid of the Criollo and Forastero; its sensory attributes are typically a combination of the two genera.

Cocoa Pod Harvesting

Chocolate production starts with the harvesting of the cacao pods. Mature pods from the cacao tree are harvested over a period of several months because the trees simultaneously bear mature pods, flowers, and growing pods (Minifie, 1989). Once the mature pods are removed from the cacao tree, they are taken to a fermentary where the cacao beans are separated from the pod and pulp by cracking the pod open with machetes or wooden clubs. Each pod may contain between 30-45 cocoa beans, which are hand-separated from the surrounding white pulp (mucilage). The cocoa beans consist of an outer shell or testa which surrounds two cotyledons (nibs) and a small germ (embryo) (Beckett, 2000). The cotyledons store the food for the developing seedling in the form of fat, called cocoa butter.

Fermentation and Drying

Cacao bean fermentation and drying are vital steps in cocoa processing that directly affect the flavor of the final cocoa or chocolate product (Beckett, 2000). Fermentation kills the live cocoa bean; it is unable to be spoiled by germination. The fermentation process of cocoa is technically not fermentation because microorganisms do not come directly in contact with the cotyledons, which is the part of the cocoa bean used to make chocolate (Minifie, 1989). For that reason, the fermenting process may be referred to as 'curing'.

Chemically, many changes take place in the cacao pod during fermentation. Within the first three days of heat treatment, the temperature of the beans rises to about 45°C (Beckett, 2000). Yeasts which are naturally present on the cocoa bean break down the sugars that are present in the pulp. The yeasts cause the amount of sugars present to decrease from 11% to 2%, thereby converting the sugars to ethanol. The formation of ethanol stimulates acetic acid and lactic acid bacteria to oxidize the alcohol and produce their respective acids, acetic acid and lactic acid. As the temperature of the beans is maintained between 45-50°C, due to the bacterial activity, some acetic acid is absorbed by the cotyledon, causing proteins and peptides to react with polyphenols to produce the customary "cocoa brown" color (Hoskin & Dimick, 1988). These reactions cause the polyphenol levels to significantly decrease during fermentation (Adamson et al., 1999). Other important chemical reactions occur between sucrose and proteins, which form cocoa flavor precursors. After the fermentation process is complete, it is necessary to dry the cocoa beans to a 7-8% moisture level, which helps retard mold growth (Beckett, 2000). The heap method and the box method are two methods used to ferment the beans.

Heap fermentation is used for small batches which can range from 25-2500 kg of fresh beans, combined with a small amount of attached white pulp (Beckett, 2000). The beans and pulp are covered with banana leaves and allowed to ferment for 5-6 days outside and are rotated every 2-3 days. Variations in fermentation time and heap size can both attribute to the final flavor and smaller heap sizes tend to produce better flavors.

Box fermentation is used for larger batches of beans, ranging from 1-2 tons (Beckett, 2000). The beans are placed in large wooden boxes which have outlet draining slits at the base of the box, where excess water from the beans and pulp drains. Boxes can

be as deep as 1 meter; however, more shallow boxes produce a better flavor due to enhanced ventilation. The beans are typically rotated from one box to another to increase aeration and provide a more uniform fermentation. The fermentation time period is between 5-8 days. Fermentation is subsequently followed by cocoa bean roasting.

Roasting

The roasting process is the most vital step in the development of the traditional cocoa flavor of chocolate. The cocoa beans are roasted in order to intensify the complex interaction between the flavor precursors which result in chocolate flavors (Hoskin & Dimick, 1988). Roasting can occur by three different methods: whole bean roasting, nib roasting, or liquor roasting (Beckett, 2000). The following flow diagram outlines the general chocolate processing procedure, demonstrating variances in the roasting procedure (adapted from Beckett, 2000).

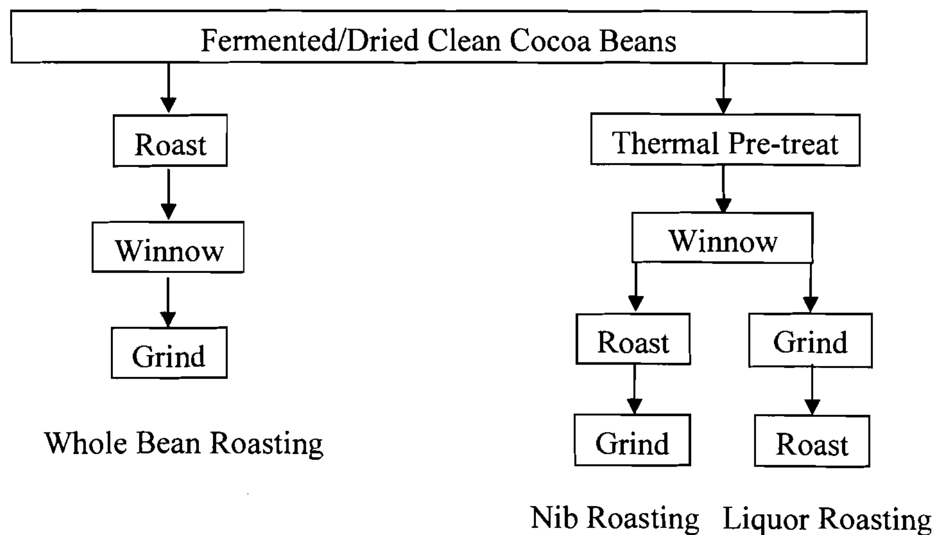

Figure 1. Cocoa Bean Roasting Flow Diagram.

Source: Becket, 2000

Alkalization

Alkalization (also referred to as Dutching) is a process that refers to the treatment of cocoa powder, liquor, beans, or nibs with an alkali solution, potassium or sodium carbonate, in order to develop a darker color of the cocoa product and adjust the taste (Bixler & Morgan, 1999). Alkalization results in products that tend to be less acidic, and products with less harsh sensory characteristics. The alkalization process is not a mandatory processing step, but some chocolate producers use an alkalized liquor to produce the final product, while others use natural, unalkalized liquor.

During the alkalization process, many chemical reactions take place involving procyanidins, a group of epicatechin oligomers (Adamson et al., 1999). The alkalization process is known to cause chemical alterations to the polyphenols, thereby decreasing the total polyphenol content of Dutched chocolate products.

FDA Regulations on the Composition of Dark Chocolate

Chocolate is regulated by the US FDA to ensure that consumers receive a standardized product and are not deceived by terminology. In the US Code of Federal Regulations, dark chocolate produced in the US is referred to as "sweet chocolate" and is described in section 163.123 of Code of Regulations, Title 21, Volume 2 (Food and Drug Administration, 2003). Sweet chocolate is defined as a solid or semi-plastic food prepared by mixing and grinding chocolate liquor with one or more optional nutritive carbohydrate sweeteners, and may contain one or more of the following: 1) cacao fat; 2) nutritive carbohydrate sweeteners; 3) spices, natural and artificial sweeteners, ground whole nut meats, dried malted cereal extract, salt, and other seasonings that do not either singly or in combination impart a flavor that imitates the flavor, chocolate, milk or butter;

4) dairy ingredients such as cream, milk fat, butter, milk, concentrated milk, evaporated milk, sweetened condensed milk, dried milk, skim milk, concentrated skim milk, evaporated skim milk, sweetened condensed skim milk, nonfat dry milk, concentrated buttermilk, dried buttermilk, and malted milk; and 5) emulsifying agents in which the total weight does not exceed 1% by weight. In addition to these requirements, the finished dark chocolate product must not contain less than 15% by weight of chocolate liquor and must contain less than 12% by weight of total milk solids as described in 4) dairy ingredients.

The amount of polyphenols present in a finished dark chocolate product depend on the cacao bean variety, amount of chocolate liquor, amount of milk solids, alkalization processing, and fermentation, all of which may affect the final polyphenol content of the dark chocolate (Adamson et al., 1999; Natsume et al., 2000).

Ingredients of Dark Chocolate

Chocolate liquor. Chocolate liquor can be defined as the cocoa nibs which are finely ground (Apgar & Tarka, 1999). Chocolate liquor is also known as baking chocolate or unsweetened chocolate. All chocolate and cocoa products are produced from chocolate liquor. Since chocolate liquor is derived directly from the cacao pod, without the addition of other ingredients, it has the highest amount of polyphenols when compared with other chocolates with other ingredients added (Natsume et al., 2000).

Cocoa butter. Cocoa butter is the fat component that is extracted from the cocoa bean cotyleydon and is used in solid chocolate products. Cocoa butter is extracted from either cocoa liquor or from whole cocoa beans (Kleinert, 1988). Cocoa butter is a triglyceride in which 95% of the fatty acids present are palmitic, stearic, and oleic acids

(Beckett, 2000). The composition of these fatty acids is what gives chocolate its unique property—a small temperature range between its solid and liquid state.

Nutritive carbohydrate sweeteners. The most common type of sweetener used to produce chocolate is sucrose (Krüger, 1988). Sucrose is a disaccharide, composed of two chemically linked monosaccharides, glucose and fructose. Crystallized sucrose is the most favorable sugar used in chocolate manufacturing due to its physical properties, which include low moisture content (0.06%), a low percentage of invert sugar (0.04%), and relative sweetness rating of 1.0. Invert sugar is typically undesirable due to its aqueous nature.

The low moisture content of sucrose is necessary during storage before chocolate manufacturing to prevent physical, chemical, and microbiological damage (Krüger, 1988). Sucrose it recommended to be stored between 20 and 60% relative humidity and 20°C to maintain a moisture content of 0.06%. Glucose and fructose are typically not used to manufacture chocolate because of their high moisture content.

Other sweeteners often used in chocolate manufacturing include monosaccharide sugar alcohols, such as sorbitol, mannitol, and xylitol. Sugar alcohols are used in chocolate manufacturing because they affect the blood glucose levels less significantly than sucrose and therefore are desirable products for diabetics and dieters (Krüger, 1988). The combination of different sugar alcohols such as maltitol and xylitol can produce a sweetening effect in chocolate undistinguishable from sucrose.

Dairy ingredients. Chocolate producers may choose to incorporate milk/dairy products into dark chocolates, in amounts less than 12% by weight (Food and Drug Administration, 2003). The optional dairy ingredients that may be incorporated into dark

chocolate were previously listed in *FDA Regulations on the Composition of Dark Chocolate* (Food and Drug Administration, 2003).

Milk products and milk solids are used to a greater extent in milk chocolate manufacturing as opposed to dark chocolate manufacturing (Würsch & Finot, 1999). The addition of milk products/solids to chocolate adds protein, calcium, lactose, and fats. Beyond its nutritive value, the addition of milk products/solids to chocolate tends to improve its taste and add desirable sensory descriptors to the final product.

Emulsifiers. Lecithin is the traditional emulsifier used in chocolate production (Beckett, 2000). Lecithin has the ability to blend together immiscible substances (polar and non-polar) that are in contact with one another. In chocolate, the role of an emulsifier is to blend together polar and non-polar ingredients, namely sugar and lipids (cocoa butter and/or milk fats). Typically, these substances would not blend together, but instead create an immiscible liquid. When an emulsifier is added, it coats the sugar surface with a polar end, and the non-polar end of the lecithin molecule will remain in the lipid portion, which allows the sugar to be dispersed throughout the lipid components. The FDA allows a maximum level of total emulsifiers to be no more than 1% by weight (Food and Drug Administration, 2003).

Optional flavoring ingredients. Optional flavoring ingredients are defined by the FDA as any spice, artificial or natural flavorings, nuts, coffee, malted cereal, etc., that does not imitate the flavor of chocolate, milk, or butter (Food and Drug Administration, 2003). Chocolate manufacturers can make flavored, coated, or types of chocolate products, providing that they adhere to the FDA regulations.

Methylxanthines in Dark Chocolate

Caffeine (1,3,7-trimethylxanthine) (Figure 2) and theobromine (3,7-dimethylxanthine) (Figure 3) are purine alkaloids naturally present in chocolate and belong to group of chemical compounds referred to as methylxanthines (Tarka & Hurst, 1998). Theophylline (1, 3-dimethylxanthine), also a methylxanthine, is only present in chocolate in trace amounts.

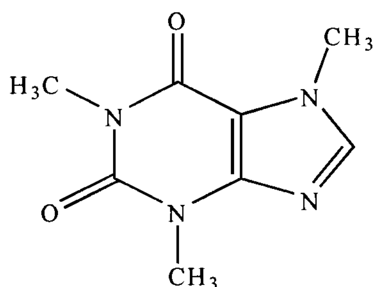

Figure 2. Caffeine.

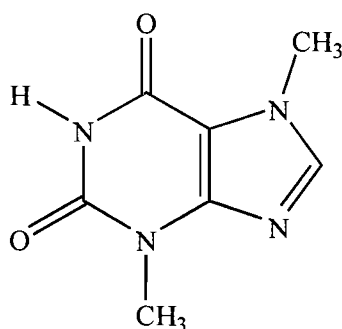

Figure 3. Theobromine.

Theobromine is present in dark chocolate in quantities six to seven times greater than caffeine (Apgar & Tarka, 1999). Dark chocolate must contain at least 15% chocolate liquor by law, but may contain more. Therefore, the caffeine and theobromine content of dark chocolate can be affected by the quantity of chocolate liquor present chocolate product. In commercial brand chocolates, a 40 g serving size of dark chocolate contains on average 185 mg of theobromine and 30 mg of caffeine. Many other factors can affect the level of theobromine and caffeine in a finished chocolate product, including cacao bean variety, maturity, and fermentation conditions (Apgar & Tarka, 1999).

Caffeine and theobromine have many physiological actions in the body, including central nervous system stimulation, cardiac muscle stimulation, relaxation of smooth muscle (especially bronchial muscle), and diuretic effects (Apgar & Tarka, 1999; Gilbert, 2004). Each of these two methylxanthines differs in the intensity of their actions on different body systems. For example, caffeine has strong effects on skeletal and brain muscle, while theobromine, although less effective physiologically than caffeine, may exhibit anti-cough properties by inhibiting sensory nerve functions (Usmani et al., 2004).

Central Nervous System and Methylxanthines

Caffeine is a central nervous system (CNS) stimulant, whereas theobromine has virtually no effect on the CNS (Apgar & Tarka, 1999). Stimulatory effects of caffeine consumed at levels between 150-200 mg results in reduced fatigue, shortened reaction time, sensory stimulation, and increased motor activity. Average literature values of caffeine and theobromine in a 40 g serving size of dark chocolate is 28 mg and 185 mg, respectively. For comparison, the average cola soft drink contains about 40 mg of caffeine per 12 ounce serving (Spiller, 1998a).

This topic has been studied extensively, and evidence indicates that caffeine produces a stimulatory effect by blocking the adenosine receptor from up-taking adenosine, which acts as a neuromodulator (Benowitz, 1990; Spiller, 1998b). Health effects associated with high doses of caffeine can include insomnia, anxiety, tremors, and seizures (Benowitz, 1990).

Cardiovascular System and Methylxanthines

Both theobromine and caffeine stimulate cardiac muscle (theobromine to a greater degree) by modifying the contractility of muscles in the heart and blood vessels and by influencing neurotransmission peripherally and centrally (Apgar & Tarka, 1999; Fredholm, 1984; Spiller, 1998b). The effects of methylxanthines tend to be the most strong in those persons who do not regularly consume caffeine or theobromine (Apgar & Tarka, 1999; Dews, 1982). Methylxanthine consumers tend to build a resistance to cardiovascular effects after a one week time period of regular consumption.

Caffeine and theobromine stimulate cardiac muscle by increasing the rate of contraction, which leads to increased heart rate and cardiac output. Conversely, methylxanthines can also stimulate the medullary vagal nuclei, resulting in a decrease in heart rate. The net result indicates that there may be no overall change in the heart rate due to methylxanthine consumption. In a recent study conducted by Barry et al. (2005), no significant changes were seen in the heart rate, respiration (breaths/min), or blood pressure in healthy adults given a single dose of 250 mg of caffeine versus a placebo.

The effects of methylxanthines on blood pressure are a controversial topic due to inconsistent results from many studies (Apgar & Tarka, 1999; Nawrot et al., 2003). Some studies have implicated caffeine as a cause of high blood pressure, while others have not.

For instance, in non-habitual methylxanthine consumers, caffeine and theobromine tend to increase blood pressure readings after a single caffeine dose; whereas, no change in blood pressure occurred in habitual methylxanthine consumers. It appears that one mechanism of action by methylxanthines is the stimulation of the central vasomotor and myocardium tend to increase blood pressure, whereas central vagal stimulation and peripheral vasodilatation tend to decrease blood pressure (Apgar & Tarka, 1999).

Respiratory System and Methylxanthines

Methylxanthines are recognized for their effectiveness in stimulating respiration and relaxing smooth muscles of the bronchi, especially theophylline and theobromine (Apgar & Tarka, 1999). Theobromine has been shown to display an antitussive (anti-cough) action that is unique to any other methylxanthine, and the mechanism of action appears not to be related to bronchodilator capabilities, but to its ability to inhibit sensory nerve activation (Usmani et al., 2004). Theobromine has insufficient bronchodilator capabilities, and has been excluded from much research involving the respiratory system.

A recent study (Usmani et al., 2004), has demonstrated suppression of capsaicin induced sensory nerve depolarization on the human vagus nerve by theobromine, therefore suggesting that theobromine plays an inhibitory role on afferent nerve activation. No adverse cardiovascular or central nervous system side effects were reported. The sensory nerve inhibition is believed to function by a peripheral mechanism.

Renal Effects of Methylxanthines

Methylxanthines are also associated with the renal system and diuretic effects, especially caffeine, since it exhibits a stronger effect on the renal system than does theobromine (Spiller, 1998b). The diuretic effect displayed by methylxanthines is due to

an increased renal blood flow and glomerular filtration rate. An increased diuretic effect causes increased calcium loss from excretion (Massey & Whiting, 1993). Increased calcium excretion may be considered a potential risk factor for developing osteoporosis, a disease in which bones mass deteriorates, rendering bones more fragile and more likely to break or fracture (Bruce & Spiller, 1998).

