The Effects Of Roasting Time and Temperature On The Antioxidant Capacity Of Cocoa Beans From Dominican Republic, Ecuador, Haiti, Indonesia, and Ivory Coast.

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ABSTRACT

Roasting is an important processing step for developing cocoa flavor, color, and aroma. Cocoa beans contain polyphenolic compounds, which can be desirable antioxidants. Oxygen Radical Absorbance Capacity (ORAC) values can be used as an indicator of health benefits of antioxidants in foods. ORAC values measure total antioxidant capacity of different foods by measuring antioxidant scavenging activity against peroxyl radical induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). This measurement of total antioxidant capacity gives a complete assessment during which the inhibition time and inhibition degree are measured as the reaction comes to a completion. ORAC values were determined as Trolox Equivalents (TE). Cocoa beans were also measured for antioxidants using Total Phenolics assay and DPPH assay and measured as gallic acid equivalents (GAE). Cocoa beans from the Ivory Coast were roasted at varying times (10-40 minutes) and temperatures (100ºC -190ºC). It was determined that cocoa beans from the Ivory Coast, roasted at 130ºC for 30 minutes resulted in an ORAC value of 522,789 µmol (micromoles) TE/g, GAE value of 2.46 mg/L as determined by Total Phenolics, and GAE value of 1.48 mg/L as determined by DPPH assay. The analyzed values tended to decrease at the highest temperatures and times of roasting. Cocoa beans from different countries were roasted at 130ºC for 30 minutes and antioxidants were analyzed. It was determined that Dominican Republic and Ecuador had the highest TE values (487,913 and 463958 µmol (micromoles) TE/g respectively). GAE differed and Total Phenolic assay found Haiti had the highest GAE (3.26 mg/L) and DPPH assay found Ivory Coast and Dominican Republic had the highest (0.623 and 0.610 mg/L respectively). If an acceptable flavor, color, and aroma of cocoa can be developed at a roasting temperature closer to 130ºC than to 160ºC, then a greater antioxidant content should occur in dry cocoa powder.

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CHAPTER I
LITERATURE REVIEW

In the past few years, antioxidants have been gaining popularity as part of our diet for their numerous health benefits. Antioxidants are abundant in our food sources and can be found in a multitude of foods such as berries, oranges, apples, and cocoa. Research has shown that antioxidants are responsible for increasing cardiovascular health, balancing cholesterol, and even decreasing the risks of certain cancers (Manach and others 2004). Chocolate products are known to contain antioxidants and studies have shown that cocoa powder has more antioxidants then red wine or green tea (Lee and others 2003).

Before chocolate products can be consumed, cocoa beans must be processed through fermentation, drying, and roasting. Processing steps, such as roasting, are known to decrease the antioxidant capacity of cocoa beans, but the roasting process can be manipulated to increase the antioxidant potential. The current research will look at the effects of different roasting times and temperatures on the antioxidant capacity of cocoa beans from five different countries: Dominican Republic, Ecuador, Haiti, Indonesia, and Ivory Coast.

Introduction

Chocolate is an important commodity of the United States. The average person in the United States consumes about 11 pounds of chocolate a year (Alberts and Cidell 2006). The chocolate that we are accustomed to is a relatively new product. The Aztecs first used cocoa beans for their own versions of chocolate before the 1300s (Chatt 1953). When cocoa beans were first

introduced to Europe, the only product that was associated with cocoa was a fatty chocolate liquid, which was prepared with cocoa beans, sugar, and spices. The cocoa press was invented in 1828, which allowed cocoa butter (fat) to be removed from the bean. This produced a cocoa powder with 23 percent fat. In the 1840's, Fry and Cadbury produced the first chocolate bars and in 1876, milk chocolate was invented in Switzerland. In 1909, Milton Hershey started the Hershey Chocolate Company, and since then, chocolate manufacturing has had roots in the United States (Minifie 1989).

Today, there are three main types of chocolate: milk chocolate, white chocolate, and dark chocolate. The FDA has a standard of identity for milk and white chocolate. The FDA states that milk chocolate must contain no less than 10% chocolate liquor, and the finished product cannot have less than 3.39% milk fat or less than 12% total milk solids. White chocolate must not contain less than 20% of cacao fat (by weight) and when finished, white chocolate must not contain less than 3.5% (by weight) of milk-fat or 14% of total milk solids. The FDA does not have a standard of identity for dark chocolate because it is usually used to describe semisweet and bittersweet chocolate (Code of Federal Regulations 2010).

Cocoa Beans

Cocoa beans come from the seeds of the cacao trees, which are native to the tropical forests of the Amazon. Cacao trees produce 20 to 30 pods a year, and each of these pods contain 25 to 40 seeds that are surrounded by a mucilaginous pulp. Each pod produces about 1.5 ounces of fermented and dried

cocoa beans (Chatt 1958). The cacao tree has specific growth requirements such as semi-shade, warmth, and high humidity. These strict growth requirements account for 75% of the cocoa beans being cultivated within eight degrees of the equator. These locations, such as the Ivory Coast and Indonesia, offer the perfect climate for cocoa beans to thrive (International Cocoa Assoc 2010).

Cocoa beans are in the genus *Theobroma* and species *Cacao*. There are over 22 species of Theobroma, but *Theobroma cacao* is the most common. Within the species *Theobroma cacao*, there are different varieties and the varieties Criollo and Forastero are the most commercially available (Minifie 1989). There is also a hybrid of the Criollo and Forastero called Trinitarios.

The Criollo cocoa beans give a delicate chocolate flavor and are typically grown in Ecuador. When the Criollo cocoa pods are ripe, they are still soft and have an oval shape. The Criollo cocoa beans have a higher economic value then Forastero cocoa beans. The higher value is associated with the cocoa beans unique flavor characteristics and the fact that this variety is more rare. When the Criollo cocoa beans are properly processed then they are worth more than other cocoas (Dand 1996).

The Forastero are mainly grown in West Africa and Brazil and produce stronger cocoa flavor then Criollo cocoa beans. The Forastero cocoa beans are also smaller and have a darker color then the Criollo. The cocoa pods also differ. When the Forastero pods are ripe, they are hard and have a more rounded shape like a melon (Dand 1996).

Trinitarios are a cross between Forastero and Criollo cocoa beans, though they are typically classified as Forastero. This variety is a product of manipulation from man and is not found in the wild. This variety was developed because the Criollo cocoa beans are vulnerable to many diseases, but also intolerant to droughts. The Trinitarios are produced on a cocoa tree that is less vulnerable to diseases and produces higher yields (Dand 1996).