In a comprehensive literature review of epidemiological and experimental studies conducted on caffeine intake and bone loss, no consistent results have been found to implicate caffeine as a key factor resulting in bone fracture (Bruce & Spiller, 1998). However, long-term patterns of high caffeine consumption and low calcium intake in women appear to contribute negatively to calcium and bone metabolism and are correlated with a loss of bone mass and increased bone fracture. Age is also implicated in the caffeine consumption and calcium loss debate: younger women appear to be able to compensate for calcium loss due to moderate caffeine consumption, while older women are less able to compensate for calcium loss (Bruce & Spiller, 1998; Massey & Whiting, 1993). No specific age is associated with calcium loss compensation, but it may be caused by the inability to absorb calcium due to increased age. Due to conflicting data, more studies need to be conducted to determine the effect of caffeine on calcium metabolism and osteoporosis. Persons who may be affected by osteoperosis should be aware of the caffeine content of dark chocolate.

Reproduction and Caffeine

Caffeine intake and pregnancy has been extensively studied since 1980, when the FDA issued a warning to pregnant women to restrict their caffeine intake after animal studies demonstrated that caffeine had harmful effects on reproduction (Winick, 1998). It

should be noted that animals metabolize caffeine differently than humans, and conclusions from animal studies on this topic cannot be directly applied to humans.

Caffeine consumption appears to have conflicting results related to human fertility and spontaneous abortion (Winick, 1998). Some studies have shown that high does of caffeine (greater than 300 mg/day) are associated with infertility and spontaneous abortion. However, other studies have demonstrated that consumption of caffeine in doses greater than 300 mg/day display no effect on fertility and spontaneous abortion. In an epidemiological review of caffeine consumption and fertility, Nawrot et al. (2003), recommends that women should reduce their caffeine level to less than 300 mg/day to increase their fertility and to decrease the risk of spontaneous abortion. Pregnant women who are restricting their caffeine intake should be aware of caffeine concentrations present in dark chocolate.

Free Radicals

Free radicals are often implicated as being co-factors to the development of many diseases. Free radicals can damage essential molecules in the body such as proteins, fat and DNA, which are responsible and necessary for cellular processes (Smythies, 1998). Once cellular functions are incapable of working properly, stress occurs, which can subsequently lead to disease.

A free radical is defined as any atom or molecule (chemical species) capable of independent existence that contains at least one unpaired electron (Morello, Shahidi, & Ho, 2002). Most biological molecules are non-radicals, containing only paired electrons. The formation of a free radical occurs when a non-radical species loses one electron, leaving one unpaired electron, which now allows the free radical the ability to gain an

electron. When a free radical gains an electron, the free radical species is terminated, and ceases all free radical activity.

Some, not all free radicals can act as oxidizing agents (Pryor, 1994). Oxidation refers to the transfer of electrons from one molecule to another. Oxidizing agents are molecules that are missing an electron and will steal an electron from a neighboring molecule, which is physically detrimental to the neighboring molecule (Smythies, 1998). Reactive oxygen species (ROS) is a term often used to describe free radicals that are oxidizing agents (Halliwell, 2001). ROS are derived from oxygen and non-radical derivatives of oxygen, such as hydrogen peroxide. For example, hydrogen peroxide is a cellular by-product designed to undergo degradation after it degrades fatty acids. Under certain conditions, the cellular hydrogen peroxide degradation can fail, leaving hydrogen peroxide in the cell, which can quickly lead to DNA oxidation (Frei, 1994).

It is important to note that not all oxidizing agents found in the body are harmful. In fact, many biochemical processes actually create reactive oxygen species in order to carry out mechanisms by which they work. For example, the immune system, specifically white blood cells, create reactive oxygen species, used to kill pathogenic bacteria and viruses (Smythies, 1998). However, dietary polyphenols have displayed strong antioxidant capabilities against free radicals, which may help prevent chronic diseases (Keeny et al., 2004; Arteel, Schroeder, & Sies, 2000).

Oxidative Stress and Antioxidants

ROS are believed to be a major contributor to the onset of oxidative stress (Halliwell, 2001), which can cause cellular damage. Oxidative stress, as defined by Sies (1991), is a disturbance in the prooxidant-antioxidant balance, in favor of the former,

leading to potential damage (as cited in Halliwell, 2001). Oxidative stress can result from the following situations, as described by Halliwell:

1) Diminished levels of antioxidants, due to mutations in antioxidant defense enzymes, toxins that deplete antioxidant defenses, or deficiencies in dietary antioxidants; and
2) Increased production of reactive species, such as toxins that are themselves reactive species or are metabolized to generate reactive species, or an excessive activation of natural biological systems producing reactive species.

The role of oxidative stress on cellular components can result in 1) adaption of the cell or organism by up-regulation of defense systems, which can protect against damage, protect against damage to some extent, or overprotect, which enables the cell to resist higher levels of oxidative stress; 2) tissue injury, such as protein, lipids, carbohydrates and DNA damage; and 3) cell death, by apoptosis or necrosis.

An antioxidant, as defined by Halliwell and Gutterridge (as cited in Halliwell, 2001), is any substance that when present can significantly delay or prevent oxidation of molecule in the human body. Typically, oxidative stress is thought to be an end result and not the primary cause of disease process; however, oxidative stress is believed to play an important role in the development of human diseases (Halliwell, 2001).

The human body creates its own antioxidants in order to combat the free radicals it generates; however, dietary antioxidants also contribute to scavenging free radicals. The primary role of antioxidants in the body is to prevent or inhibit cellular degradation initiated by free radical reactions (Morello et al., 2002).

Polyphenol Antioxidants

Polyphenols are a class of antioxidants most universally found in the human diet (Scalbert et al., 2005). They are widely distributed throughout the plant kingdom and typical dietary sources include fruits, vegetables, tea, coffee, red wine, grains and chocolate. Polyphenols are defined as a compound comprised of two or more aromatic rings, with each ring containing of one or more hydroxyl groups (Lazarus et al., 2001). Polyphenol antioxidant capacity can be influenced by various factors. One important structural factor involves the degree of hydroxylation on the B ring (Figure 4). The more hydroxyl groups the polyphenol has on its B ring, the more potent the antioxidant activity (Bors, Michel, & Stettmaier, 2001).

The antioxidant activity of polyphenols involves the donation of a hydrogen atom and transfer metal binding. Polyphenols are noted to exhibit strong antioxidant effectiveness, and the aroxyl radicals created as a product of free radical termination are sufficiently stable to avoid chain-propagation reactions (Bors et al., 2001).

Polyphenols have the ability to donate a hydrogen atom to the free radical. Once it is donated to the free radical, it becomes inactivated, preventing further reactions. When the polyphenol antioxidant loses its hydrogen from an attached hydroxyl group, the radical becomes an aroxyl radical, which is sufficiently stable enough to avoid chain-propagation reactions (Bors et al., 2001).

Polyphenols also have the ability to bind to transition metals, therefore inhibiting oxidation of cells potentially affected by the metals (Morello et al., 2002). The binding of metal ions by antioxidants is very important, as transition metal ions reacting with ROS are implicated in many chronic diseases (Fuchs, 2001). Therefore, the incorporation of

dark chocolate polyphenols into one's diet may be beneficial by helping to prevent free-radical mediated diseases.

Flavonoid Chemistry of Dark Chocolate

Flavonoids are a category of polyphenols that are considered to be very important because they are the most commonly occurring and widely distributed throughout the plant kingdom, produced as secondary plant metabolites (Bloor, 2001; Bravo, 1998). Flavonoid polyphenols are recognized for their ability to affect numerous enzymatic, intercellular, and intracellular functions, including: immune function modulation, inflammatory processes, vascular reactivity, antioxidant mechanisms, cell proliferation, and platelet function (Middleton, Kandaswami, & Theoharides, 2000).

Flavonoids typically exist in the diphenylpropane form (C_6-C_3-C_6), which involves the linkage of two aromatic rings through three carbons, which usually form an oxygenated heterocycle (Figure 4) (Bravo, 1998). Flavonoids can be further categorized into different classes. Structurally, changes will occur in ring C, such as the presence of a double bond, a 3-hydroxy group, and/or a 4-oxo group and positioning of hydroxyl and methoxyl groups in rings A and B (Pietta & Mauri, 2001).

Flavanols are a sub-class of flavonoids. Flavanol monomers such as (+)-catechin and (−)-epicatechin (Figures 5 & 6), and their oligomers, are found in cocoa and chocolate products (Adamson et al., 1999). Catechin and epicatechin are classified as flavon-3-ol monomer units (flavanols); their oligomers are classified as procyanidins (Lazarus et al., 2001). Numerous procyanidins can be formed from the catechin and epicatechin monomers, bonded through a 4→6 linkage or 4→8 linkage (Figure 5), (Hammerstone, Lazarus, & Schmitz, 2000).

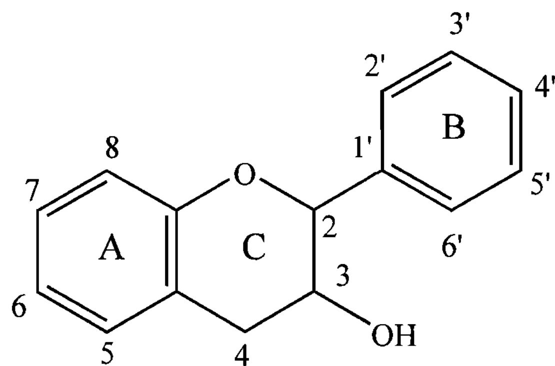

Figure 4. Flavonoid Diphenylpropane Structure.

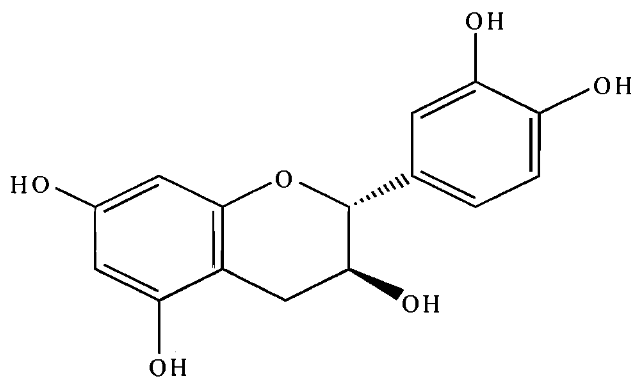

Figure 5. (+)-catechin.

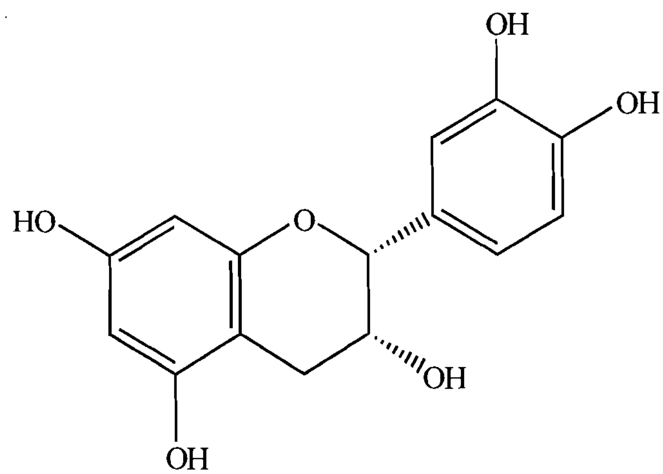

Figure 6. (−)-epicatechin.

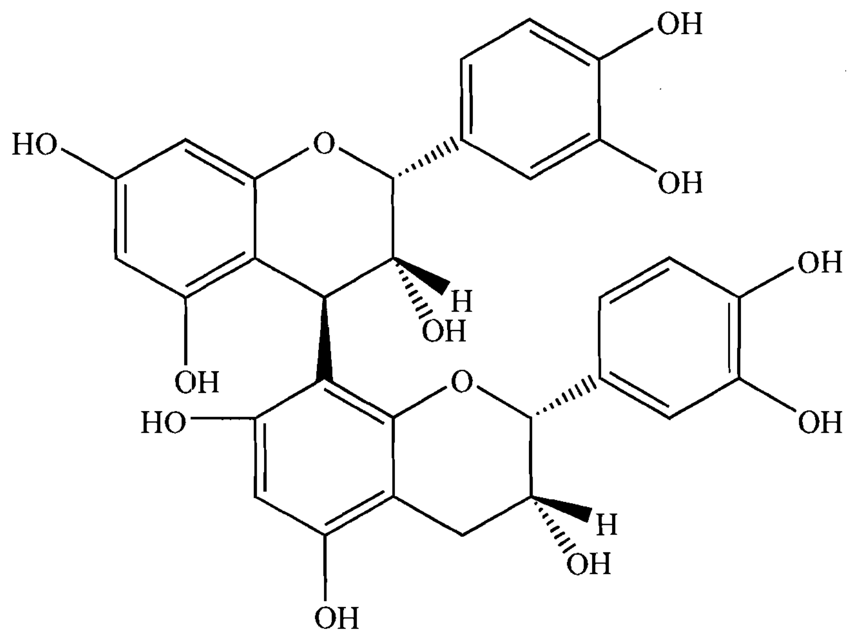

Figure 7. Procyanidin Dimer (4→8 linkage, epicatechin monomers).

As reported by Hammerstone et al. (2000), monomers (flavan-3-ols) contribute the most to the total procyanidin content in chocolate, 1.08 mg/g monomers in 4.45 mg/g total procyanidins. However, procyanidins with three or more epicatechin or catechin units display better antioxidant capabilities than monomers or dimers (Counet & Collin, 2003). Dark chocolate contains a combination of monomers and procyanidins, both of which have shown to display effective antioxidant properties.

Dark Chocolate & Oxidative Damage

Oxidative damage has been considered a risk factor in the development of cardiovascular diseases (Rein et al., 2000a). The flavonoids present in dark chocolate (catechin, epicatechin, and procyanidins), are believed to provide a protective effect on the cardiovascular system due to their free radical scavenging capabilities and their ability to inhibit lipid oxidation (Wang et al., 2000).

A study conducted by Rein et al. (2000b) investigated the epicatechin concentration and antioxidant capacity in human subjects at 2 and 6 hours after dark chocolate consumption. Each dark chocolate sample was 80 g (40 g is typical serving size), containing 557 mg of total procyanidins, of which137 mg were epicatechin. Within 2 hours of the dark chocolate consumption, epicatechin plasma levels in subjects raised 12 fold and the mean plasma antioxidant capacity significantly rose 36% greater than the original baseline readings. Plasma 2-thiobarbituric acid reactive substances (TBARS) are plasma oxidation products that were measured to determine chocolate's effect on plasma oxidation. After 2 hours, the TBARS were 40% lower than at baseline and 30 % lower after 6 hours.

Wang et al. (2000) conducted a similar study, in which they investigated the antioxidant capacity and chocolate procyanidin dose concentrations. Participants were divided into groups that consumed 0, 27, 53, or 80 g of dark chocolate in one day. Blood samples were collected at baseline, 2, and 6 hours from ingestion of the chocolate samples. The results were similar to Rein et al. (2000b), in that as epicatechin plasma concentrations increased, plasma antioxidant capacity increased, while plasma TBARS concentrations decreased.

Other studies have also demonstrated that cocoa flavonoids delay lipid and LDL oxidation by increasing the oxidation lag time *in vitro* (Lotito & Fraga, 2000; Osakabe et al., 2002), and the consumption of cocoa powder and dark chocolate by human subjects has displayed favorable effects by moderately preventing LDL oxidation susceptibility, while increasing serum antioxidant capacity (Wan et al., 2001). These studies suggest that the consumption of cocoa and/or dark chocolate may act against plasma oxidation, thereby preventing the development of cardiovascular diseases.

Dark Chocolate & Blood Pressure

Elevated blood pressure is a risk factor for the development of cardiovascular diseases (Haffner & Taegtmeyer, 2003). Many recent studies on dark chocolate consumption have investigated the relationship between dark chocolate and blood pressure.

Two studies on dark chocolate consumption and blood pressure and cholesterol have shown similar results. In the first study, (Grassi et al., 2005), healthy participants were placed in two different diet groups with the first group consuming 100 g of dark chocolate/day, and the second group consuming 90 g of white chocolate/day for 15 days.

Both types of chocolate bars contained similar amounts of macronutrients, cocoa butter, vitamins, fiber and electrolytes. The difference between the chocolate bars was that the dark chocolate contained about 500 mg of total flavonoids, while the white chocolate bar was presumed to have 0 mg of flavonoids. Baseline and 15-day blood pressure readings were recorded.

At day 15, results demonstrated that although blood pressures were within normal range for both chocolate groups, the dark chocolate group displayed a significant decrease in systolic blood pressure.

In a similar study conducted by Taubert, Berkels, Roesen, and Klaus (2003), the same experimental factors were used for chocolate bar groups and doses, with the exception that the study was only conducted for 14 days. The study was conducted on elderly individuals with isolated systolic hypertension.

At day 14, the dark chocolate group showed a decrease in systolic and diastolic blood pressures. Interestingly, after the discontinuation of the 100 g of dark chocolate per day, blood pressure levels returned to the pre-intervention levels within two days.

Dark Chocolate and Endothelial Cell Function

Growing evidence recognizes that vascular endothelium cell dysfunction may be a factor in the clinical expression of cardiovascular disease (Engler et al., 2004; Vita & Keaney, 2002). Endothelial cells are responsible for maintaining a level of homeostasis in blood vessel wall and lumen by producing nitric oxide, which acts as a vasodilator, prevents platelet adhesion and aggregation, and inhibits vascular smooth muscle cell proliferation. Nitric oxide has also shown to exhibit anti-inflammatory, anti-

atherosclerotic, and vasodilatory actions, all of which aid in the prevention of heart diseases (Sies, Schewe, Heiss, & Kelm, 2005).

Endothelial cell dysfunction causes lower levels of nitric oxide to be produced, resulting in the cells to adapt a phenotype that facilitates inflammation, thrombosis, vasoconstriction, and atherosclerotic lesion (Levine, Keaney, & Vita, 1995). The new endothelial cell phenotype is associated with cardiovascular risk factors such as high cholesterol levels, hypertension, and type II diabetes (Vita & Keaney, 2002).