The Chocolate Process

Fermentation

Fermentation is a vital step in developing specific volatile fractions such as alcohols, esters, and fatty acids. Fermentation is also responsible for the development of chocolate flavor and aroma precursors such as amino acids and reducing sugars (Counet and others 2002). Studies have shown that unfermented cocoa beans do not contain the aroma precursors that are present in the fermented cocoa beans (Knapp 1937).

Once the cocoa pod is harvested from the tree, it is cracked open and the beans and pulp are removed and placed into wooden fermenting boxes. The initial introduction of microorganisms, which is necessary for the fermentation to take place, comes from the microorganisms on the hands of the farmers and the skins of the pods. The main function of the microorganisms during fermentation is to produce acids and alcohols that will penetrate the cocoa bean and start the chemical reactions that will form the precursors of chocolate flavor (Dand 1996).

Once the beans are placed in wooden fermenting boxes, they are covered with banana leaves and left in the forest to ferment. The fermentation length

varies depending on the variety of the cocoa beans. Forastero beans are left to ferment for five to six days, while Criollo beans only ferment for one to three days (Afoakwa 2008). The pulp inside of the cocoa bean is 85% water and 11% sugar, with a pH of 3.5, which is from citric acid. The anaerobic conditions, low pH, and high sugar content, offers an ideal conditions for naturally present yeast to convert sugar from the cocoa beans into alcohol (Dand 1996).

During the fermentation process, lactic acid and acetic acid increase. The increases in lactic acid are from lactic acid bacteria, which thrive in anaerobic conditions and convert sugar to lactic acid. The increase in acetic acid comes from acetic acid bacteria, which produce acetic acid from the alcohol and organic sugars (Dand 1996). As the fermentation process continues, the heat increases (up to 122°F), which causes the pulp to liquefy and drain off (Minifie 1989). The combination of heat and acid prevents germination, an undesirable characteristic from occurring in cultivated cocoa beans. During the bean death process, the cell membranes also begin to degrade, which causes enzymes to spread throughout the bean. These enzymes later play an important role in roasting (Hoskin 1994).

During fermentation there are also significant color changes that occur. Unfermented Forastero beans are originally a slate gray color and become purple/brown during fermentation, and then a rich dark brown once the fermentation is complete. Unfermented Criollo beans are a slate gray and then becomes a light brown after fermentation (Minifie 1989).

Drying

Once fermentation is completed, the beans must be dried. If cocoa beans are not properly dried after fermentation, mold can develop, which will cause unpleasant flavors. Drying is also needed to develop the cotyledon (the embryo) of the seeds into nibs. During fermentation, the cotyledon gains moisture and during drying, the texture changes from a wet mass to a brittle structure known as the nib (Minifie 1989). Most cocoa beans are dried in the sun and are covered during the night or rainstorms. During drying the beans are spread two inches deep and are periodically raked to expose new beans. In areas of the world that are too humid for sun drying, artificial dryers must be used, but these have been known to cause smoky off-flavors (Minifie 1989). Once the beans are dried, they are packed in 140 lb bags and are then transported to buyers.

Roasting

Once the cocoa beans are sold, they must be roasted before further processing can occur. Roasting is necessary to develop flavor, color, and aroma of chocolate. During roasting, flavor precursors that have developed during fermentation interact to produce the desired chocolate flavor (Ramli and others 2006). The flavors produced during roasting come from a combination of 400-500 compounds (Dimick 1983). These compounds include aldehydes and pyrazines, which are formed during roasting, through the Maillard reaction and Strecker degradation of amino acids and sugars (Heinzler and Eichner 1992).

The Maillard reaction is a non-enzymatic browning reaction that requires an amino acid and a reducing sugar, such as glucose or fructose. The ideal

conditions for the Maillard reaction to occur are high temperatures and low moisture content and these conditions can be found in roasting (Fennema 1996).

The Strecker degradation occurs when carbonyl derivative from the Maillard reaction reacts with free amino acids in the food product. This causes a degradation of amino acids to aldehydes, ammonia, and carbon dioxide. The aldehydes that are produced contribute to the aroma. Strecker degradation of each specific amino acid produces a unique aldehyde with a unique aroma (Fennema 1996).

Standards

Grading standards for cocoa beans are hard to regulate, especially when the countries that produce the beans and the countries that consume the beans are different. In recent years, the Federation of Cocoa Commerce (FCC) and Cocoa Merchants Association of America (CMAA) have developed standards for sellers and buyers to follow. According to the standards set by FCC, there are two grades; good fermented cocoa beans and fair fermented cocoa beans. Good fermented cocoa beans need to have less than 5% mold, 5% slate, and less than 1.5% of foreign matter. Fair fermented cocoa beans need to have less than 10% mold, 10% slate, and less than 1.5% foreign matter. These standards are determined by the cut test (Trading and shipping 2011).

The cut test was developed by the International Office of Cocoa and Chocolate (Dand 1996). It uses a points system, which gives or subtracts points for the dimensions, color, odor, and absence of imperfections in the beans. It is a relatively quick test that does not require a lot of training or equipment. The cut

test gives an evaluation of the cocoa beans and this evaluation is used to infer certain quality aspects of the cocoa (Dand 1996). The FDA uses the cut test to determine if imports of cocoa beans are of a high enough standard to enter the country.

The evaluation is conducted by thoroughly mixing a sample of cocoa beans. The beans are then divided into a pile of about 300 randomly selected cocoa beans. The cocoa beans are then cut lengthwise through the middle and examined. The beans are then separated into groups of different defects such as mold, slaty, insect damage, germination, or flatness. If the bean has more than one defect, only one is chosen. The defects are then recorded as a percentage of the overall bean count (Minfie 1989). Definitions of the different defects that are found in cocoa beans can be found in the appendix on page 99.

In countries that produce cocoa beans, local traders and sellers are responsible for preparing the beans for export. In these countries, traders, buyers, and brokers work together to export cocoa beans. They also work together to agree on the quality of the cocoa beans being sold (Cocoa story 2011).

Cocoa beans are an important source of income for many countries that produce the product, but the availability of cocoa beans is dependent on climate changes, and the yields can vary depending on the year. Since cocoa beans are such an important commodity, the governments of these countries are heavily involved in cocoa production and exports and some governments have established international treaties with countries that buy their products. One

example, the Treaty of Lomé, was established to prevent the European Union from levying import duties on cocoa imports from a number of producer countries. These treaties have been established to stabilize the price of cocoa by including long-term price agreements in the treaties between producers and consuming countries. These treaties do not always work, and in the early 1990s, some West African nations privatized their raw cocoa production process (Cocoa story 2011).

Health Benefits

Chocolate is primarily viewed as a dessert with lots of sugar and fat, but it is gaining popularity as a nutritionally dense product. Cocoa beans contain polyphenolic compounds, which can be further classified as flavonoids and these flavonoids contain subgroups of flavanols and procyanidins (Othman 2007). Flavonoids are composed of two aromatic rings linked by an oxygenated heterocycle and are subdivided into thirteen different classes, which are determined by the degree of hydroxylation and oxidation of the rings (Steinberg and others 2003). The classes of flavonoids that are most commonly found in cocoa beans are flavan-3-ols, catechins, epicatechins, and proanthocyanidins. Proanthocyanidins have been found to compose 12% to 48% of the dry weight of the cocoa beans (Bravo 1998). Studies have also found that chocolate products have a stronger total flavan-3-ol concentration (per weight basis) than most other plant based foods and beverages that contain flavan-3-ols (Hammerstone and others 2000).

Though processing decreases the flavonoid content in chocolate products, a significant amount of the compounds can still be found in chocolate and especially in cocoa powder, which can contain as much as 10% flavonoids on a dry-weight basis. Dark chocolate also has a higher amount of flavonoids, compared to milk chocolate, because more cocoa bean liquor is used to make dark chocolate, so it contains more flavonoids (Steinberg and others 2003). Flavonoids are important because they offer potential cardiovascular health benefits, antioxidant protections, and help balance cholesterol in the body (Steinberg and others 2003). Flavonoids have also been found to contain anti-allergy, anti-viral, anti-inflammatory, and anti-carcinogenetic properties (Yao and others 2004).

Polyphenolic compounds have gained attention in health and nutrition because of their antioxidant capacity (Adamson 1999). A study conducted by Adamson and coworkers used HPLC to separate and quantify procyanidins in cocoa. The quantified information was correlated to the antioxidant capacity of the samples as measured by Oxygen Radical Absorbance Capacity (ORAC), which can be used as an indicator of health benefits of procyanidins in foods (Adamson 1999). Researchers found that procyanidin content in cocoa samples are the primary contributors to the antioxidant capacity in cocoa. The study found that the procyanidin content in cocoa correlated with their high ORAC values, which may help explain some health benefits of chocolate (Adamson 1999). Though cocoa beans are known to have polyphenolic compounds, the total content varies depending on the country of origin. For example, cocoa

liquor from Ecuador has a total polyphenolic compound content of 4.11%, while beans from the Ivory Coast contain 3.13% (Natsume 2000).

Processing Cocoa Beans

There are many different ways to roast cocoa beans and much of it depends on the bean being roasted and the type of chocolate product that is being made (International Cocoa Assoc 2010). Cocoa beans that are not roasted have a bitter, acidic, astringent, and nutty flavor (Afoakwa and others 2008). By roasting the cocoa beans, the acidity is decreased by reducing the concentration of volatile acids, such as acetic acid (Ramli and others 2006). Cocoa beans are normally roasted whole, which loosens their shell. The shell is then easily removed during the winnowing process, leaving only the cocoa nib. Winnowing is the process of blowing air to cause the lighter portion of the cocoa bean (the shell) to be blown away, leaving the heavier nibs.

Cocoa beans can be roasted in a conventional oven (Arlorio and others 2008), a Barth automatic gas roaster (Rohan and Stewart 1967), or a convection oven. Convection heating is the most common method of roasting cocoa beans (Krysiak 2006). In addition to roasting methods, the times and temperatures that the beans are heated also vary. Studies, which looked at roasting times and temperatures of cocoa beans, varied from a low of 70ºC, up to, a high of 180ºC, and with times ranging from five minutes to forty-five minutes.

A study conducted by Krysiak roasted 200 g of Ivory Coast cocoa beans in a convection oven, which was set to 110ºC, 135ºC, and 150ºC. The cocoa beans were roasted for 15 minutes and 20 minutes. The study concluded that

the ratio of anthocyanins to yellow and brown pigments in the cocoa bean was dependent on the roasting condition, and the color continued to drop as the roasting temperature increased (Krysiak 2006). This is an important study for the intended research because it showed a decrease in one type of antioxidants as the temperature increased.

Another study conducted by Rohan and coworkers roasted cocoa beans in a Barth automatic gas roaster at a temperature of 182ºC for a maximum of 28 minutes. The cocoa beans were removed at five-minute increments (5, 10, 15 etc) until 28 minutes had passed (Rohan 1965). The research concluded that during roasting there were early loses of amino acids and their degradation followed a linear line with the time spent in the oven (Rohan 1965).

A third study roasted 100 g of cocoa beans for thirty minutes, but manipulated the temperature of the convection oven to 70ºC, 100ºC, 125ºC, and 150ºC (Reineccius 1972). The researchers concluded that the pyrazine content increased as the temperature increased (Reineccius 1972). Though the total pyrazine content increased, the specific type and amount of pyrazines varied depending on carbon, nitrogen, and pH. This led researchers to believe that in cocoa beans, both chemical and physical conditions influence the rate of pyrazine accumulation during roasting (Reineccius 1972).

A fourth study conducted by Ramli and coworkers roasted cocoa beans at 120°C, 130°C, 140°C, 150°C, 160°C, and 170°C for 20, 30, 40, and 50 minutes. Among other things, the study was measuring the pyrazine compounds using HPLC. It concluded that there is a linear correlation between roasting

temperature and pyrazine compound formation. They found that cocoa beans roasted 160°C for 40 minutes produced the highest amount tetramethylpyrazine and cocoa beans roasted at 140°C for 40 minutes produced the highest amount of trimethylpyrazine. This study is important because it illustrates the importance of roasting cocoa beans and that the flavor compounds from pyrazines are increased during roasting temperatures (Ramli and others 2006).

Antioxidant Capacity Assay

Antioxidants have been of great interest to the food industry because of their effectiveness in preventing rancidity in foods. They are also of interest to the medical fields because they are known to protect the human body against damage from reactive oxygen species (ROS) (Halliwell and others 1995). Antioxidants can be defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell 1990)." A compound may show antioxidant properties by inhibiting the formation of ROS or by scavenging free radicals (Halliwell and others 1995).