Flavanols and procyanidins in dark chocolate may improve endothelial cell function in human subjects (Engler et al., 2004). In a study recently conducted by Engler et al. (2004), human subjects who consumed 46 g of dark chocolate (Dove® Dark)/day (213 mg total procyanidins, 46 mg epicatechin) demonstrated a significant increase in endothelium-dependant flow-mediated dilation and increased epicatechin plasma concentrations as opposed to the non-chocolate eating group. Increased flow-mediated dilation is associated with increased plasma epicatechin concentrations. Therefore, elevated epicatechin levels from dark chocolate consumption may be responsible for the increased endothelium derived vasodilatation. However, it should be noted that it is uncertain how flavanols and procyanidins interact with biological systems in the body to produce these results; possible mechanisms are being investigated.

Dark Chocolate and Platelet Function

Platelet aggregation and overall increased platelet activity are known risk factors in the development of many coronary artery diseases (Awtry & Loscalzo, 2000). Many studies have shown flavonoids to display platelet anti-aggregation properties *in vivo* in animal and human trials (Demrow, Slane, & Folts, 1995; Freedman et al., 2001).

In a study conducted by Rein et al. (2000a), groups consuming cocoa flavonoids have displayed acute inhibition of epinephrine-induced platelet activation *in vitro*. In a similar study, Rein et al. (2000c) replicated the inhibition of platelet activation in humans who consumed 897 mg of total epicatechin and procyanidins from a procyanidin-enriched cocoa powder.

A study conducted by Innes, Kennedy, McLaren, Bancroft, & Belch, (2003) demonstrated similar results in that they noted an inhibition of collagen-induced platelet aggregation in platelet-rich plasma in those participants consuming dark chocolate. White chocolate and milk chocolate displayed no significant effect on platelets. Therefore, these studies all demonstrate that the consumption of cocoa and/or dark chocolate helps to reduce platelet aggregation, thereby reducing the risk of developing cardiovascular disease.

Dark Chocolate and Insulin Sensitivity

Resistance from insulin receptors to uptake insulin cause excess blood glucose levels; insulin resistance is a known contributing factor for the development of type II diabetes (Haffner & Taegtmeyer, 2003). While the role of insulin sensitivity as an independent risk factor for the development of cardiovascular diseases is still under debate, a study by Balletshofer et al. (2000) has demonstrated that insulin sensitivity may act as an independent risk factor.

The effects of dark chocolate on insulin sensitivity were studied by Grassi et al. (2005). Participants in the cross-over study were placed in two different diet groups, with the first group having consumed 100 g of dark chocolate/day, while the second group consumed 90 g of white chocolate/day for 15 days. The only difference between the

chocolate bars was that the dark chocolate contained about 500 mg of total flavonoids, while the white chocolate bar was presumed to have 0 mg of flavonoids. At the end of the 15 day trial, oral-glucose-tolerance tests were administered to calculate the homeostasis model assessment of insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check (QUICKI), which are indicators of insulin resistance and insulin sensitivity, respectively. Blood pressures of each participant were measured daily.

Results indicated that the dark chocolate consumption group had decreased fasting insulin and glucose concentrations, in addition to decreased oral testing insulin and glucose values. The dark chocolate group displayed statistically lower HOMA-IR and statistically greater QUICKI. Blood pressure results indicated that the dark chocolate consumption group displayed a statistically significant decrease in systolic blood pressure after 15 days as opposed to the white chocolate group. No significant differences were seen in the diastolic blood pressure values.

This lower insulin resistance and increased insulin sensitivity displayed are hypothesized by Grassi et al. (2005) to be caused by dark chocolate flavonoids. Although the experiment did not test for nitric oxide concentrations, it can be hypothesized the insulin sensitivity displayed by dark chocolate consumption may have been due to a decrease in the group's systolic blood pressure, since elevated nitric oxide uptake by endothelial cells is known to decrease blood pressure (Vita & Keaney, 2002). However, flavanol consumption is also attributed to other cellular process which can affect insulin sensitivity (Grassi et al., 2005). Although no mechanism is yet clear, compounds present in dark chocolate appear to lower blood pressure and insulin resistance. By lowering

blood pressure and insulin resistance, dark chocolate consumption may help prevent the onset of cardiovascular diseases and type II diabetes.

Dark Chocolate, Oxidative Stress, Inflammation, & Cancer

Oxidative stress and inflammation may be factors which can aid in the development of cancers by causing direct damage to genomic DNA, alter intracellular signaling, causing abnormal cell growth, and promoting damaged cells to undergo promotion and progression of cancerous cells (Surh, Kundu, Na, & Lee, 2005). Cells are equipped with certain mechanisms of defense against oxidative stress, protecting themselves from reactive oxygen species (ROS) free radicals. ROS and other free radical species are believed to be a major contributor to the onset of oxidative stress, and thereby cause cellular damage (Halliwell, 2001), which can subsequently lead to carcinogenesis (Surh et al., 2005).

The effects of ROS and other radicals, such as peroxynitrite, have been studied due to their ability to induce oxidative stress that may be caused by inflammation (Arteel et al., 2000). A study by Arteel et al. (2000) has shown that cocoa flavonoids such as epicatechin and a tetramer of epicatechin, have inhibited oxidative reactions involving peroxynitrite *in vitro*. The tetramer displayed a more potent ability to prevent oxidative damage than did epicatechin. This study demonstrates the potential chemo-preventive benefits from chocolate flavonoids.

Dark Chocolate, Angiogenesis, and Cancer

Angiogenesis is the formation of new capillaries from an existing blood vessel (Harper & Moses, 2006); the process of which is always implicated in the development of cancer because new capillaries are formed with tumor growth in order to supply

nutrients and remove metabolites (Dhanabal, Jeffers, & Larochelle, 2005). By preventing or stopping the ability of new capillaries to grow, the ability for tumors to develop and grow would also be impaired.

Keeny et al. (2004) has recently investigated the effects of cocoa flavonoids on the proliferation of human dermal microvascular endothelial cells (HDMECs) following angiogenic stimulation. This study showed that pentameric and octameric procyanidins inhibited induced HDMEC proliferation. Furthermore, the pentameric procyanidin fraction was shown to modulate the activity of signaling enzymes in angiogenic signaling, regulating their expression and down-regulating their receptors.

This study, although *in vitro*, implies that cocoa procyanidins may have a large impact on factors related to tumor growth. Keeny et al. (2004) noted that the concentrations of procyanidins used in their study most likely exceed the level obtainable *in vivo*. However, their findings on angiogenesis regulation warrant further studies involving cocoa procyanidins and cellular signaling.

Dark Chocolate Flavonoids and Inhibition of Human Colon Cancer Cell Growth

The effects of cocoa powder and flavanol-rich extracts were investigated on the growth of Caco-2 cell lines, a line of human colonic cancer cells in a study conducted by Carnésecchi et al. (2002). Results indicated that the cocoa flavanol extracts inhibited cell proliferation in a dose-dependant manner. Flavonoids extracted from cocoa powder with a concentration of 141.2 mg/g demonstrated the least effective cancer cell inhibition. However, extractions containing 510.5 mg/g of crude procyanidin extract and 940.6 mg/g of procyanidin-enriched extract demonstrated a 25% and 75% growth inhibition, respectively. Non-apoptosis cell death was also observed from the procyanidin-enriched

extract. The authors hypothesize that the observed decrease of two key enzymes in the polyamine biosynthesis might be the major target of the anti-proliferative effects of cocoa flavonoids.

These results indicate that cocoa flavanol extracts in high concentrations have anti-proliferation capabilities *in vitro*. Although flavanol concentrations used in this study are unattainable from cocoa/chocolate consumption, other types of flavanol treatments may be created to help prevent human colon cancer proliferation.

Dark Chocolate Flavanol and, Mammary and Pancreatic Cancers

In a study conducted by Yamagishi et al. (2002a), the effects of cacao liquor procyanidins were investigated *in vitro* and *in vivo* on induced mutagenesis in female Sprague-Dawley rats. *In vitro*, the cacao liquor procyanidins inhibited induced mutagenesis in a non-dose dependent manner (100, 200, and 400 μg/plate), warranting further *in vivo* testing on pancreatic and mammary cancers in rats.

In vivo, induced carcinogenesis of the pancreas and mammary glands was performed on the female rats. Cacao procyanidins were incorporated into the basal diet of the rats at doses of 0.025% and 0.25%. Results indicated that the incidences, multiplicities, and volume of mammary tumors in the 0.25% cacao liquor procyanidins group was less than the control group, but with no statistical significance ($p < 0.05$). The incidences of pancreatic tumors decreased in a dose dependent manner in rats fed cacao liquor procyanidins ($0.25\% < 0.025\%$). These results indicate that cacao liquor procyanidins may help to inhibit pancreatic carcinogenesis when ingested in amounts of 0.25% and 0.025% of the basal diet.

Dark Chocolate Flavonoids and Lung Cancer

In a second study, Yamagishi et al. (2002b) investigated the relationship between multi-organ induced carcinogenesis and cacao liquor procyanidins on male F344 rats. Cancerous cells were found in the pituitary gland, zymbal gland, forestomach, kidney, urinary bladder, testis, small intestine, colon, thyroid gland, and lung after induced carcinogenesis. One week after initiation, the rats were fed cacao liquor procyanidins at doses of 0.025% and 0.25% of the basal diet. Results indicated that the survival rate of the 0.25% cacao liquor procyanidin group was significantly greater. A statistically significant reduction ($p < 0.05$) in the incidence and multiplicity of lung carcinomas was observed in the 0.25% cacao liquor procyanidin group as compared to the control group. A dose-dependent relationship was associated with a decrease in quantitative values of lung cancer. No significant reductions of the other types of cancers were observed.

This study confirms the cacao liquor procyanidin dose-dependent results of the previous study (Yamagishi et al., 2002a). In addition to preventing pancreatic cancer proliferation, cacao liquor procyanidins also help to prevent lung cancer instance and multiplicity. Furthermore, no adverse side effects were observed in any of the major organs studied, implying that cacao liquor procyanidins are safe.

Conclusion

The experiments reviewed use dark chocolate/cocoa/chocolate liquor flavonoids as potential cardio and chemo preventative compounds. The studies have demonstrated that flavanols and procyanidins originating from the *T. cacao* tree and related chocolate products provide numerous health benefits both *in vitro* and *in vivo.*

In addition to dark chocolate's polyphenol content, methylxanthines are also compounds of interest because of their prevalence in chocolate and their related central nervous system stimulation, cardiac muscle stimulation, relaxation of smooth muscle, and diuretic effects.

These studies offer sufficient evidence to conclude that flavonoids and methylxanthines from cocoa and/or dark chocolate may have health benefits directly related to their consumption; therefore, the above presented literature review validates the basis of this experiment to investigate Legacy Chocolates and commercially available dark chocolates for flavanols and methylxanthines.

Chapter III: Methodology

Flavanols such as catechin and epicatechin, and methylxanthines such as caffeine and theobromine are unique because both categories of compounds have similar UV-VIS spectra; therefore, they are able to be simultaneously extracted and analyzed via high performance liquid chromatography (HPLC). HPLC is the method of choice for flavonoid analysis because most instruments offer multi-wavelength capabilities and the ability to record multiple spectra (Bloor, 2001).

Reversed-phase gradient HPLC was used to quantify levels of catechin, epicatechin, caffeine, and theobromine in four commercial dark chocolates and chocolate samples obtained from Legacy Chocolates with weight percentages of chocolate liquor ranging from 41% to 100%.

Fresh chocolate samples were analyzed within one week of being acquired. The chocolate samples were refrigerated at 3°C.

Subject Selection and Description

Raw chocolate samples used to make medallions and truffle shells were obtained from Legacy Chocolates of Menomonie, Wisconsin. The weight percentages of chocolate liquor in the finished products, alkali processing information, and *Theobroma cacao* genera from which the chocolate originated from are shown in Table 1 (M. Roberts, personal communication, March 30, 2006).

Table 1

Legacy Chocolate Variety Corresponding to Percent Chocolate Liquor, Alkali Processing, and Tree Genus

Legacy chocolate variety	Percent chocolate liquor (%)	Alkali processed	*T. cacao* genus
41.0%	41.0	Yes	Criollo
58.5%	58.5	Yes	Criollo
60.0%	60.0	Yes	Forastero
73.5%	73.5	Yes	Criollo
Chocolate liquor (100.0%)	100.0	Yes	Criollo & Forastero

Brands of commercial dark chocolates obtained from local grocery stores included: Ghirardelli 60% cacao, Hershey's® Dark, Dove® Dark, and Lindt 70% cocoa. The weight percentages of chocolate liquor in the finished products and alkali processing information are shown in Table 2. *Theobroma cacao* genera from which the commercial dark chocolates originated were unavailable from Ghirardelli, Hershey's®, Dove®, and Lindt.

Table 2

Commercial Chocolate Variety Corresponding to Percent Chocolate Liquor, Alkali Processing, and Tree Genus

Commercial chocolate variety	Percent chocolate liquor (%)	Alkali processed	*T. cacao* genus
Dove® Dark[a]	40.0-50.0	Yes	Unknown
Hershey's® Dark[b]	45.0	Yes	Unknown
Ghirardelli 60% cacao[c]	60.0	No	Unknown
Lindt 70% cocoa[d]	70.0	No	Unknown

[a] (L. Lisa, personal communication, May 26, 2006).
[b] (R. Green, personal communication, June 1, 2006).
[c] (S. Ortiz, personal communication, May 24, 2006).
[d] (Lindt, 2006).

Standard Solutions Preparation

The polyphenolic compounds, (+)-catechin and (−)-epicatechin, and methylxanthine compounds, caffeine and theobromine, were used to prepare stock solutions. Catechin, caffeine, and theobromine standards were obtained from Sigma (St. Louis, MO). The epicatechin standard was obtained from Aldrich (Milwaukee, WI). Stock solutions of caffeine (1000 mg/L) and theobromine (800 mg/L) were prepared by dissolving the 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 before being diluted to volume. Stock solutions

of catechin and epicatechin (1000 mg/L) were prepared by dissolving the compounds in methanol. Standard mixture solutions of catechin, epicatechin, caffeine and theobromine were prepared by volumetric transfer of the stock solutions into 100-mL volumetric flasks and diluted to volume with methanol. The concentrations of the stock solutions are listed in Table 3.

Table 3

Standard Mixture Solutions Concentrations

Standard mixtures	Concentration (mg/L)			
	Catechin	Epicatechin	Caffeine	Theobromine
Standard mix 1	10	10	25	160
Standard mix 2	20	20	50	240
Standard mix 3	30	30	75	320
Standard mix 4	40	40	100	400
Standard mix 5	50	50	125	480

Polyphenol and Methlyxanthine Extraction

Chocolate samples were stored in a refrigerator until analyzed. Each sample was finely ground using an Osterizer blender. Approximately two grams of each ground sample was weighed on an analytical balance and was transferred to an Erlenmeyer flask. To each sample, 25 mL of Milli-Q® water warmed to 95° C was added. The samples were stirred on a stir plate at a medium setting for 40 minutes. All samples were then transferred via a funnel into 50-mL volumetric flasks and allowed to cool to room temperature; the stir bar, Erlenmeyer flask, and funnel were rinsed with Milli-Q® water to

ensure quantitative transfer. Once room temperature was reached, each flask was diluted to volume with Milli-Q® water. Next, each sample was filtered by a funnel lined with Whatman® # 1 (110 mm diameter) filter paper to separate the water-soluble liquid component from lipids and other insoluble components. Before transferring to autosampler vials, each sample was filtered with a new Whatman® 0.45 μm polypropylene membrane filter.

Instrumentation

A Waters high performance liquid chromatography (HPLC) system with Millennium® software was used to identify and quantify (+)-catechin, (−)-epicatechin, caffeine and theobromine in the chocolate samples from a modified HPLC method (Yang, 1988). The column used in the analysis was a Waters Radial Compression™ 10 cm x 8 mm ID Novapak C_{18} column with a NovaPak GaurdPak in an RCM-100 radial compression module. Gradient conditions are described in Table 4. Solvent A consisted of Milli-Q® water/glacial acetic acid 99.5:0.5 (v/v) and solvent B consisted of Milli-Q® water/acetonitrile/glacial acetic acid 59.5:40.0:0.5 (v/v). The flow rate was set at 2.0 mL/minute and the run time was 35 minutes. The injection volume for each sample was 25 μL. The HPLC hardware used included a Waters 717 Plus autosampler, Waters 1525 Binary HPLC Pump, and a Waters Photodiode Array Detector. The system was controlled with a PC using a Windows® NT operating system and Waters Millennium® 4.0 software.

Table 4

Mobile Phase Gradient Separation Conditions

	Time (min)	Flow (mL/min)	%A	%B	Curve
1	---	2.00	100.0	0.0	---
2	30.00	2.00	0.0	100.0	7
3	31.00	2.00	100.0	0.0	6
4	35.00	2.00	100.0	0.0	6

The UV-Vis spectra of the analytes were measured with an Aligent© UV-Vis spectrophotometer with UV-Vis Chem Station software. At concentrations of 10 mg/L, the UV absorption maxima of catechin and epicatechin were determined to be 280 nm; caffeine and theobromine were 272 nm (Figures 8, 9, 10, and 11, respectively). Therefore, chromatograms were collected using analytical wavelengths of 278 and 290 nm; these wavelengths are close to the λ_{max} for the analytes being investigated. An example chromatogram for a standard mixture containing catechin (20 mg/L), epicatechin (20 mg/L), caffeine (50 mg/L), and theobromine (240 mg/L) is displayed in Figure 12; an example of a chromatogram for a Legacy Chocolates product, Legacy Chocolate 73.5%, is displayed in Figure 13.