Antioxidants can deactivate radicals by two major mechanisms, Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET). Both tests have the same end results, but the mechanisms differ (Prior and others 2005). The HAT method measures the ability of an antioxidant to supply free radicals by hydrogen donation, while the SET method detects the capability of a potential antioxidant to transfer an electron to reduce a compound (Wright and others 2001). In this

study, three different antioxidant assays were used to detect the antioxidant capacity: ORAC, DPPH, and Folin-Ciocalteu.

Oxygen Radical Absorbance Capacity (ORAC)

ORAC measures the total antioxidant capacity of different foods by measuring the antioxidant scavenging activity against peroxyl radical induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) at 37ºC (Ou 2001, Ortega 2008, and Miller 2006). The ORAC assay is based on the free radical damage to a fluorescent probe (Fluorescein). The damage to the Fluorescein causes a decrease in the fluorescent intensity. It is assumed that the decrease in fluorescent intensity is an indicator of the amount of radical damage. When antioxidants are present the free radical damage is decreased, which can be measured by the preservation of the fluorescent values.

The protection from the antioxidants in the samples can be quantified by calculating the area under the curve (AUC) from the experimental sample. The AUC is a relative value, which means the initial reading of the raw data from the ORAC machine is considered 1 and all of the other raw data points range from 0 to 1. The AUC is then calculated as the sum of many trapezoids under the curves.

When the AUC of the phosphate blanks are subtracted from the sample, the values (Net AUC) are the protections that are provided by the antioxidants in the experimental samples (Held 2005). By comparing the net values to known standards (Trolox), the equivalents can be calculated and compared to different experimental samples. In the ORAC assay, the assay is run until completion, so

the values are a combination of the inhibition time and inhibition percentage of free radical damage by the antioxidant that is reported as a single quantity (Ou 2001). Foods with higher ORAC values indicate that they are more effective at neutralizing free radicals, which in turn slows down the oxidative processes and free radical damages (Fennema 1996).

The chemical Fluorescein (FL; 3'6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) is used as a stable fluorescent probe. The probe reaction with peroxyl radicals and causes a loss of fluorescence over time (Prior and others 2005). The ORAC assay follows the reactions of the antioxidants over an extended time (> 30 minutes). This time period helps accounts for possible effects of secondary antioxidant products.

There are some limitations to using the ORAC assay. The assay as described above is only capable of measuring hydrophilic antioxidants. This test does not take into account lipophilic antioxidants. The ORAC assay can be changed to account for hydrophilic and lipophilic antioxidants, but that method was not used for this experiment. Another limitation is that the ORAC assay reaction is temperature sensitive, so the temperature must be controlled and kept at 37°C (Prior and others 2005).

There are benefits though to using the ORAC assay. The ORAC assay gives a controllable source of peroxyl radicals that mimic reactions of antioxidants in food and physiological systems. The assay is also easily automated through micro-plate readers. A machine called the Fluostar Optima can be used to run the ORAC assay. The Fluostar Optima is a micro-plate

reader that can perform a multitude of applications for fluorescence intensity, time-resolved fluorescence, and absorbance.

DPPH

1,1-diphenyl-2-picrylhydrazly (DPPH) is a stable free radical that can be used to estimate the activity of antioxidants by measuring their scavenging ability (Sánchez-Moreno 2002 and Molyneux 2003). When DPPH, a purple radical, comes in contact with antioxidants, it receives a proton and converts to a light yellow/colorless protonated DPPH molecule. Through this color change, the free-radical scavenging ability can be determined through spectrophotometry (Shahidi and Wanasundara 1997).

The reduction of the DPPH in the presence of phenolic compounds is a popular assay among food scientists. The DPPH has a stable absorbance over a wide pH range and resists oxidation (Nenadis and Tsimidou 2002). This assay is commonly used because it is a stable, simple, and easily reproduced (Katsube and others 2004).

The radical form of DPPH has an absorption band around 515 nm, which disappears when it is reduced by an antiradical compound (Brand-Williams and others 1994). The reactions of the DPPH assays are based on the decrease of the absorbance of the radical solution. The reaction is: DPPH + HA → DPPH-H + A (Nenadis and Tsimidou 2002).

Folin-Ciocalteu

The Folin-Ciocalteu reagent is used in the colorimetric assay of phenolic and polyphenol antioxidants. This reagent measures the amount of the

substance being tested that is needed to inhibit the oxidation of the reagent (Singleton and others 1999, Vinson and others 2005). This reagent will also react with any reducing substance, so the reagent measures the total reducing capacity of a sample, not only the phenolic compounds (Ikawa and others 2003). In the assay, total polyphenols are measured by the Folin-Ciocalteu oxidation-reduction colorimetric method, with gallic acid as a standard (American Chemical Society 1999). The total phenolic content is then expressed in Gallic Acid Equivalents (GAE) (Kahkonen and others 1999). The Folin-Ciocalteu assay is not a complete assessment of the quantity and quality of the phenolic compounds in extracts, which is why other antioxidant assays are used (Singleton and Rossi 1965).

Gallic Acid

Gallic acid (GA 3,4,5-trihydroxybenzoic acid) is a naturally occurring polyphenol that is found in plants such as gallnuts, sumac, tea leaves, grapes, strawberries, and lemons (Senapathy and others 2011). As with other antioxidants, gallic acid has been found to have cytotoxicity against cancer cells, and anti-inflammatory and antimutagenic properties (Senapathy and others 2011, Lu and others 2006). Gallic acid is used as a food additive to help prevent oxidation (Lu and others 2006). In the United States, the average intake of gallic acid is about 1 g/day and toxicological studies have been conducted that show the “no-observed-adverse-effect-level” (NOAEL) of gallic acid is at least 120mg/kg/day for rats (Lu and others 2006).

Polyphenols

Over the past years, many researchers and food manufacturers have become more interested in polyphenols. This interest stems from polyphenol's antioxidant properties and the data showing they may play a role in the prevention of a variety of diseases such as cancer, cardiovascular, and neurodegenerative diseases (Manach and others 2004). There are thousands of molecules that have a polyphenol structure, which is characterized as having several hydroxyl groups on aromatic rings. The polyphenol compounds can be classified into different groups based on the number of phenol rings and the structural elements that bind the rings together (Manach and others 2004). One of the classes, flavonoids consists of two aromatic rings that are bound together by three carbon atoms. These carbon atoms form an oxygenated heterocycle, which can be further divided into subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanins, and flavanols (catechins and procyanthocyanidins (Manach and others 2004).

Flavanol compounds exist in monomer forms (catechins/epicatechins) and polymer forms (procyanthocyanidins). Catechins (two isomers in the trans configuration) and epicatechin (two isomers in the cis configuration) are the main flavonols found in fruits, and epicatechins have been found to be very stable in heat as long as the pH was acidic (Zhu and others 1997). Proanthocyanidins, or condensed tannins, are dimmers, oligomers, and polymers of catechins that are bound together between C4 and C8 (Guyot and others 1998). These

proanthocyanidins are responsible for the bitterness in chocolate (Santos-Buelga and Scalbert 2000).

Antioxidant Content in Cocoa

Cocoa is known to contain a large amount of flavonoids (Adamson and others 1999) and many studies have been conducted that look at the phenolic compounds in more detail. A study, which looked at the phenolic and anthocyanin content in cocoa beans, found that phenolic compounds ranged from 67 mg of Epicatechin Equivalents (ECE) to 149.2 mg of ECE in fresh picked beans to 102.3 mg of ECE to 139.6 mg of ECE in beans that had been fermented for one day. In the same study, HPLC was used to isolate specific compounds in cocoa beans. The results showed that Catechins and Epicatechins were the predominant polyphenolic compounds. The compound Epicatechin made up 2% to 4% of the dry mass of defatted cocoa powder and Catechins made up 0.05% to 0.1% of the defatted cocoa powder (Niemenak and others 2006).

Another study was conducted that looked at the phenolic compounds and antioxidant capacity of cocoa powder, tea, and red wine. The study found that cocoa powder contains more total phenolics, with 611 mg of Gallic Acid Equivalents (GAE) and more flavonoids, with 564 mg of Epicatechin Equivalents (ECE) then black tea (124 mg of GAE and 34 mg ECE), green tea (165 mg of GAE and 47 mg ECE), and red wine (340 mg of GAE and 163 mg of ECE). The researchers also found that cocoa powder had the highest overall antioxidant capacity as measured by Vitamin C Equivalent Antioxidant Capacity (VCEAC). Overall it was found that the antioxidant capacity of cocoa powder is four to five

times more powerful then black tea, two to three times more powerful then green tea, and two times more powerful then red wine (Lee and others 2003).

Differences Among Cocoa Beans from Different Countries

Many factors can account for the variances among cocoa beans from different countries. These factors such as climate, bean maturity, fermentation, and variety can contribute to the flavor and aroma of the final product. A study conducted by Zoumas and others found that the theobromine and caffeine content in cocoa liquor vary depending on the country of origin. Chocolate liquor from Haiti contains 1.26% theobromine and 0.105% Caffeine, for a 16.8 to 1 ratio of theobromine to caffeine ratio. Ecuador liquor contains 1.07% theobromine and 0.234% Caffeine, for a 4.6 to 1 ratio of theobromine to caffeine ratio. Dominican Republic liquor has 1.57% theobromine and 0.177% caffeine, for an 8.9 to 1 ratio of theobromine to caffeine ratio (Zoumas and others 1980). The variation in the Theobromine and caffeine content can be caused by difference in the maturity of the harvest cocoa bean, the degree of fermentation, and the genetic make-up of the specific cocoa beans. (Zoumas and others 1980)

Another study evaluated cocoa beans from Ecuador, Dominican Republic, Ivory Coast, and Indonesia on properties such as pH, and volatile and nonvolatile acids. After evaluation, Ecuador was classified as having a high pH (5.50 – 5.80), while Ivory Coast, Indonesia, and Dominican Republic beans were classified as having a medium pH (5.20 – 5.49). The study pointed out that even though Indonesia and Dominican Republic were classified as having a medium pH, the values had high mean standard deviation. This information leads

researchers to conclude that the cocoa beans from Dominican Republic and Indonesia went through a very short fermentation period (Jinap and Dimick 1990). The researchers also pointed out that Indonesia cocoa beans were less acidic and this could be contributed to the beans being washed before fermentation. Some cocoa growers in Indonesia practice this method (Forsyth and Quesnel 1957).

Intended Research

The intended research will study the effects of roasting time and temperature on the antioxidant capacity of cocoa beans from five different countries. The cocoa beans originate from the Dominican Republic, Ecuador, Haiti, Indonesia, and Ivory Coast. The optimal roasting time and temperature of will first be determined through trial runs with cocoa beans from Ivory Coast. The Ivory Coast beans will be roasted at different set temperatures and removed at specified times. The beans will then have their shells removed, leaving their nibs. The nibs will be crushed and the lipids will be extracted. The defatted nibs will be analyzed for antioxidants using ORAC, Total Phenolics Assay, and DPPH Assay. It is hypothesized that the optimal antioxidant capacity will be from cocoa beans roasted at the lowest time and temperature and that cocoa beans from the Ecuador will have the highest antioxidant capacity.

L* a* b* Values

The L* a* b* values were developed to give a standardized color scale that could be used to easily compare color values. The color scale is known as a

uniform color scale. In this color scale, the differences between points plotted in the color space correspond to visual differences between the colors plotted. In the scale the L* values range from zero to one hundred, where zero is black and 100 represents white, or a reflecting diffuser. The a* and b* can be positive or negative numbers, but do not have specific numerical ranges. The ranges depend of the color being measured. When a* is a positive number, it is measuring red/magenta. When b* is a positive number it is measuring yellow. When a* is a negative number it is measuring green and when b* is negative, it is measuring blue. The a* and b* values for each sample reading can be multiplied together to measure how brown in color a sample is (HunterLab 2008).

CHAPTER II
MATERIALS AND METHODS

Ivory Coast Cocoa Beans

The first objective of the experiment was to determine an optimal roasting time and temperature of cocoa beans from the Ivory Coast. The Ivory Coast cocoa beans were roasted at different times and temperatures and then the roasted cocoa beans were analyzed for antioxidants by ORAC assay, Total Phenolics Assay, and DPPH assay. Through statistical analysis, the optimal roasting time and temperature was determined and then applied to the second objective of the experiment.

Processing

Sample Collection

Unroasted Cocoa beans were selected based on their quality. Samples of cocoa beans were chosen that were free of holes and cracks in the shell, were a good color, and were not flat. Once the cocoa beans were chosen from the large sample bags they were sorted into groups for roasting and these groups were separated into three reps for roasting. Each rep, for each roasting time and temperature, contained 20 g of cocoa beans.