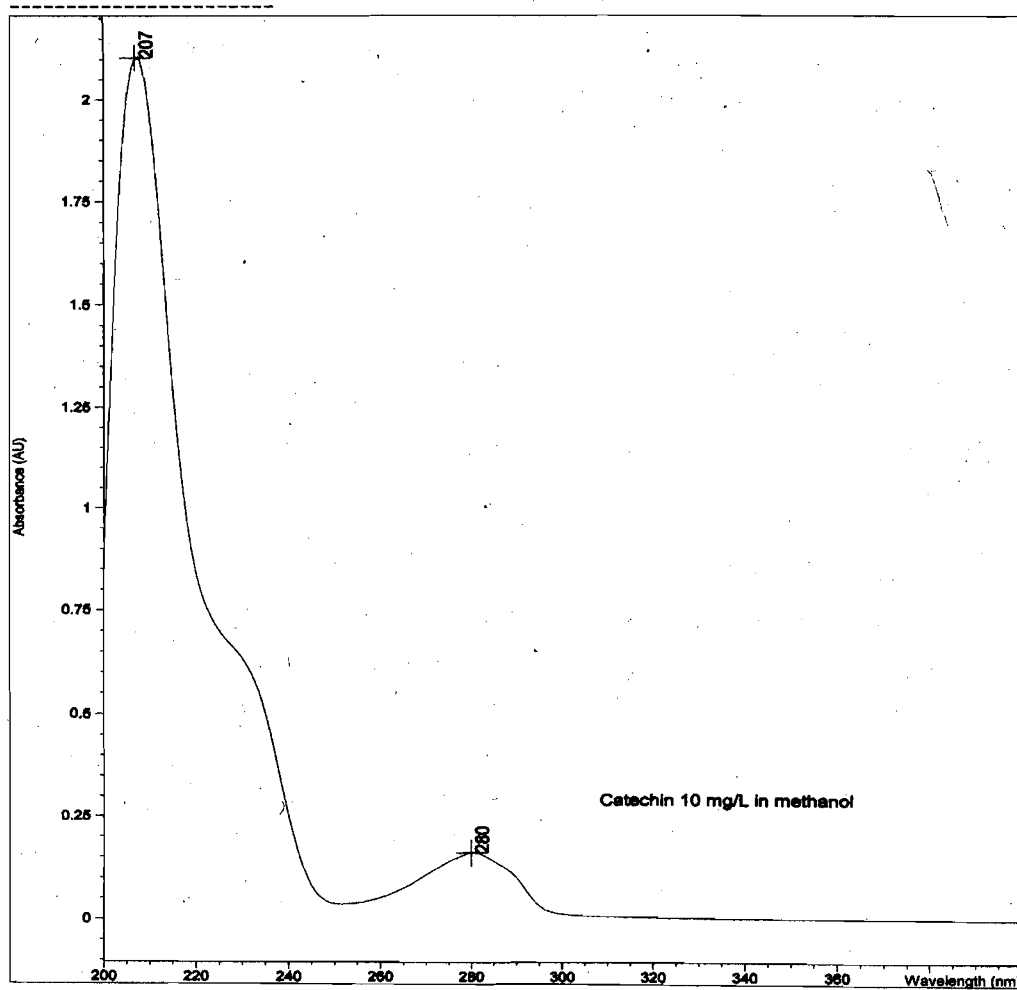

Figure 8. Catechin UV-spectra.

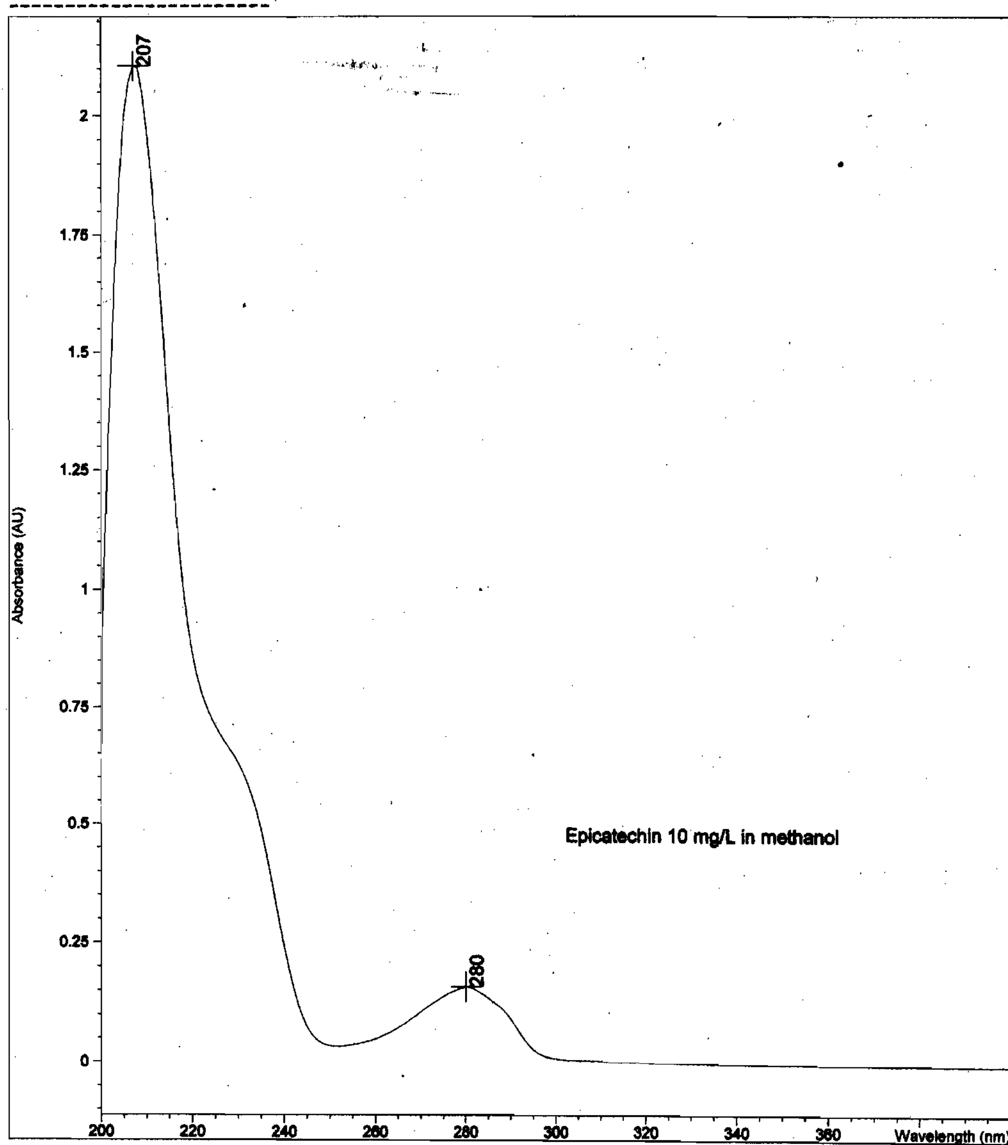

Figure 9. Epicatechin UV-spectra.

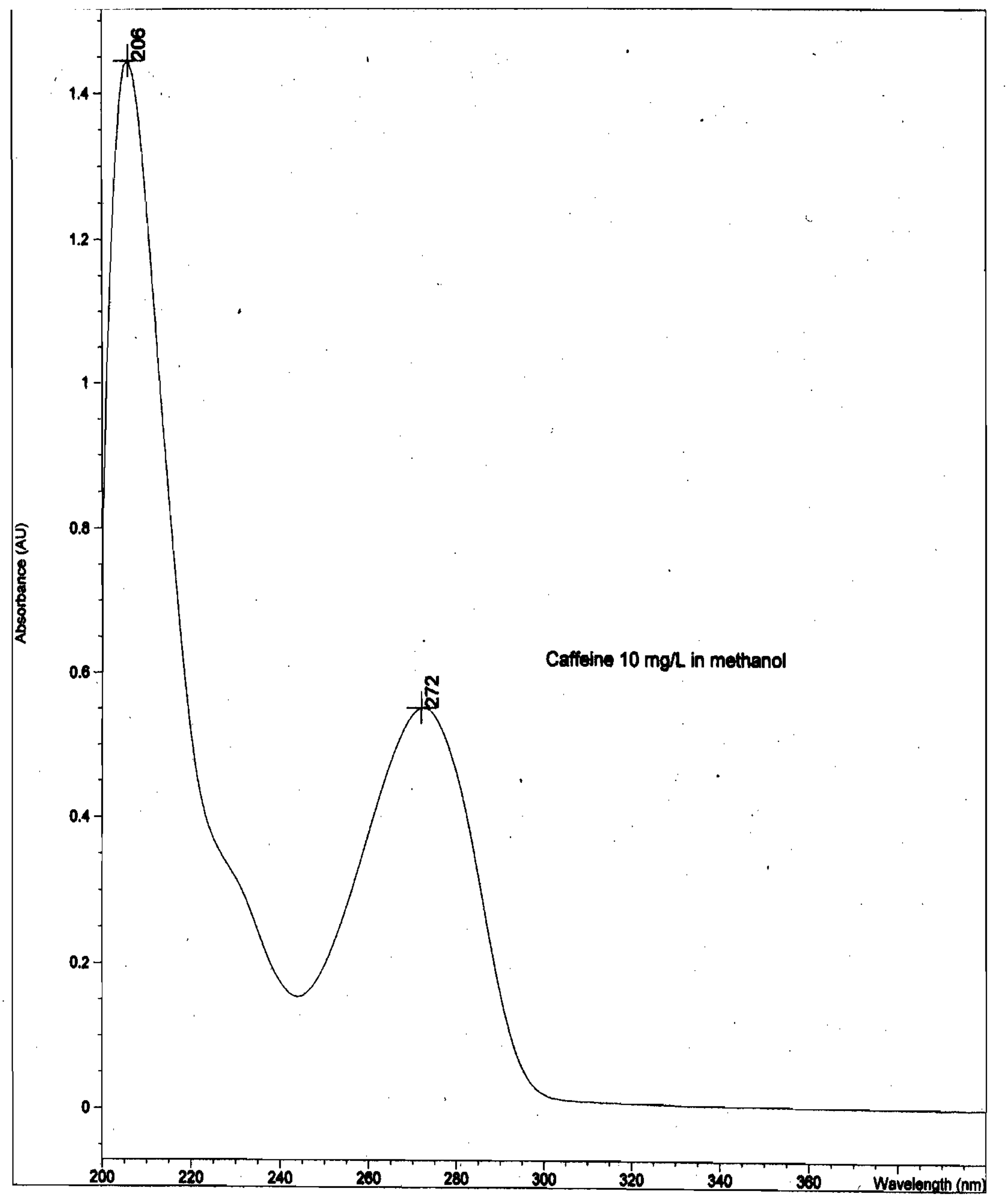

Figure 10. Caffeine UV-spectra.

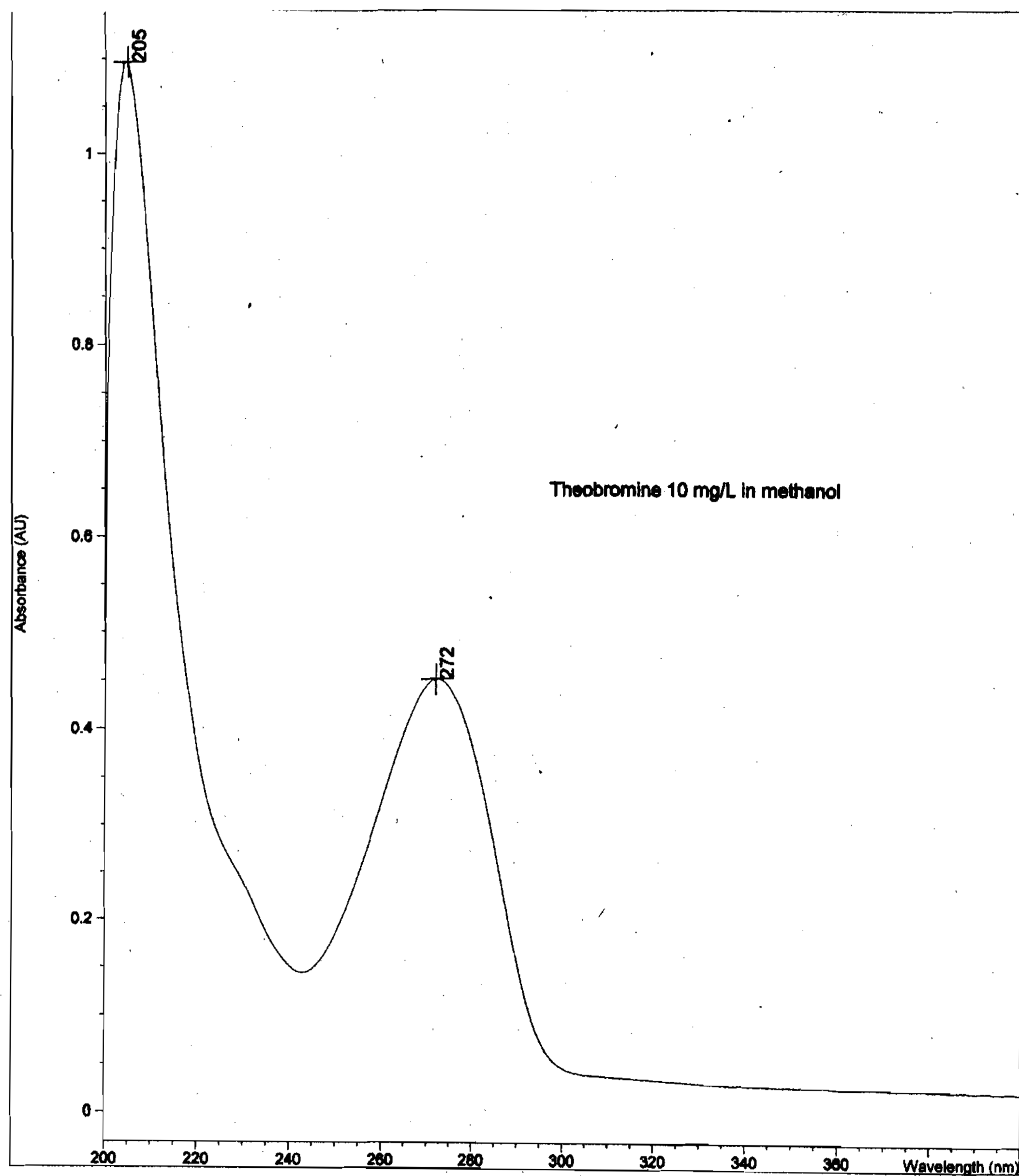

Figure 11. Theobromine UV-spectra.

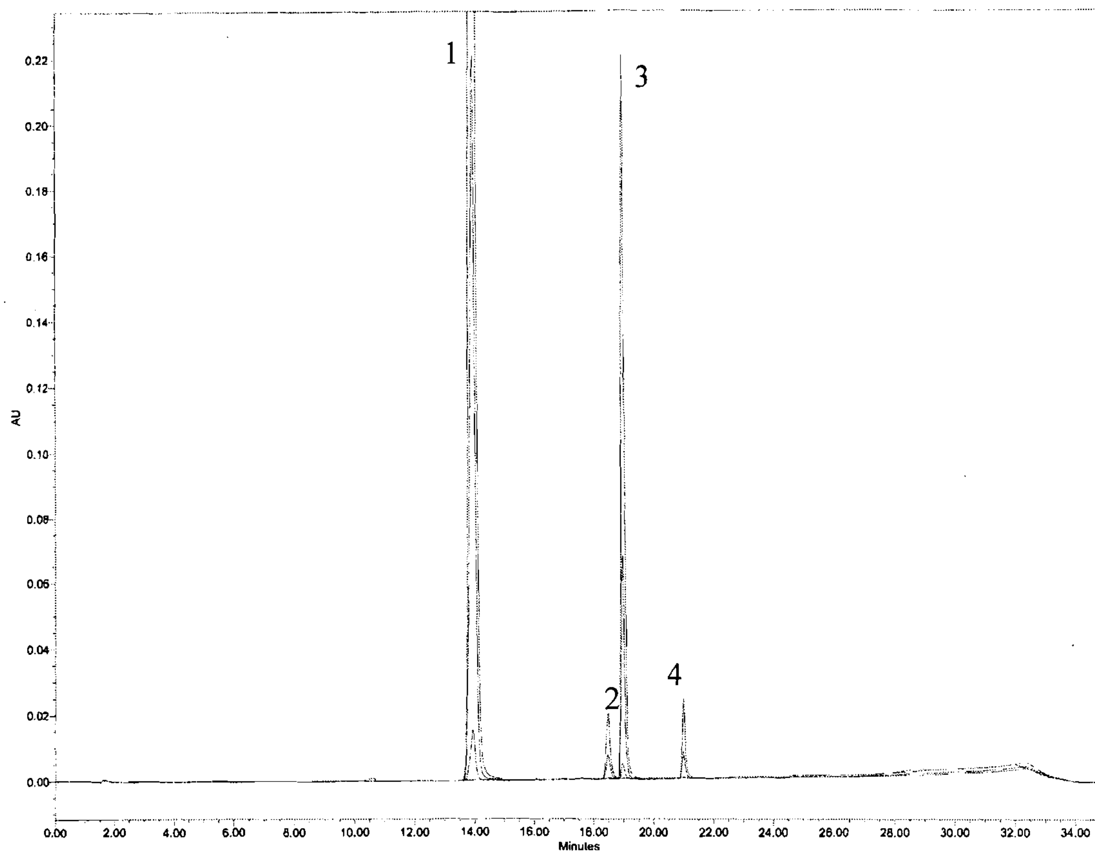

Figure 12. Chromatogram of Standard Mixture. †

† 1. theobromine (240 mg/L); 2. catechin (20 mg/L); 3. caffeine (50 mg/L); and 4. epicatechin (20 mg/L)

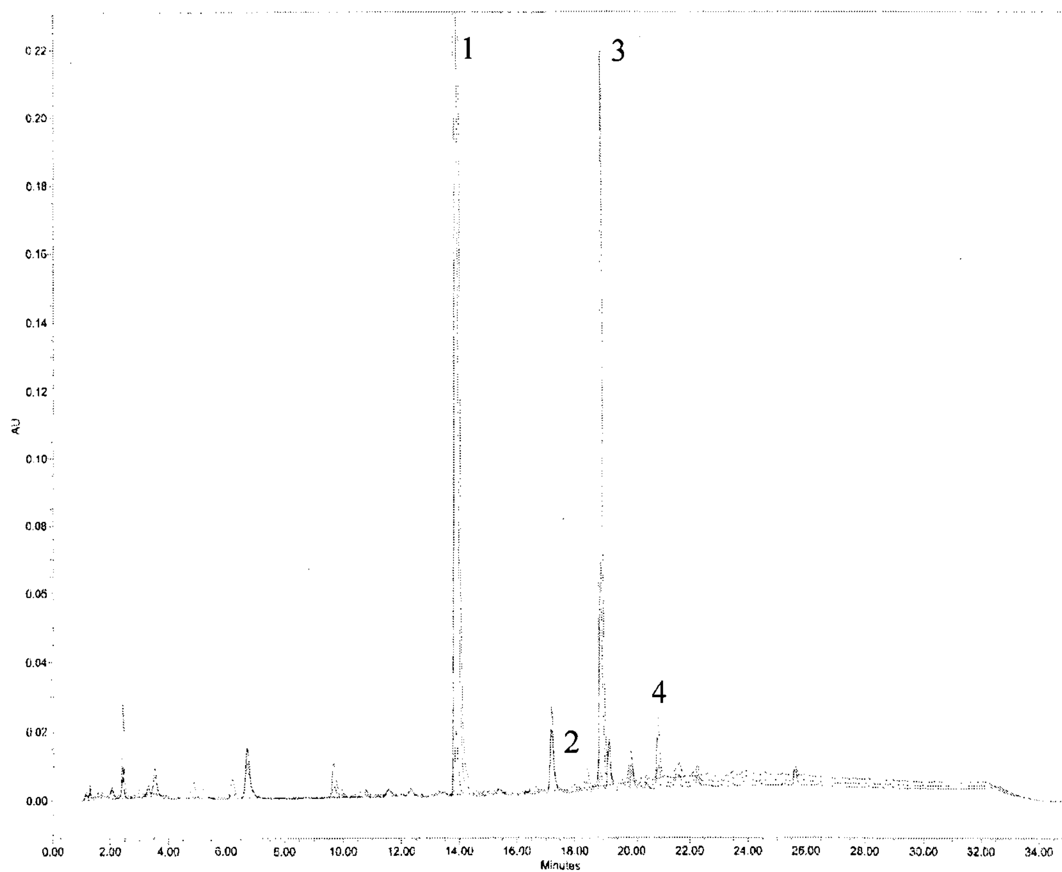

Figure 13. Legacy Chocolates 73.5% Chromatogram. †

† 1. theobromine; 2. catechin; 3. caffeine; and 4. epicatechin

Data Analysis

Statistical differences between the mean concentrations (mg/40g) of catechin, epicatechin, caffeine, and theobromine for each chocolate variety were examined by one-way ANOVA with post-hoc testing. The statistical analysis was performed using the computer statistical package SPSS version 12.0. Differences between mean concentrations were assessed by a Student-Newman-Keuls t-test. $P<0.05$ was considered statistically significant.