Roasting Procedure

The cocoa beans were then placed in between two mesh trays. These trays allowed for air to circulate around the cocoa beans, but prevented them from moving around in the oven. The cocoa beans were placed in a convection oven (Blodgett, Ann Arbor MI) that was preheated to the appropriate roasting

temperature, either 100°C, 130°C, 160°C, or 190°C. The temperature within the oven was measured to assure the correct temperatures were obtained. The beans were then placed in the oven and left to roast for 10, 20, 30, or 40 minutes. The oven door was only opened once to remove the beans. This ensured that heat was not loss during the roasting process. Every roasting time and temperature was repeated two more times for a total of three replications. The average temperature in the oven at the different roasting temperatures can be found in figure 1 of the appendix.

When the beans were done roasting at each time and temperature, they were removed from the oven and left to cool at ambient temperature (20°C - 25°C). Once the beans were cool, they were weighed again in order to measure the moisture loss. The average moisture loss for Ivory Coast cocoa beans roasted at different times and temperatures can be found in table 1 of the appendix. After the weight had been recorded, the beans were placed in freezer bags and labeled with the oven temperature, roasting time, and rep. The cocoa beans were placed into a freezer and kept at -2°C until needed.

Removing the Shell

In order for the cocoa beans to be analyzed, the shell was removed from the nib as described. The cocoa beans were removed from the freezer and placed on a wooden cutting board. The beans were then gently tapped with a rolling pin, which cracked the shell open. The shell was easily removed and discarded. If there were any shell pieces left in the nib pile, the nibs were winnowed, and the light shell was blown away. The nib was then crushed on the

cutting board with the rolling pin. The crushed nibs were stored in the freezer and kept at -2°C until needed for analysis.

Lipid Extraction

The lipid extraction performed on the cocoa nibs was adapted from a procedure by Adamson et al (1999). The previously crushed cocoa nibs were weighed with a digital scale (Denver Instrument) and the nibs were transferred to a 150 mL beaker. Next, forty-five milliliters of hexane (Fisher Scientific, Fair Lawn, NJ) was added to the glass beaker, which contained the cocoa nibs. The beaker was placed on a stir plate with a magnetic stir bar, and mixed on high for one-hour under the fume hood. The hexane was poured out and the cocoa nibs were drained over Miracloth™ (Calbiochem La Jolla, CA). The cocoa nibs were added back into the beaker, and another 45 mL of hexane was added and the process was repeated two more times. The cocoa nibs were then left to air dry for at least 24 hours. Once the cocoa nibs were completely dried, they were reweighed and the percent of lipid loss was calculated. If less than 20% of the lipids had been removed, the cocoa nibs were extracted with hexane two more times to ensure proper lipid removal. The defatted cocoa nibs were stored in a clean vial (Greiner Bio-One, Frickenhausen), which was labeled with the time, temperature, and rep, and was stored in a -2°C freezer until it was analyzed.

Procyanidin Fraction

After lipid extraction was complete, the procyanidin fraction was collected according to the procedure of Adamson et al (1999). One gram of defatted and

dried cocoa nib was placed in a 25 mL beaker and 7 mL of methanol (Fisher Scientific Fair Lawn, NJ), 2.95 mL of deionized water, and 0.05 mL formic acid (Acros Organics, New Jersey) was added to the breaker for a 70:29.5:0.5 ratio. The beaker with the solution was placed on a stir plate along with a magnetic stir bar, and mixed for two hours at room temperature underneath a fume hood. After two hours, the liquid portion was drained through Miracloth™ and poured into a 50 mL centrifuge tube (Fisher Scientific Suwanee, GA). The liquid was centrifuged (Sorvall RC 5B Plus, Duluth, GA) at 5000 rpm for 10 minutes. The supernatant was taken up by a plastic pipette and deposited in a clean vial. The vial of supernatant was stored in a -2°C freezer until it was analyzed for ORAC using a Fluostar Optima (BMG Labtech, Durham, NC).

Phenolics Fraction

The phenolic fractions were collected as described by Folin and Ciocalteu with slight modifications (Folin and Ciocalteu 1927). One gram of defatted and dried cocoa nib was placed in a 25 mL beaker and extracted with 5 mL of ethanol. The beaker was placed on the stir plate, with a magnetic stir bar, and mixed for one hour. After one hour, the solution was poured over Miracloth™ into a graduated cylinder and the ethanol volume was measured. If it was less than the original five milliliters, more ethanol was added to bring all of the solutions to the same original volume. The ethanol solution was then poured into a 50 mL centrifuge tube and centrifuged at 3000 rpm for 15 minutes. The supernatant was collected with a plastic pipette and deposited in a clean vial. The vial was

labeled with the roasting time, temperature, and rep, and was stored in a -2°C freezer until it was analyzed.

Chemical Reagents

Phosphate Buffer

To make the phosphate buffer solution, 10.21g of KH_2PO_4 (Acros Organics, New Jersey) was added to a 1000 mL volumetric flask. The flask was filled to volume (1000mL) with DI water and the solution was placed on a stir plate with a magnetic stir bar, and mixed until the monopotassium phosphate was dissolved. This produced a 75 mM monopotassium phosphate solution. Next, 13.06g of K_2HPO_4 (Fisher Scientific, Fair Lawn, NJ) was added to a 1000mL volumetric flask. The flask was filled to volume (1000mL) with DI water and the solution was placed on a stir plate with a magnetic stir bar and mixed until the dipotassium phosphate was dissolved. This produced a 75 mM dipotassium phosphate solution.

To make the phosphate working solution, 800mL of the 75 mM dipotassium phosphate solution was added to a 1000mL beaker. The beaker was placed on a stir plate with a magnetic stir bar in the beaker and set to stir on low. A pH electrode (Fisher Scientific) was placed into the beaker and the monopotassium phosphate solution was slowly added to the 75 mM dipotassium phosphate solution (about 200 mL) until a pH of 7.4 was reached. The buffer solution was stored in an amber glass bottle at 2.7°C (37° F) until it was ready to be used. The write up for the phosphate buffer can be found in the appendix on page 91.

Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid)

The Trolox solution was prepared by adding 25 mg of Trolox (Acros Organics, New Jersey) to 100 mL of working phosphate buffer. The solution was then mixed on a stir plate, using a magnetic stir bar until the Trolox was dissolved. This gave a 1mM solution of Trolox. The solution was then diluted with 100mL of working phosphate buffer (0.05mM Trolox). The final solution was divided into 1.5 mL Eppendorf tubes, with 1.5 mL of solution being added to each. The solution was stored at -85°C until it was needed for the ORAC analysis.

When the Trolox solution was ready to use for the ORAC assay, one tube was removed from the freezer and left to thaw at room temperature. The Trolox solution was used to make a Trolox working solution, which served as the standards for the ORAC assay. To make the Trolox working solution, 1 mL of Trolox solution was removed from the tube and added to a 25 mL flask that contained 9mL of phosphate buffer to make a 50 μm solution. Two milliliters were then removed from the first flask and added to a 10 mL flask that contained 2 mL of phosphate buffer to make a 25 μm solution. Two milliliters were then removed from the second flask and added to a 10 mL flask that contained 2 mL of phosphate buffer to make a 12.5 μm solution. Two milliliters were then removed from the third flask and added to a 10 mL flask that contained 2 mL of phosphate buffer to make a 6.25 μm solution. The write-up for the Trolox solution can be found in the appendix on page 93.

Fluorescein Solution

The Fluorescein stock solution was prepared by adding 22.5 mg of Fluorescein (Acros Organics, New Jersey) to 50 mL of working phosphate buffer. The solution was then mixed on a stir plate, using a magnetic stir bar. Fifty micro-liters of the Fluorescein solution and ten milliliters of phosphate buffer (75 mM) were put into a 10 mL centrifuge tube and vortexed to make the final stock solution. Then, 1.5 mL of the final stock solution was placed into 1.8 mL Eppendorf tubes and stored at -2° C until used.

The Fluorescein working solution was made fresh each day before running the ORAC analysis. A tube of Fluorescein stock solution was removed from the freezer and left to thaw at room temperature. Then, 800 μl of the stock solution was added to a 50 mL of the phosphate buffer. The solution was placed into a water bath at 37°C until needed. The write-up for the Fluorescein solution can be found in the appendix on page 92.

2, 2' –Azobis (2-amidino-propane) dihydrochloride (AAPH)

AAPH (Sigma-Aldrich, St Louis) solution was prepared right before each ORAC analysis. Right before the start of the ORAC assay, 0.108 g of AAPH (from the refrigerator at 4°C) was added to five milliliters of room temperature phosphate buffer. This produced an AAPH solution containing 79.6 μmol/mL. The 20μl of AAPH aliquot into each well gives a 1.6 μmol AAPH per micro-plate well. The write-up for the AAPH solution can be found in the appendix on page 94.

Gallic Acid

To make the Gallic Acid solution, 50 mg of Gallic Acid (Sigma Aldrich, St. Louis, MO) was added to a 100 mL volumetric flask and 3 mL of ethanol (Acros Organics, New Jersey) was added to aid in dissolving the solution. The flask was then filled to volume (100 mL) with DI water. The solution was poured into a storage bottle and stored in the refrigerator for up to nine days. This solution was used as the gallic acid standards for the Folin-Ciocalteau and DPPH Assays. The write-up for the Gallic Acid solution can be found in the appendix on page 95.

Folin-Ciocalteu

To make the Folin-Ciocalteu solution, 12.5 mL of 2N Folin-Ciocalteu (Sigma Aldrich St. Louis, MO) was added to a 100 mL volumetric flask. The flask was then filled to volume (100 mL). The solution was poured into a storage bottle and kept at room temperature (up to two months). The write-up for the Folin-Ciocalteu solution can be found in the appendix on page 96.

Sodium Carbonate

To make the Sodium Carbonate solution, 12.4 g of Na_2CO_3 (Fisher, Fair Lawns NJ) were dissolved into 100 mL of DI water. The solution was then poured into a storage bottle and kept at room temperature (up to two months) until it was used for the Folin-Ciocalteu assay. The write-up for the Sodium Carbonate can be found in the appendix on page 97.

1,1-diphenyl-2-picrylhydrazyl (DPPH)

The DPPH solution was prepared by dissolving 4mg of DPPH (Sigma Aldrich, St. Louis MO) in 100 mL of methanol. The solution was put on a stir

plate with a magnetic stir bar, and set to medium until the DPPH was dissolved in the methanol. The solution was poured into a storage bottle and kept in the refrigerator for up to two days. The write-up for the DPPH can be found in the appendix on page 98.

ORAC Assay Procedure

Procedure

Cocoa bean samples were analyzed based on hydrophilic oxygen radical absorbance capacity (H-ORAC). The procedure was based on the method of Prior and coworkers, with slight modifications (Prior and others 2005).

One sample of Trolox solution and one sample of Fluorescein solution were removed from the freezer the night before the ORAC analysis was run. On the day of the procedure, the cocoa samples were removed from the freezer. To start the procedure, the water bath was heated to 37°C. Next, the Fluostar Optima was turned on and the temperature control was set to 37°C. The Fluorescein working solution was made and stored in a 50 mL centrifuge tube and the centrifuge tube was place into the water bath that was now heated to 37°C. The Fluorescein working solution was kept in the water bath until it was ready to be used. The Fluorescein working solution procedure can be found in the appendix on page 92.

Next, the Trolox working solution was made. The Trolox working solution was diluted to make four solutions: 50 µm, 25 µm, 12.5 µm, and 6.25 µm. These dilutions were used as the Trolox standards. Twenty micro-liters of each

standard of Trolox were aliquoted into separate wells. The Trolox working solution procedure is can be found in the appendix on page 93.

Next, the cocoa sample that contained the procyanidin fraction was diluted to a 1:1000. Ten micro-liters of the procyanidin fraction were placed into 9.99 mL of phosphate buffer. Twenty micro-liters of the diluted procyanidin fraction were aliquoted into the micro-plate. Every cocoa sample was analyzed four times in the micro-plate. An example of the micro-plate can be found in figure 15 of the appendix.

Once all of the samples were in the micro-plate, the Fluorescein working solution was removed from the water bath and 200µl were placed into the top left well of the plate. The plate was then placed into the Fluostar Optima machine so it could warm up. While the plate was warming up, the AAPH solution was made. The Fluorescein working solution and AAPH solution were placed in the Fluostar Optima where they were used to prime pumps one and two, respectively. Pump one injected 200 µl of Fluorescein and pump two injected 20 µl of AAPH solution. An example of the micro-plate layout is found in figure 14 of the appendix.