Limitations of the Study

The extraction process of the analytes is a limitation of the study. The quantification of flavanols and methylxanthines by HPLC is dependant on the proficiency of the extraction process. The HPLC instrumentation is another limitation. Flavanol and methylxanthine concentrations are based on the HPLC's capability to quantify those components.

Chapter IV: Results and Discussion

The HPLC method used was developed to allow detection of flavanols (catechin and epicatechin) and methylxanthines (caffeine and theobromine) simultaneously from solid chocolate samples. The concentration of flavanols and methylxanthines was determined in the four chocolate medallion products manufactured by Legacy Chocolates and in the chocolate liquor used in their preparation. Other commercial brand dark chocolate manufactured by Dove®, Ghirardelli, Hershey®, and Lindt were also analyzed.

Using the average retention times obtained by standards, peaks were identified in chocolate samples (Table 5) so that tentative peak identities could be verified by comparing their 278/290 nm peak area ratios to those of standards. Each component analyzed (catechin, epicatechin, caffeine, and theobromine) had similar peak area ratios at 278 and 290 nm as compared to each standard. The chocolate samples had similar peak areas at 278 nm and 290 nm (Table 6), which confirms that the analyte of interest was present in each dark chocolate sample. Concentrations (mg/L) of catechin, epicatechin, caffeine, and theobromine displayed in Table 6 were determined from individual compound standard curves (Appendixes A-D).

Table 5

Retention Times and Peak Area Ratios[†]

Compound	Retention time (min)	Peak area ratios (278/290)
Theobromine	13.97 ± 0.07	2.77 ± 0.03
Catechin	18.52 ± 0.07	2.94 ± 0.02
Caffeine	18.99 ± 0.06	3.28 ± 0.00
Epicatechin	21.02 ± 0.06	3.32 ± 0.02

[†] Mean of three trials ± standard deviation

Table 6

Average Methylxanthine and Polyphenol Concentrations (mg/L) at 278 nm & 290 nm†

Chocolate sample	Concentration (mg/L) at 278 nm††				Concentration (mg/L) at 290 nm††			
	TB	C	CAF	EC	TB	C	CAF	EC
Dove® Dark 40-50%[a]	177 ± 3	6.3 ± 0.3	24.8 ± 0.3	6.6 ± 1.2	177 ± 4	6.8 ± 0.4	25.2 ± 0.2	6.9 ± 2.0
Legacy 41%[a]	64.8 ± 2.4	2.5 ± 0.4	23.4 ± 0.9	6.5 ± 1.5	66.1 ± 1.9	2.0 ± 0.7	23.4 ± 0.5	7.4 ± 1.8
Hershey's® Dark 45%[a]	185 ± 4	8.7 ± 0.9	17.8 ± 0.6	12.0 ± 1.7	185 ± 4	9.7 ± 0.3	18.0 ± 0.2	13.3 ± 1.6
Legacy 58.5%[a]	160 ± 6	4.3 ± 0.3	40.7 ± 1.4	18.4 ± 2.6	160 ± 6	3.4 ± 1.1	41.5 ± 1.7	18.4 ± 3.6
Legacy 60%[a]	302 ± 5	8.9 ± 0.9	25.2 ± 0.2	29.2 ± 2.2	303 ± 4	8.2 ± 3.8	27.7 ± 1.5	38.8 ± 10.2
Ghirardelli 60%[a]	206 ± 4	7.9 ± 0.7	34.4 ± 0.9	27.2 ± 4.6	206 ± 4	9.4 ± 1.0	34.4 ± 0.7	24.8 ± 5.9
Lindt 70%[a]	284 ± 10	10.4 ± 2.1	35.5 ± 0.8	32.7 ± 2.2	284 ± 10	15.2 ± 5.5	37.2 ± 1.9	38.7 ± 14.2
Legacy 73.5%[a]	207 ± 1	8.7 ± 1.6	49.9 ± 0.8	24.7 ± 6.3	207 ± 1	7.6 ± 0.3	50.3 ± 0.1	21.0 ± 1.8
Chocolate liquor (100%)[a]	414 ± 7	18.6 ± 1.2	59.4 ± 1.6	69.9 ± 4.9	417 ± 6	24.4 ± 3.2	62.5 ± 1.8	77.4 ± 6.8

† mean of three trials ± standard deviation
†† TB = theobromine, CAF = caffeine, C = catechin, and EC = epicatechin
[a] percentage of chocolate liquor

Calculation of Catechin, Epicatechin, Caffeine, and Theobromine

The concentrations of each compound per 40 g serving size were calculated using the following equation:

Concentration (mg/L) * 0.05 L * 1/2.00 g* 40 g/serving size = mg/40 g serving size

The average concentration and standard deviation of each compound (mg/40 g serving size) is displayed in Table 7 for each dark chocolate sampled. The compound concentrations were given in mg/40 g serving size for ease of comparison with current human studies that typically denotes the catechin/epicatechin or total polyphenols in milligrams per serving size. Methlyxanthine values (mg/40 g serving size) from dark chocolates in this study agreed with literature values published by Apgar and Tarka (1999). Apgar and Tarka reported "dark" or "sweet" chocolate as containing 185 mg of theobromine per 40 g serving size and 28 mg of caffeine per 40 g serving size. These values are similar to values obtained from dark chocolates tested in this study, such as Dove® Dark 40-50% (177 mg/40 g theobromine and 24.8 mg/ 40 g caffeine) and Hershey's® Dark 45% (185 mg/40 g theobromine and 17.8 mg/40 g caffeine) (Table 7). Therefore, comparable values to Apgar and Tarka are found in the current study.

The average concentration and standard deviation of each compound (mg/g) are displayed in Table 8. Flavanol monomer concentrations in chocolate samples labeled "dark chocolate" and "high liquor chocolate" as reported by Adamson et al. (1999) were 0.8 mg/g and 4.0 mg/g, respectively. In comparison, all chocolates sampled in this study (Table 8) were within the range of flavanol monomers (mg/g) published by Adamson et al (1999). For example, Hershey's® Dark 45% contained 0.52 mg/g of total monomer flavanols and Lindt 70% contained 1.08 mg/g of total monomer flavanols (Table 8).

Table 7

Methylxanthine and Polyphenol Concentrations per 40 g serving size[†]

Chocolate sample	Concentration (mg)/40 gram serving size					
	Catechin	Epicatechin	Total flavanol monomers	Caffeine	Theobromine	Total methyl-xanthines
Dove® Dark 40-50%[a]	6.3 ± 0.3	6.56 ± 1.1	12.9 ± 1.4	24.8 ± 0.3	177 ± 3	202 ± 3
Legacy 41%[a]	2.5 ± 0.4	6.5 ± 1.5	8.91 ± 1.9	23.4 ± 0.9	65 ± 2	88 ± 3
Hershey's® Dark 45%[a]	8.7 ± 0.9	12.0 ± 1.7	20.7 ± 2.6	17.8 ± 0.6	185 ± 4	203 ± 5
Legacy 58.5%[a]	4.3 ± 0.3	18.4 ± 2.6	22.7 ± 2.9	40.7 ± 1.4	160 ± 6	201 ± 8
Legacy 60%[a]	8.9 ± 0.9	29.2 ± 2.2	38.1 ± 3.1	25.2 ± 0.2	302 ± 5	327 ± 5
Ghirardelli 60%[a]	7.9 ± 0.7	27.2 ± 4.6	35.1 ± 5.3	34.4 ± 0.9	206 ± 4	240 ± 5
Lindt 70%[a]	10.4 ± 2.1	32.7 ± 2.2	43.1 ± 4.3	35.5 ± 0.8	284 ± 10	320 ± 11
Legacy 73.5%[a]	8.7 ± 1.6	24.7 ± 6.3	33.4 ± 7.9	49.9 ± 0.8	207 ± 1	257 ± 2
Chocolate liquor (100%)[a]	18.6 ± 1.2	70.0 ± 4.9	88.6 ± 6.1	59.4 ± 1.6	414 ± 7	473 ± 8

[†] mean of three trials ± standard deviation
[a] percentage of chocolate liquor

Table 8

Methylxanthine and Polyphenol Concentrations (mg/g) of Dark Chocolates[†]

Chocolate sample	Concentration (mg/g)					
	Catechin	Epicatechin	Total Flavanols	Caffeine	Theobromine	Total Methyl-xanthines
Dove® Dark 40-50%[a]	0.16 ± 0.01	0.16 ± 0.03	0.32 ± 0.04	0.62 ± 0.01	4.43 ± 0.08	5.05 ± 0.09
Legacy 41%[a]	0.06 ± 0.01	0.16 ± 0.04	0.22 ± 0.05	0.59 ± 0.02	1.62 ± 0.06	2.21 ± 0.08
Hershey's® Dark 45%[a]	0.22 ± 0.02	0.30 ± 0.04	0.52 ± 0.06	0.45 ± 0.01	4.63 ± 0.11	5.08 ±0.12
Legacy 58.5%[a]	0.11 ± 0.01	0.46 ± 0.07	0.57 ± 0.08	1.02 ± 0.04	4.00 ± 0.16	5.02 ± 0.20
Legacy 60%[a]	0.22 ± 0.02	0.73 ± 0.06	0.95 ± 0.08	0.63 ± 0.01	7.55 ± 0.12	8.18 ± 0.13
Ghirardelli 60%[a]	0.20 ± 0.02	0.68 ± 0.12	0.88 ± 0.14	0.86 ± 0.02	5.15 ± 0.10	6.01 ± 0.12
Lindt 70%[a]	0.26 ± 0.05	0.82 ± 0.06	1.08 ± 0.11	0.89 ± 0.02	7.10 ± 0.26	7.99 ± 0.28
Legacy 73.5%[a]	0.22 ± 0.04	0.62 ± 0.16	0.84 ± 0.20	1.25 ± 0.02	5.18 ± 0.03	6.43 ± 0.05
Chocolate liquor (100%)[a]	0.47 ± 0.03	1.75 ± 0.12	2.22 ± 0.15	1.49 ± 0.04	10.35 ± 0.17	11.8 ± 0.21

[†] mean of three trials ± standard deviation

[a] percentage of chocolate liquor

Legacy Chocolates Medallions and Truffle Shells

The mass of individual medallions and truffle shells (with ganache removed) was used to calculate the amount of catechin, epicatechin, caffeine, and theobromine per serving size. Results (mg/ Legacy Chocolates serving size) are shown in Table 9 to demonstrate the amount of flavanols and methylxanthines per truffle or 2 medallions, as sold by Legacy Chocolates.

The chocolate liquor used to produce the truffles and medallions came from the same chocolate source (M. Roberts, personal communication, March 30, 2006); therefore, it was expected that as the percentage of chocolate liquor increased, the concentration of flavanols and methylxanthines should have also increased. Table 9 demonstrates that the greater the percentage of chocolate liquor added to the finished product, the greater the flavanol and methylxanthine concentration. For example, comparing a serving size of Legacy 41% and 73.5% medallions, Legacy 41% had 4.4 mg/serving size of epicatechin and 16.0 mg/serving size of caffeine, while legacy 73.5% had 16.9 mg/serving size of epicatechin and 34.0 mg/serving size of caffeine (Table 9). The medallions provided more flavanols and methylxanthines because the serving size for medallions is larger than the truffles. For example, Legacy's 73.5% medallion had a greater epicatechin content (16.9 mg/27.18 g serving size) (Table 9) as compared to Legacy's 73.5 % truffle shell (4.0 mg/6.38 g serving size) (Table 9). These differences can be attributed to the weight difference between the two samples. Also, since only the truffle shell was analyzed, and not the ganache, which is typically prepared with dark chocolate, it is anticipated that the flavanol and methylxanthine content of the entire truffle would increase.

Table 9

Flavanol and Methylxanthine Concentration per Legacy Chocolates Medallion and Truffle Shell Serving Sizes[†]

Legacy Chocolate Type	Serving Size	Concentration (mg/serving size)			
		Theobromine	Caffeine	Catechin	Epicatechin
41%[a] medallion	2[b]	44.0 ± 1.6	16.0 ± 0.5	1.6 ± 0.3	4.4 ± 1.1
58.5%[a] medallion	2[b]	109 ± 4	27.7 ± 1.1	3.0 ± 0.3	12.5 ± 1.9
73.5%[a] medallion	2[b]	141 ± 1	34.0 ± 0.5	6.0 ± 1.1	16.9 ± 4.4
41%[a] TS[c]	1[d]	10.3 ± 0.4	3.8 ± 0.1	0.4 ± 0.1	1.0 ± 0.3
58.5%[a] TS[c]	1[d]	25.5 ± 1.0	6.5 ± 0.3	0.7 ± 0.1	2.9 ± 0.5
73.5%[a] TS[c]	1[d]	33.1 ± 0.2	8.0 ± 0.1	1.4 ± 0.3	4.0 ± 1.0

[†] mean of three trials ± standard deviation

[a] Percentage of chocolate liquor.

[b] Medallions (27.18 g ± 0.98).

[c] Truffle shell.

[d] Truffle shell only, no ganache (6.38 g ± 0.69).

Means Comparison and Statistical Analysis

The Student-Newman-Keuls t-test ($p < 0.05$) was used to determine statistical differences between the means of the analyte concentrations (mg/40g).

Legacy Chocolates flavanols. Results for statistical differences between Legacy chocolate varieties for catechin and epicatechin concentrations are shown in Figures 14 and 15, respectively. The total amounts of monomer flavanols for all chocolate varieties are shown in Figure 16. In general, for Legacy Chocolates the greater the percentage of chocolate liquor that was added to the finished product, the greater the flavanol antioxidants present. For example, Legacy 60% chocolate had 8.90 mg/40 g catechin, while Legacy 40% had only 2.46 mg/40 g catchin. And, as anticipated, Legacy's chocolate liquor (100%) had significantly greater concentrations of catechin (18.6 mg/40 g) (Figure 14) and epicatechin (70.0 mg/40 g) (Figure 15) than any other chocolate type attributed to the chocolate liquor as the source of dark chocolate polyphenols. Therefore, chocolate liquor at 100% was demonstrated to be the greatest source of total monomer flavanols (88.6 mg/40 g) (Figure 16) as compared to other lower chocolate liquor containing samples. A recent study demonstrated that the consumption of 46 mg of epicatechin and 213 mg of procyanidins from dark chocolate over a two week period resulted in a significant increase in endothelium-dependant flow-mediated dilation and increased epicatechin concentrations, both of which help prevent the development of cardiovascular diseases (Engler et al., 2004). This current finding would indicate that at these levels of epicatechin, Legacy's chocolate liquor (100%) would be an ideal chocolate product to consume in order to attain a substantial content of dietary flavonoids; however, chocolate liquor is not commonly consumed due to its bitter

sensory attributes. However, the consumption of Legacy dark chocolates having a large total flavanol concentration, such as with Legacy 60% (38.1 mg/40 g) and Legacy 73.5% (33.4 mg/40 g) (Figure 16) may also increase endothelium-dependant flow-mediated dilation and epicatechin plasma concentrations.

Interestingly, the Legacy chocolate prepared from 60% chocolate liquor had a significantly greater concentration of epicatechin (29.2 mg/40 g) as compared to Legacy 58.5% (18.4 mg/40 g) (Figure 15). Since the percentages of chocolate liquor added to Legacy 58.5% and Legacy 60% dark chocolate were similar, it would be expected that the flavanol content would also be similar. Although there were no significant differences between catechin and epicatechin concentrations for Legacy 60% and Legacy 73.5%, the total monomer flavanols was greater in Legacy 60% (38.1 mg/40 g) as compared to Legacy 73.5% (33.4 mg/40 g) (Figure 16). It was expected that Legacy 73.5% dark chocolate would have a greater concentration of total monomer flavanols than Legacy 60% because Legacy 73.5% has a greater concentration of chocolate liquor added to the finished product. Since all of Legacy's chocolates are processed with alkali, it is unlikely that alkalization attributed to the difference in flavanols between the three chocolate types. A possible explanation for this difference may be explained by the cacao tree genera: Legacy 60% is made from the Forastero genus, while the 58.5% and 73.5% is made from the Criollo genus. The Forastero genus has been cited in literature to contain more polyphenols than the Criollo genus (Lopez, 2002).

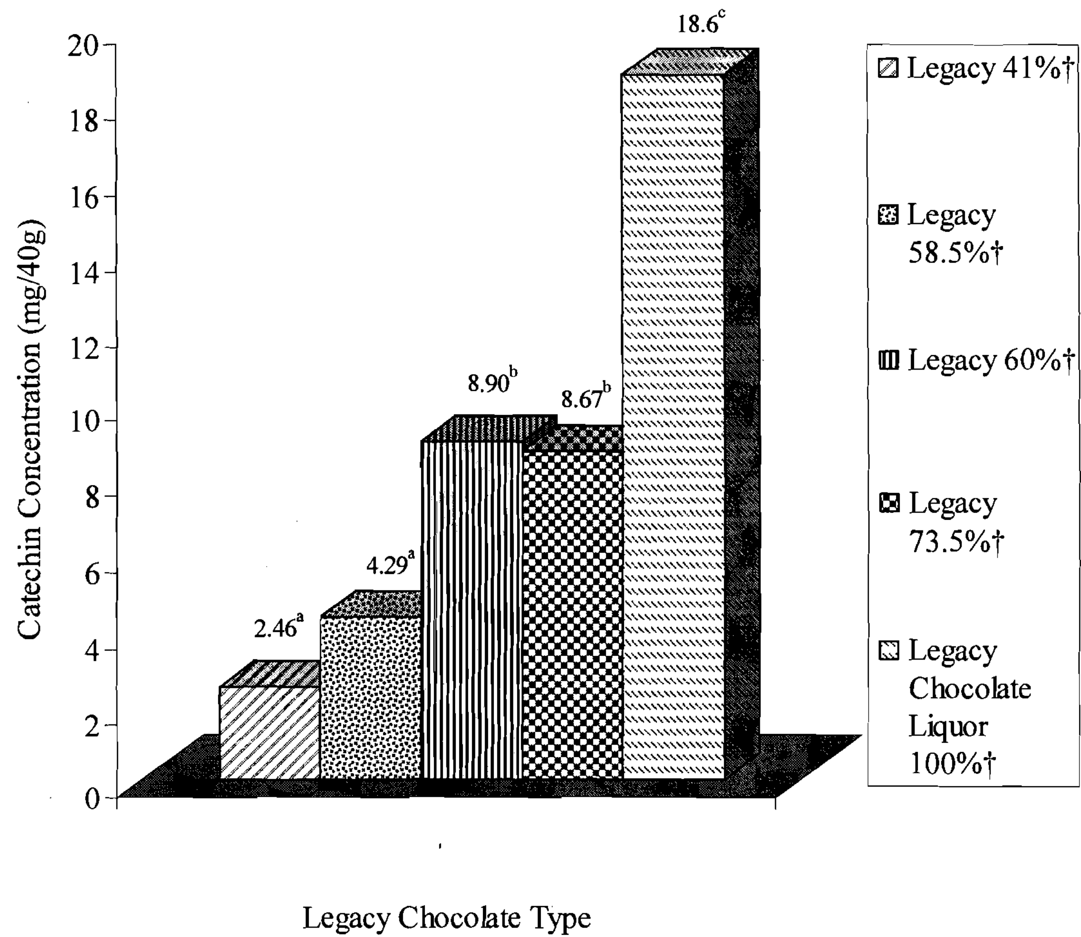

Figure 14. Mean Catechin Concentrations (mg/40g) for Legacy Chocolates.[1]

† Percentage of chocolate liquor.

[1] Values followed by different lowercase letters are significantly different ($p < 0.05$).

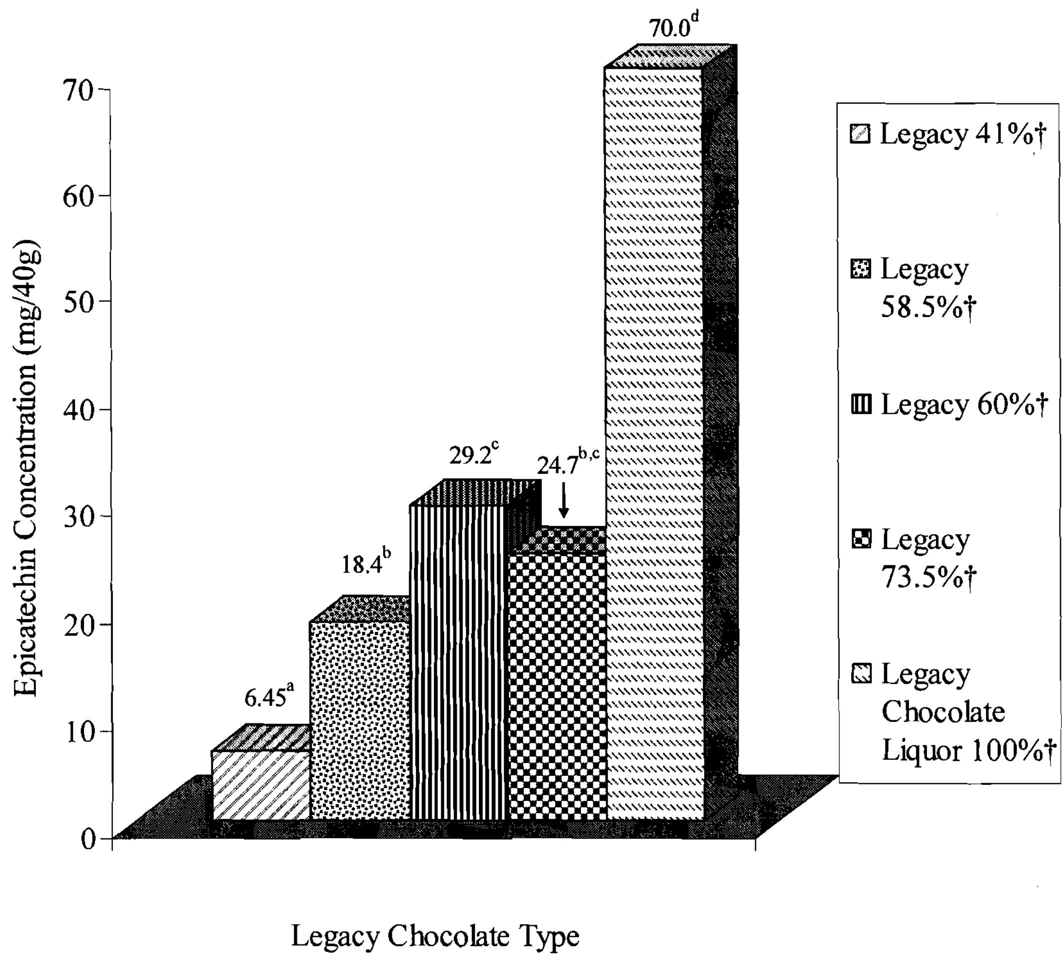

Figure 15. Mean Epicatechin Concentrations (mg/40g) for Legacy Chocolates.[1]

† Percentage of chocolate liquor.

[1] Values followed by different lowercase letters are significantly different ($p < 0.05$).

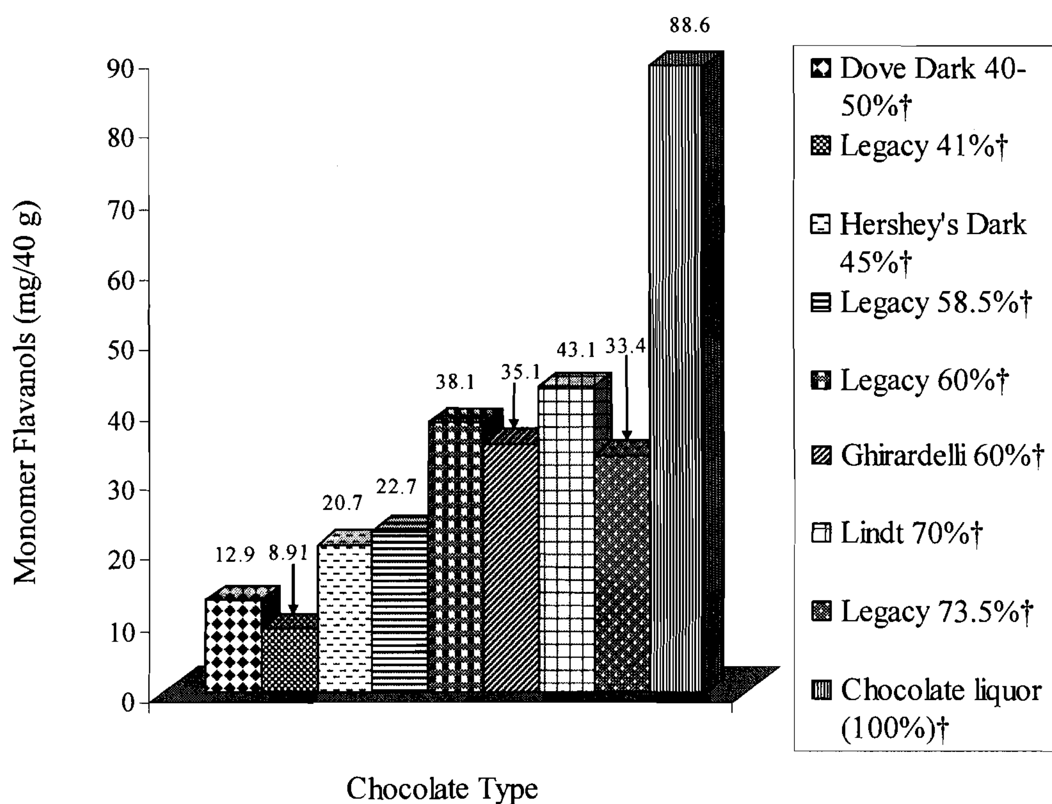

Figure 16. Mean Monomer Flavanols (mg/40 g, catechin and epicatechin) for all Chocolate Types.

† Percentage of chocolate liquor.

Flavanols, Legacy Chocolates, & commercial dark chocolates. Figures 17 and 18 demonstrate the results of catechin and epicatechin concentrations among all chocolate varieties. The percentage of chocolate liquor added to the finished commercial dark chocolate products (eg., Hershey's®, Lindt) is shown in Table 2 in order for a direct comparison of commercial brand chocolates to Legacy Chocolates based on the percentage of chocolate liquor added. Commercial chocolates and those Legacy chocolates having similar percentages of chocolate liquor may be expected to contain similar concentrations of flavanols.

The chocolate samples containing the largest levels of catechin (excluding chocolate liquor) were Lindt 70% (10.4 mg/40 g), Legacy 60% (8.90 mg/40 g), Hershey's® Dark 45% (8.72 mg/40 g), and Legacy 73.5% (8.76 mg/40 g) (Figure 17). The sample of Lindt 70% had a significantly larger ($p<0.05$) catechin concentration (10.4 mg/40 g) than all the other chocolate samples. It may be considered unusual that a chocolate sample such as Hershey's® Dark, with only 45% chocolate liquor added to the product, has comparable catechin concentrations to chocolates containing 60% chocolate liquor. Comparing Hershey's® Dark to Legacy 60% and Ghirardelli 60%, Hershey's® Dark and Legacy 60% were both processed with alkali, while Ghirardelli 60% was non-alkalized, which does not explain the catechin concentration differences. Perhaps the differences may be due to variables such as cacao tree genus, which were unknown for all commercial chocolates (Table 2).

Many commercial chocolate companies use a blend of cacao beans from around the world (L. Lisa, personal communication, May 26, 2006, R. Green, personal communication, June 1, 2006, S. Ortiz, personal communication, May 24, 2006, & Lindt,

2006), which implies that two to three cacao bean varieties may be blended into the finished chocolate product. Since the cacao tree genus (or genera) was unavailable for all of the commercial chocolates, no inferences can be made about the flavanol content regarding cacao tree genera.

Legacy 60% was derived from the Forastero genus, while the chocolate liquor origin for Hershey's® Dark and Ghiradelli were unknown (Tables 1 & 2). Since the Forastero genus has been noted to contain more antioxidants than the Criollo genus (Lopez, 2002), it may be possible that Hershey's Dark was at least partially produced with chocolate originating from the Forastero genus, which may explain the larger catechin content found in Hershey's® Dark.

Consuming chocolates with large levels of antioxidants, such as catechin, may be beneficial to help prevent cardiovascular diseases. In a recent study (Taubert et al. 2003), conducted on elderly individuals with isolated systolic hypertension, those participants who consumed 100g of dark chocolate per day decreased their systolic and diastolic blood pressure as compared to the non-chocolate control group. The consumption of chocolates with large levels of catechin, such as Lindt 70%, may be beneficial for reducing blood pressure in individuals with hypertension.

Comparing Legacy 60% and Ghirardelli 60%, Legacy had a slightly larger mean concentration of catechin (8.90 mg/40 g) than Ghirardelli (7.89 mg/40 g) (Figure 17), although not significant ($p<0.05$). In addition, both Legacy 60% and Ghirardelli 60% were found to have significantly greater mean catechin concentrations compared to Legacy 41% (2.46 mg/40 g) and 58.5% (4.29 mg/40 g) (Figure 17); therefore, it may be difficult to attain any purported health benefits associated with chocolate consumption

from chocolates containing a lower chocolate liquor content such as with Legacy 41% and 58.5%.

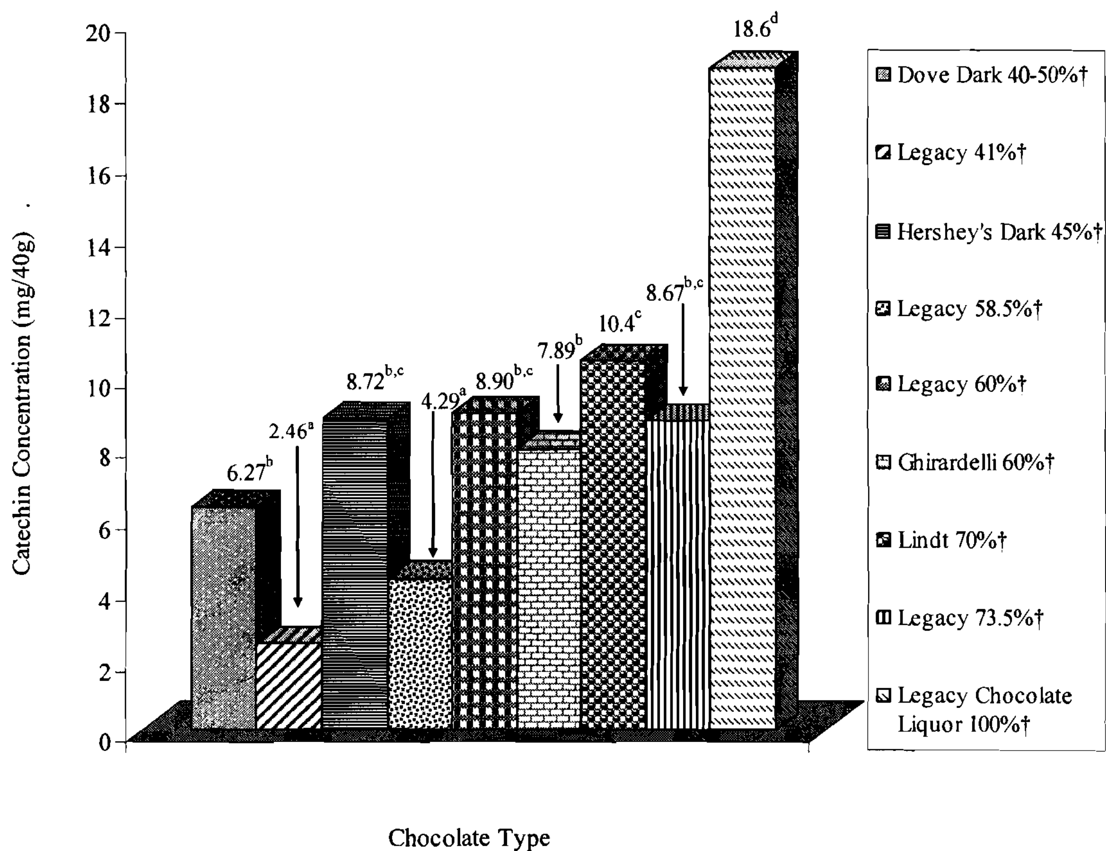

Figure 17. Mean Catechin Concentrations (mg/40 g) for all Chocolate Types.[1]

† Percentage of chocolate liquor.

[1] Values followed by different lowercase letters are significantly different ($p < 0.05$).

Figure 18 demonstrates results of epicatechin concentrations among all chocolate varieties. Lindt 70% had significantly larger epicatechin concentration (32.7 mg/40 g) than any other chocolate sampled, except for chocolate liquor (70.0 mg/40 g). Interestingly, Legacy 60% had a larger epicatechin concentration (29.2 mg/40 g) than Ghirardelli 60% (27.2 mg/40 g), and Legacy 73.5% (24.7 mg/40 g), although not significant (Figure 18). Chocolates with elevated percentages of chocolate liquor added to the finished product and smaller epichatechin concentrations than chocolates with less chocolate liquor added implies that other factors may affect the epicatechin concentration. For example, Ghirardelli 60% was not processed with alkali, while Legacy 60% and 73.5% were alkalized (Tables 1& 2). Ghirardelli had a larger epicatechin concentration than Legacy 73.5%, which implies that a factor other than percent chocolate liquor added or alkalization processing may affect the epicatechin concentration, such as cacao tree genus.

It was noted that Legacy 60% and Ghirardelli 60% had significantly greater epicatechin concentrations (29.2 mg/40 g and 27.2 mg/40 g, respectively) compared to Legacy 41% (6.45 mg/40 g), Dove® Dark (6.56 mg/40 g), and Hershey's® Dark (12.0 mg/40 g) (Figure 18). Because epicatechin concentrations were significantly lower for Legacy 41% and Dove® Dark as compared to all other chocolate products, these chocolate varieties would not be recommended for consumption to help prevent chronic diseases because of their low flavonoid content.

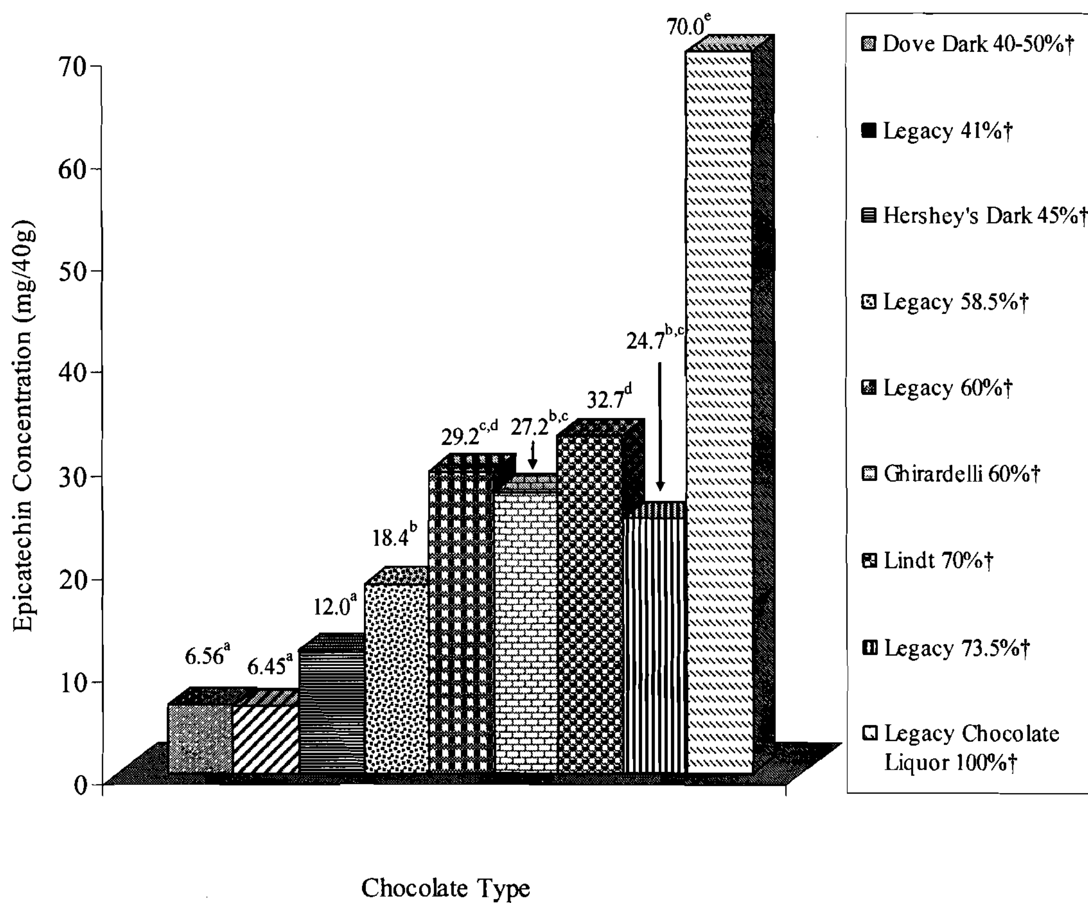

Figure 18. Mean Epicatechin Concentrations (mg/40 g) for all Chocolate Types.[1]

† Percentage of chocolate liquor.

[1] Values followed by different lowercase letters are significantly different ($p < 0.05$).

Legacy 60% and Ghirardelli 60% had similar total monomer flavanol concentrations (38.1 mg/40 g and 35.1 mg/40 g, respectively) (Figure 16). Even though Ghirardelli 60% is made with non-alkalized chocolate and Legacy 60% is made with alkalized chocolate (Tables 1 & 2), this finding may suggest that the total monomer flavanol concentration of Legacy 60% was not affected by the alkalization process or that the process was different for each chocolate product.

Comparing Legacy Chocolates (excluding chocolate liquor 100%) and commercial brands for total monomers, Lindt 70% had the greatest mean concentration of total monomer flavanols (43.1 mg/40 g) (Figure 16). Lindt 70% was also found to have a significantly greater mean concentration of catechin (10.4 mg/40 g) than Legacy 41% (2.46 mg/40 g), Legacy 58.5% (4.29 mg/40 g), and Dove® Dark 40-50% (6.27 mg/40 g) (Figure 17). Lindt also had significantly greater epicatechin mean concentrations than Legacy 41% (6.45 mg/40 g), Legacy 58.5% (18.4 mg/40 g), Dove® Dark 40-50% (6.56 mg/40 g), and Hershey's® Dark 45% (12.0 mg/40 g) (Figure 18). Although no statistical difference existed for total flavanol concentrations between Lindt 70% (43.1 mg/40 g) and Legacy 73.5% (33.4 mg/40 g) (Figure 16), Lindt 70% had a considerably greater total flavanol mean concentration and slightly less chocolate liquor added to the finished product than Legacy 73.5%. In addition, Lindt 70% was not processed with alkali, while Legacy 73.5% was processed with alkali; therefore, any flavanol differences may be attributed to alkalization processing since the percentage of chocolate liquor added was similar. Other chocolates with the same percentages of chocolate liquor added previously discussed (Ghirardelli 60% and Legacy 60%) demonstrated negligible differences in flavanol content between alkalized and non-

alkalized chocolates. Differences in total flavanol content may be attributed to factors not observed in this study, such as cacao bean genus, cacao bean fermentation, and cacao bean maturity.

The chocolates having large monomer flavanol contents, such as Lindt 70% (43.1 mg/40 g), Legacy 60% (38.1 mg/40 g), and Ghirardelli 60% (35.1 mg/40 g) (Figure 16) may be beneficial for decreasing systolic blood pressure. One study (Grassi et al., 2005) found that the consumption of 100 g of dark chocolate/day containing 500 mg of total flavonoids significantly decreased systolic blood pressure in human subjects, thereby decreasing the risk of cardiovascular diseases.

Since Lindt 70% and Legacy 73.5% had large concentrations of flavanols, specifically epicatechin, these chocolate varieties would be ideal to consume to achieve the health benefits that aid in the prevention of cardiovascular diseases. It is reported that elevated levels of epicatechin in the blood serve as antioxidants, and are believed to provide a protective effect on the cardiovascular system due to their free radical scavenging capabilities and their ability to inhibit lipid oxidation (Wang et al., 2000). Wang et al. (2000) found that the consumption of 80 g of dark chocolate containing 557 mg of total procyanidins (of which 137 mg were epicatechin) significantly increased the subjects mean plasma antioxidant capacity by 36% (two to six hours after consumption), thereby decreasing the risk of cardiovascular diseases. In the current study, the largest level of epicatechin was obtained from chocolate liquor (100%) at 70 mg/40 g (Figure 18). Even though this level is almost half of that found by Wang et al. (2000) to elicit a protective effect on the cardiovascular system, it may be postulated that some plasma benefits could be obtained, albeit at lower levels.

Caffeine, Legacy Chocolates, & commercial dark chocolates. Figure 19 demonstrates results of caffeine concentrations among all chocolate varieties. Legacy chocolate liquor (100%) and Legacy 73.5% had significantly greater concentrations of caffeine (59.4 mg/40 g and 49.9 mg/40 g, respectively) (Figure 19) than all other chocolate samples. Legacy's chocolate liquor (100%) and Legacy 73.5% would be ideal chocolate products to consume in order to attain a large content of dietary caffeine; however, chocolate liquor is not commonly consumed due primarily to its bitter sensory attributes.

In general, the statistical analyses indicated that caffeine concentrations (mg/40 g) of each product did not necessarily depend on the amount of chocolate liquor. For example, in comparison to all other chocolate samples, Legacy 60% had a caffeine concentration of 25.2 mg/40 g, while Ghiradelli 60% had 34.4 mg/40 g which was comparable to Lindt 70% that had 35.5 mg/40 g (Figure 19). This indicates that factors other than the percentage of chocolate liquor added to the finished product may affect the methylxanthine content of chocolates, such as: cacao bean genus, cacao bean fermentation, and cacao bean maturity. It was noted that Legacy 58.5% which had a significantly greater (40.7 mg/40 g) (Figure 19) caffeine content than those chocolates with larger chocolate liquor contents, such as Legacy 60%, Ghirardelli 60%, and Lindt 70%, was processed with alkali and had significantly greater caffeine concentrations than other chocolates with similar weight percentages of chocolate liquor. Therefore, in this study, alkalization processing did not appear to have adversely affected caffeine concentrations.

An interesting note was that Legacy 73.5% had a significantly greater mean caffeine concentration (49.9 mg/40 g) than Lindt 70% (35.5 mg/40 g) (Figure 19), even though the percentage of chocolate liquor added to each product was quite similar. It appears that the alkalization processing may not affect the caffeine content since Legacy 58.5% and Legacy 73.5%, both alkalized chocolates, have significantly greater caffeine concentrations than non-alkalized commercial chocolates with similar percentages of chocolate liquor.

According to the overall results for Legacy Chocolates, chocolates produced from the Criollo genus (Legacy 58.5%) had greater caffeine concentrations as compared to chocolates with similar percentages of chocolate liquor (Legacy 60%), which was produced from the Forastero genus. When comparing Legacy 58.5% and Legacy 60 %, Legacy 58.5% had a significantly greater caffeine concentration (40.7 mg/40 g) than Legacy 60% (25.2 mg/40 g) (Figure 19). Since both chocolates were processed with alkali, these results may be explained by the cacao tree genera: Legacy 58.5% originates from the Criollo genus, while Legacy 60% originates from the Forastero genus. Therefore, this suggests that consuming chocolate from the Criollo genus may possibly exhibit greater physiological stimulation (i.e., caffeine effects) than chocolate from the Forastero genus.

Since Legacy 58.5%, 73.5%, and chocolate liquor (100%) had the greatest mean caffeine concentrations, they may impart a greater level of central nervous system stimulation, cardiac muscle stimulation, relaxation of smooth muscle, and diuretic effects as compared to other chocolate types (Apgar & Tarka, 1999; Gilbert, 2004). Studies have shown that caffeine consumption increases the diuretic effect, which leads to increased

calcium loss from excretion (Massey & Whiting, 1993). Women at risk for osteoperosis may consider regulating their caffeine intake from chocolate because increased calcium excretion may be considered a potential risk factor for developing osteoporosis (Bruce & Spiller, 1998). Caffeine has also been implicated for causing infertility and spontaneous abortions (Nawrot, 2003). Although evidence is inconclusive, pregnant women are recommended to decrease their caffeine level to less than 300 mg/day; therefore, they should be aware of the caffeine concentrations present in certain dark chocolates.

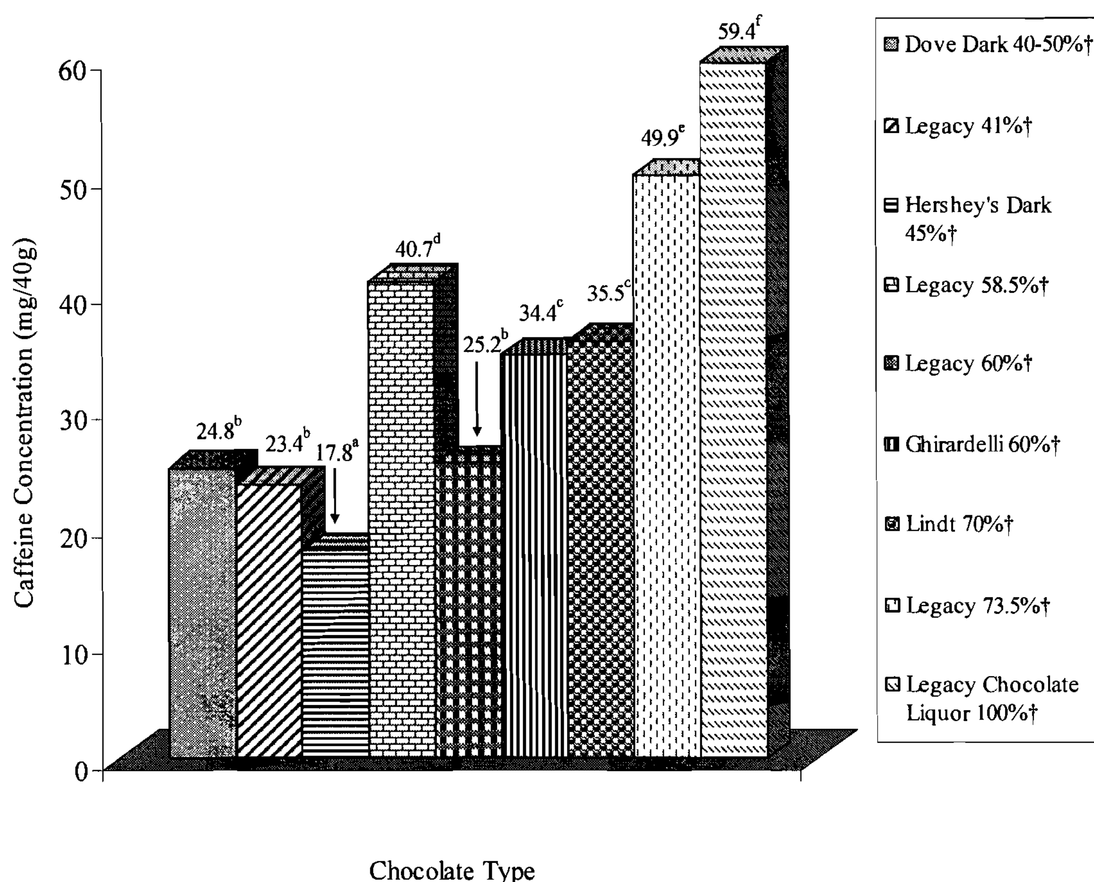

Figure 19. Mean Caffeine Concentrations (mg/40g) for all Chocolate Types.[1]

† Percentage of chocolate liquor.

[1] Values followed by different lowercase letters are significantly different ($p < 0.05$).

Theobromine, Legacy Chocolates, & commercial dark chocolates. Figure 20 demonstrates the theobromine concentrations among all chocolate varieties. Legacy chocolate liquor (100%) had a significantly greater concentration of theobromine (414 mg/40 g) (Figure 20) than any other chocolate. This large content of theobromine is due to chocolate liquor being the source of methylxanthines in dark chocolate. Legacy's chocolate liquor (100%) would be an ideal chocolate product to consume in order to

attain a large level of dietary theobromine; however, it is not commonly consumed because of its bitter sensory attributes.

Legacy 60% had the second greatest mean theobromine concentration (302 mg/40 g) (Figure 20), which was significantly greater than the other Legacy and commercial brands. When comparing Legacy 60% and Legacy 58.5% (both having similar levels of chocolate liquor), Legacy 60%, it was noted to have a significantly greater theobromine concentration (302 mg/40 g) than Legacy 58.5% (160 mg/40 g) (Figure 20). The differences in theobromine concentrations between the two Legacy chocolates both processed with alkali and containing similar percentages of chocolate liquor may be attributed to the cacao tree genera: the Forastero genus has a greater theobromine concentration compared to the Criollo genus. Legacy 60% was from the Forastero genus, while Legacy 58.5% was from the Criollo genus. However, other factors that may also influence the theobromine concentration, such as cacao bean fermentation and cacao bean maturity, both of which were not investigated in this study, warrant further investigation.

Lindt 70% had third greatest theobromine concentration (284 mg/40 g), which was significantly greater than all other chocolates except Legacy 60% (327 mg/40 g) and Legacy chocolate liquor (100%), (473 mg/40 g), (Figure 20). These results indicate that dark chocolates with large theobromine concentrations such as Legacy 60% and Lindt 70% may be beneficial since their ingestion may exhibit anti-cough properties by inhibiting sensory nerve functions as demonstrated previously in one study (Usmani et al., 2004). In this study, it has been noted that concentrations of dietary theobromine ranging from 500 mg to 1000 mg are associated with displaying a bronchodilator effect in human subjects (Usmani et al., 2004).

Lindt 70 % (284 mg/40 g) had a significantly greater theobromine concentration when compared to Legacy 73.5% (207 mg/40 g), and Legacy 60% (302 mg/40 g) had a significantly greater theobromine concentration when compared to Ghirardelli 60% (206 mg/40 g) (Figure 20). Even though the percentage of chocolate liquor added to the compared samples is similar, and the theobromine varies between them, other factors may be contributing to the differences in the theobromine concentration. For instance, Lindt 70% and Ghirardelli 60% were not processed with alkali, while Legacy 60% and Legacy 73.5% were processed with alkali. Comparing Lindt 70% and Legacy 73.5%, it was found that Lindt 70%, a non-alkalized chocolate, contained a significantly greater theobromine concentration than Legacy 73.5%, an alkalized chocolate. However, comparing Legacy 60% and Ghirardelli 60%, Legacy 60% (an alkalized chocolate) had a significantly greater theobromine concentration than Ghirardelli 60% (a non-alkalized chocolate). This conflicting evidence may suggest that alkalization does not play a significant role in reducing the theobromine content in chocolates with similar percentages of chocolate liquor. The differences may be explained by other factors, such as cacao bean genera, cacao bean fermentation, or cacao bean maturity. Therefore, more studies need to be conducted in order to determine the difference in theobromine content of dark chocolates containing similar percentages of chocolate liquor in the finished product.

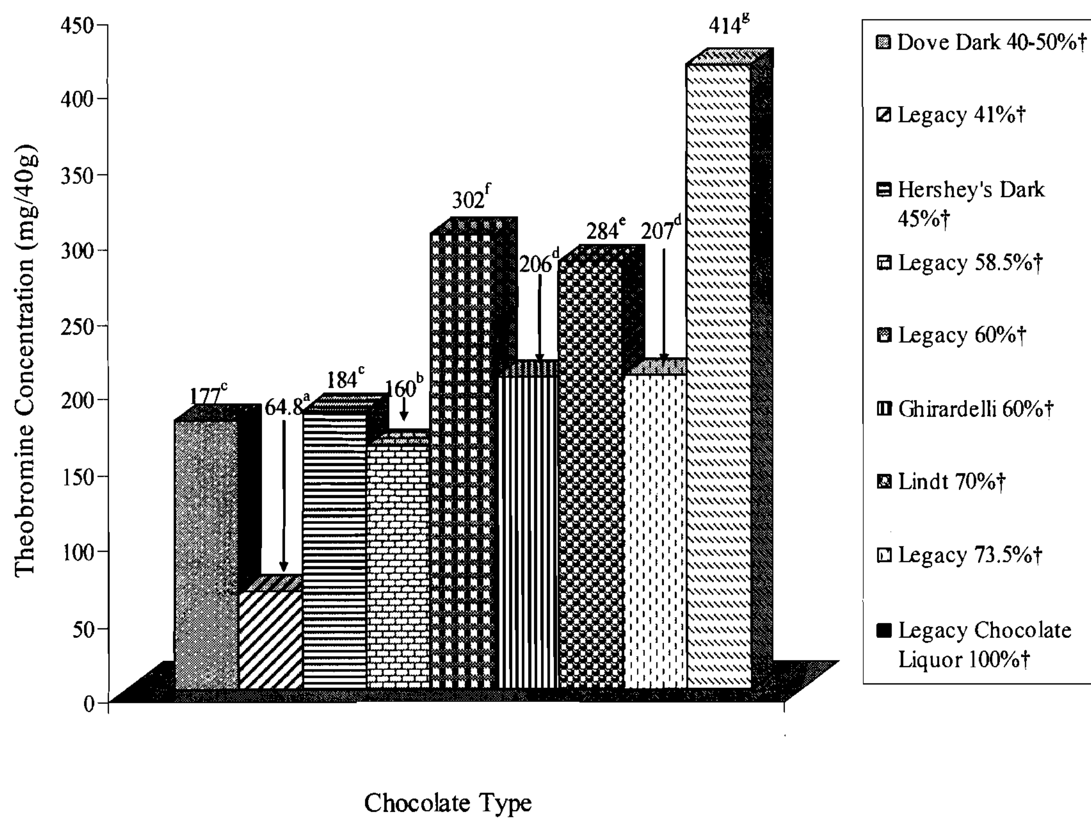

Figure 20. Mean Theobromine Concentrations (mg/40g) for all Chocolate Types.[1]

† Percentage of chocolate liquor.

[1] Values followed by different lowercase letters are significantly different ($p < 0.05$).

Chapter V: Conclusion

The purpose of this study was to identify and quantify monomer flavanols and methylxanthines in chocolates obtained from Legacy Chocolates with different weight percentages of chocolate liquor and to then compare these findings to commercially available dark chocolates. Flavanols and methylxanthines were extracted from the chocolate samples and analyzed by HPLC. The concentrations of each compound were calculated per 40 gram sample for comparison to health effects attained from human studies.

Statistical analysis was performed on catechin, epicatechin, caffeine, and theobromine concentrations (mg/40 g serving size) to determine if differences existed between the weight percent of chocolate liquor added to each product, flavanol content, and methylxanthine content.

The cacao bean genera were unknown blends for all of the commercial products, and known for Legacy chocolates. No information was known concerning the cacao bean fermentation processes used for any of the chocolates. The only commercial chocolates known to contain alkalized chocolate liquor were Hershey's® and Dove®, while all Legacy products were processed with alkali.

In general, the greater percentage of chocolate liquor added to a chocolate product, the greater the flavanol content. However, this study demonstrated that other factors can also influence the total flavanol content. One know factor that demonstrated its influence on flavanol concentrations in this study was cacao tree genus. For example, when comparing the flavanol content of Legacy 60% and 58.5%, Legacy 60% had a significantly greater concentration than Legacy 58.5%. Since the percent of chocolate

liquor added was very similar and both chocolates were processed with alkali, the only other known factor was the different cacao tree genera, implying that the Forastero genus contains more flavanols than the Criollo genus. Although other factors such as cacao bean fermentation and cacao bean maturity can affect the flavanol content, not enough information about these two factors was known for the chocolate products analyzed to draw definite conclusions on flavanol differences.

To achieve the flavanol antioxidant health benefits from dark chocolate consumption reported in this study, it would be recommended to consume dark chocolate with a large percent of chocolate liquor from the Forastero genus. Typically, the cacao tree genus information is not readily available for consumers. Many of the studies previously discussed involved consuming between 40-100 g of dark chocolate/day to achieve health benefits that prevent the development of chronic diseases. For many people, this recommended quantity of chocolate would be difficult to consume on a daily basis. However, dark chocolate could be incorporated into a healthy diet that includes flavonoids from different plant sources.

According to the results for Legacy Chocolates, the percentage of chocolate liquor added and tree genera had opposite influences on the caffeine and theobromine content. For caffeine, the chocolates with the greatest percent of chocolate liquor from the Criollo genus had the greatest caffeine content as compared to the Forastero genus. Conversely, the Criollo genus had the lowest theobromine concentrations, while the Forastero genus had the greatest. More studies need to be conducted to determine what effects cacao bean fermentation, cacao bean maturity, and alkalization have on caffeine and theobromine concentrations in dark chocolates.

Methylxanthines produce many stimulatory physiological effects in the body. Many people regulate their caffeine/theobromine intake to avoid these health effects. To avoid some of the stimulatory effects of caffeine and still attain the antioxidant health effects of the flavonoid polyphenols, it would be advised to consume dark chocolate from the Forastero genus. The Forastero genus has less caffeine per serving in chocolates with similar percentages of chocolate liquor compared to the Criollo genus.

This study demonstrated that dark chocolate is an excellent source of flavanols and methylxanthines. Chocolate consumers should be aware of the factors studied in this experiment that can influence the polyphenol and methylxanthine concentration in chocolates, such as the percentage of chocolate liquor added to the finished product and cacao tree genus. From the results of this study, it is recommended to consume chocolates with an elevated percentage of chocolate liquor (60% or more) from the Forastero genus to attain health benefits from flavanols that are commonly associated with chocolate consumption.

Recommendations

The following research is suggested to further explore the flavonoid/methylxanthine content of chocolate products available from Legacy Chocolates:

1) Quantify the flavanol/methylxanthine content of the truffle ganache to calculate the total concentration per truffle.
2) Quantify the flavanol/methylxanthine content of the 85% chocolate (made from 73.5% + 100% chocolate liquor).

3) Identify and quantify other polyphenols/flavonoids present from the addition of merlot wine and green tea in the merlot and green tea truffles.
4) Identify and quantify chocolate procyanidins present in the chocolates containing different percentages of chocolate liquor by HPLC and MS.
5) Determine the affect of alkalization on flavonoid/methylxanthine concentrations in dark chocolates by comparing chocolates with the same percentage of chocolate liquor from the same known cacao tree genus, alkalized and non-alkalized.

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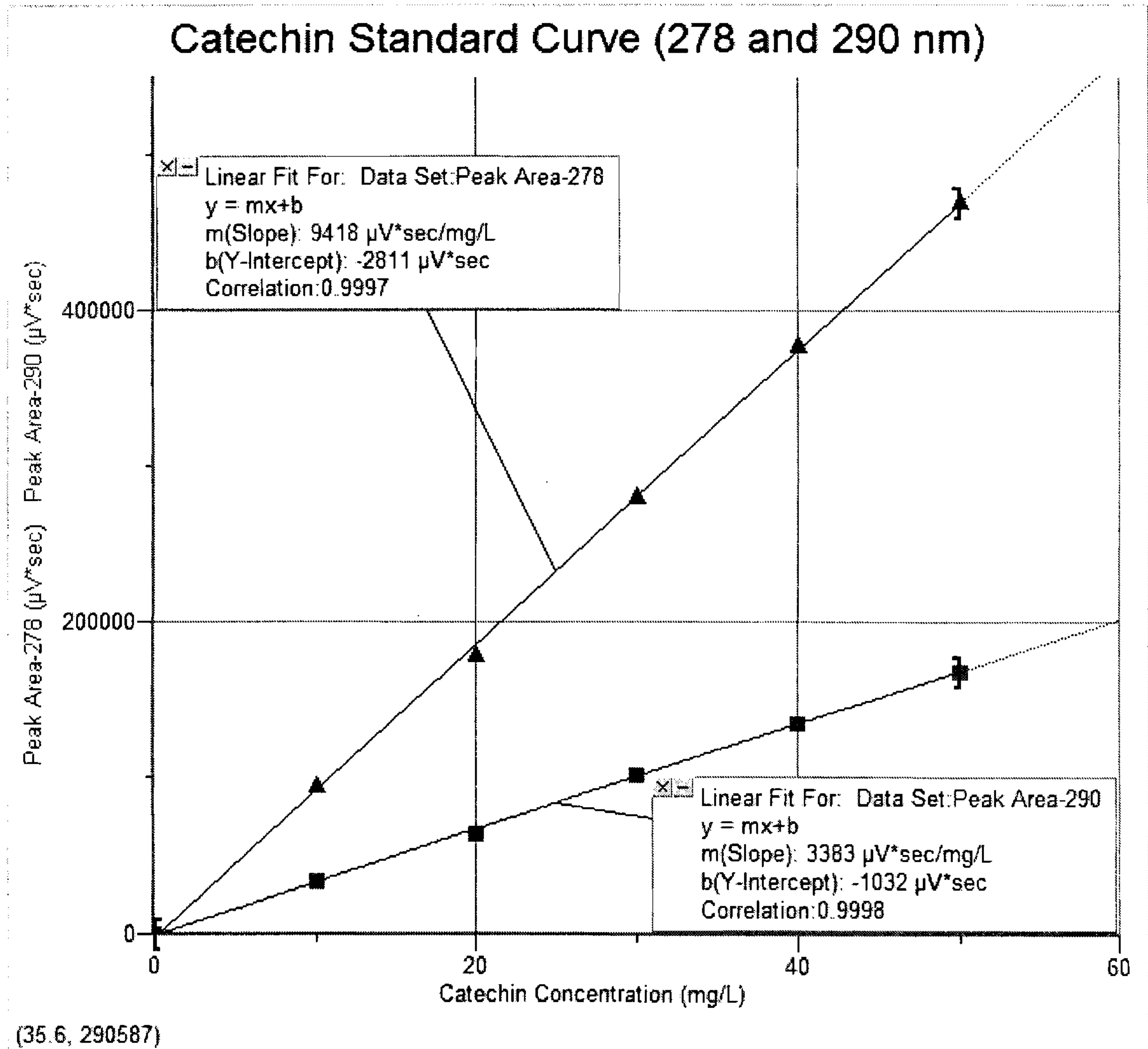
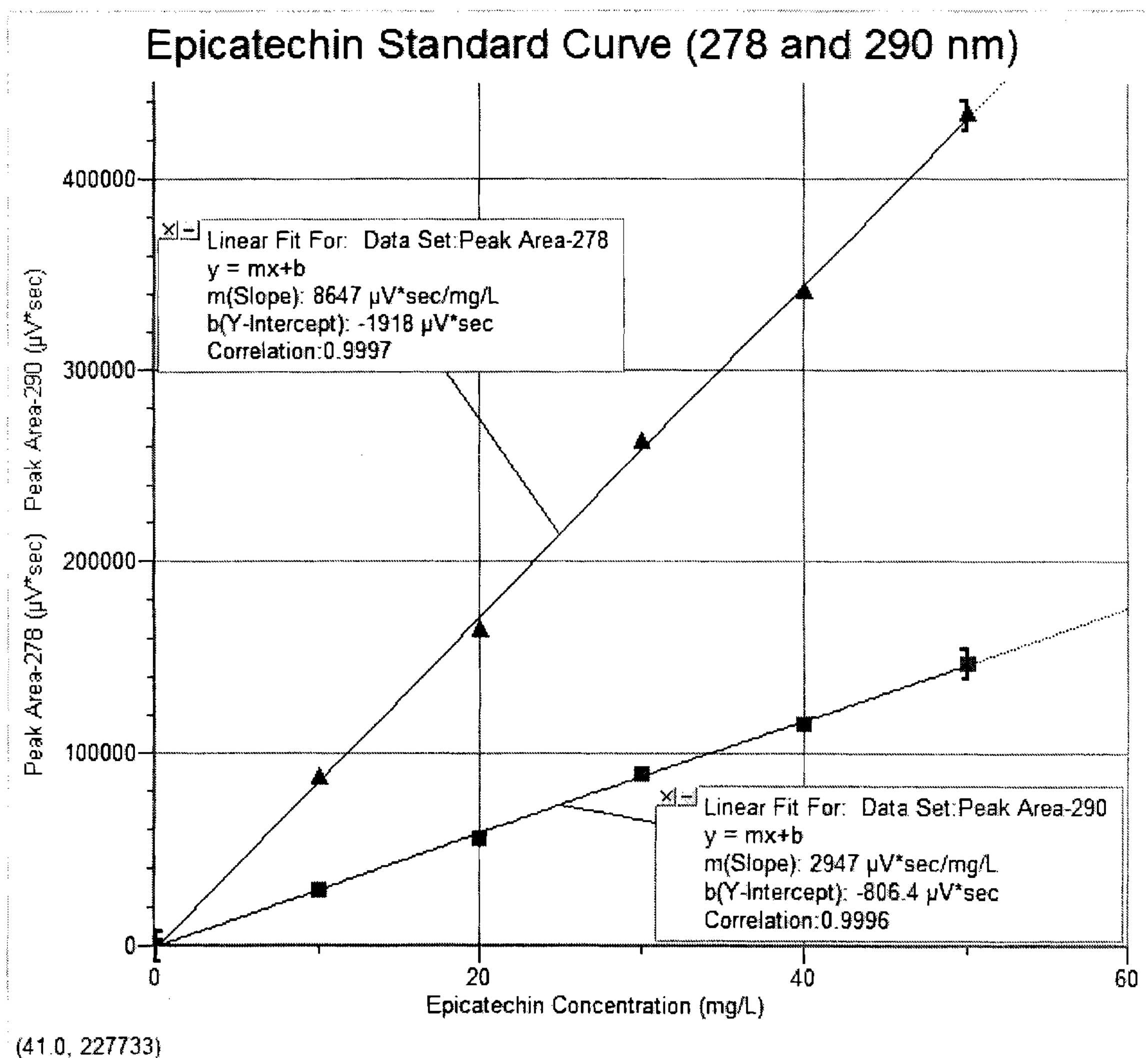
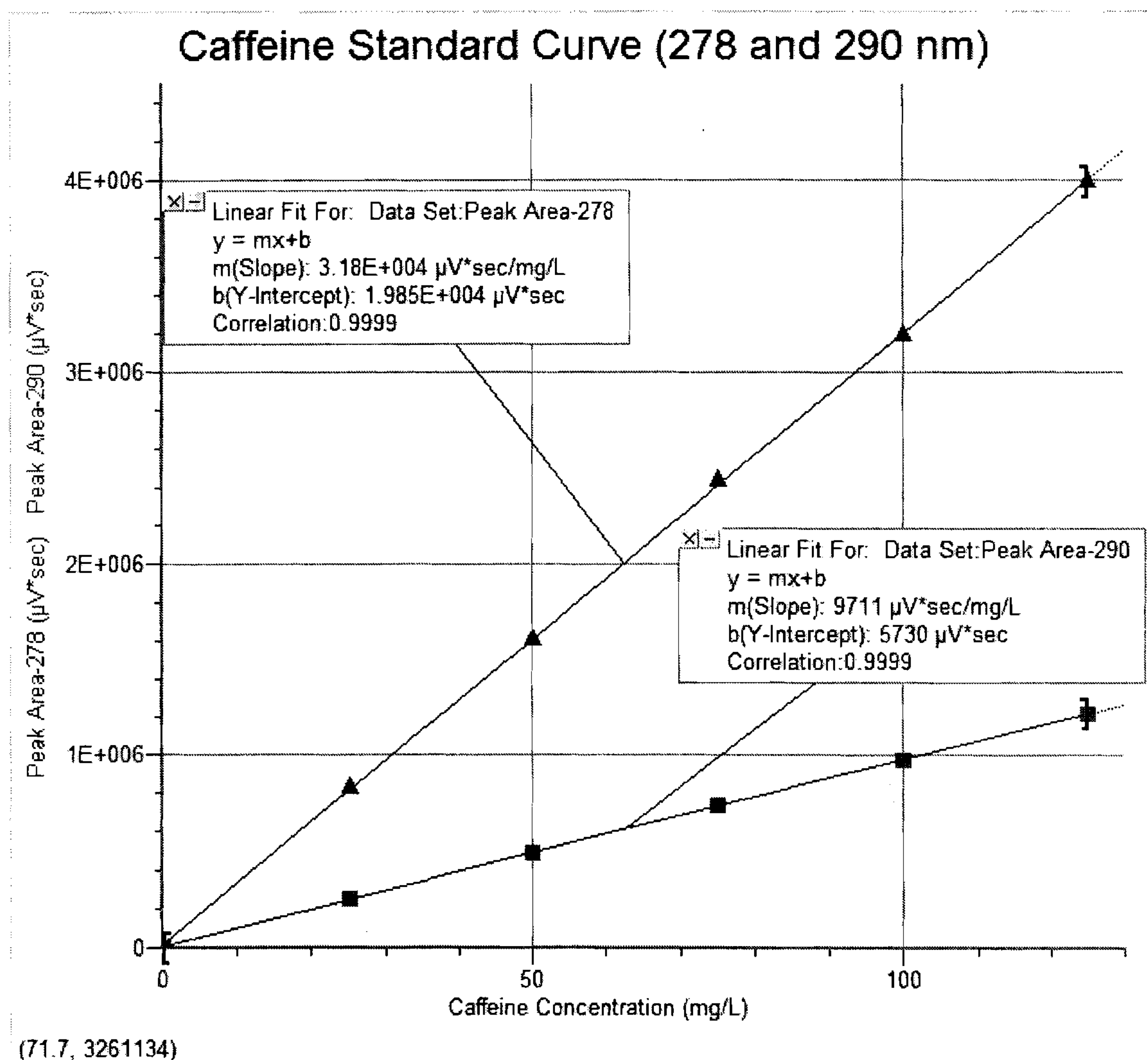
Appendix A: Catechin Standard Curve



Appendix B: Epicatechin Standard Curve



Appendix C: Caffeine Standard Curve



Appendix D: Theobromine Standard Curve