Each of the cocoa samples were measured four times and the Trolox standards were measured two times. These measurements were used to produce the Fluorescent measurements at each cycle time (minutes). The 48-well micro-plates were analyzed in the Fluostar Optima fluorescence micro-plate reader, with two automated injectors, an incubator, and Fluostar Optima software

version 2.20R1 (BMG Lab Technologies, Inc. Durham, NC). A total of 32 cycles were run, and each cycle lasted 83 seconds, for a total run time of 84 minutes.

Calculations

ORAC calculations were based on previous work by Prior and coworker (Prior and others 2005). ORAC measurements are reported in Trolox equivalents to indicate the amount of Trolox required for the same number of antioxidants as the sample being measured. First, the appropriate wells of the micro-plate are filled with a phosphate buffer solution. Next, AAPH is added to the phosphate buffer, which will begin to decompose the phosphate buffer if no antioxidants are present. To determine the amount of phosphate that has decomposed, routine measurements at the emitted wavelength of the phosphate buffer solution are obtained. Samples that contain antioxidants retain the phosphate buffer solution for an extended period of time due to the protective properties of the antioxidants.

Figure 2 illustrates the measurements values taken directly from a Fluostar Optima for a sample containing antioxidants, buffer solution, and AAPH. To account for small differences in phosphate buffer concentrations from sample runs, all measurements are scaled relative to the maximum phosphate buffer measurements. In all cases, the maximum concentration of phosphate buffer occurs at the beginning of the procedure after the solution has been thoroughly mixed throughout the well and before the AAPH begins to decompose the buffer. With most sample runs, the buffer solution reaches its peak concentration around cycle four. After scaling, all measurements have values between 0 to 1, which

indicates the percent of phosphate buffer solution remaining in the well. Figure 3 illustrates the same sample as figure 2, but with scaled measurement values.

Scaling of the measurements values takes care of the small changes in phosphate buffer solution, but does not account for the small differences in the AAPH solution that is injected during in the runs. To normalize the samples between runs with the different concentrations of AAPH solution, several blanks are measured along with the samples. Each blank contains only phosphate buffer solution and AAPH and this value gives the amount of unprotected phosphate buffer that decomposes over time. Figure 5 shows the measurements taken for a sample with cocoa nibs in a phosphate buffer solution and measurements taken of a blank sample without any cocoa nibs. To determine the difference in phosphate decomposed with and without the antioxidants, the area under the blank's curve is subtracted from the area under the sample with antioxidants' curve. To determine the area under the curves, a series of trapezoids placed side-by-side are used. Figure 4 illustrates the area under a sample's curve separated into a series of trapezoids and the formula is shown in equation 1 of the appendix.

To determine the amount of Trolox Equivalents in the sample, the sample's fluorescent-minutes are compared to that of Trolox. Measurements using the same procedure as above were taken of several different Trolox concentrations. Figure 6 shows the fluorescent-minutes of several concentrations of Trolox. A linear regression line was fit to the data points to form a least-squares solution equation. That equation can then be used to

determine the exact amount of Trolox required for a given fluorescent-minutes value.

Data Analysis

The fluorescent-minute values and Trolox Equivalents were calculated in Microsoft Excel (Version 12.2.8). A completely randomized design, with 3 replicates per treatment was used to compare four roasting temperatures, with 4 roasting times. Trolox Equivalent values were analyzed by analysis of variance, which was conducted using mixed models and run with SAS 9.2. (2002-2008)

Folin-Ciocalteu Assay Procedure

Procedure

Before the Folin-Ciocalteu assay was run, the samples of phenolics fraction from the cocoa nibs were removed from the freezer. A water bath was heated to 40°C and the Folin-Ciocalteu solution, the Sodium Carbonate solution, and the gallic acid solution were made according to the procedures mentioned previously.

Four 10mL tubes were used to make the gallic acid standards. The first standard contained 200 µl of gallic acid solution, the second contained 500 µl of gallic acid solution, the third 1000µl of gallic acid solution, and the fourth 2000µl of gallic acid solution. The tubes were then filled to volume (10mL) with D.I. water. These concentrations produced standards with 0.01 mg/mL Gallic Acid Equivalents, 0.025 mg/mL GAE, 0.05 mg/mL GAE, and 0.1 mg/mL GAE

respectively. Once the standard concentrations were made, they were placed to the side until further use.

The test tubes were then prepared for the Folin-Ciocalteu Assay. Four test tubes were labeled S1, S2, S3, and S4 and used for the gallic acid standards. These four test tubes each received one milliliter of 200 µl (S1), 500 µl (S2), 1000 µl (S3), or 2000µl (S4) of gallic acid standard. Other test tubes were labeled with the sample being tested and received 200µl of the respective phenolics fraction. Then seven milliliters of D.I. water and then one milliliter of Folin-Ciocalteu solution were added to each tube. The tubes were left to sit for three minutes and then one milliliter of Sodium Carbonate was added to the tube. The tubes were vortexed and put in a water bath that was heated to 40°C and left for 30 minutes.

While the test tubes were heating in the water bath, the Spectrophotometer (Thermo Spectronic, Genesys 2) was turned on and set to 725 nm. After 30 minutes, the samples were removed from the water bath. The samples were then poured into cuvettes and read at an absorbency of 725 nm.

Calculations

Total Phenolic values were reported as Gallic Acid Equivalents (GAE). To calculate GAE the sample's gallic acid concentration needed to be found from the linear regression line of the standard gallic acid concentrations. The gallic acid standards and their absorbance values were plotted and a linear regression line was found. The absorbance values of the samples were plugged into the equation and used to solve for the gallic acid concentration. The value from the

linear regression line was used in the following equation for C, the sample gallic acid concentration.

Gallic Acid = C*V*D/W

C= the sample gallic acid concentration from the linear regression analysis.

V= The final volume of the sample that was prepared for analysis (mL)

D= The dilution factor of the sample preparation.

W= Weight of the sample being analyzed (mg)

Data Analysis

The Gallic Acid Equivalent values were calculated in Microsoft Excel (Version 12.2.8). A completely randomized design, with three replicates per treatment was used to compare four roasting temperatures, with four roasting times. Gallic Acid Equivalent values were analyzed by analysis of variance, which was conducted using mixed models using SAS 9.2 (2002-2008).

DPPH Assay Procedure

Procedure

Before the DPPH Assay was run, the samples of phenolics fraction, from the cocoa nibs, were removed from the freezer and the DPPH solution and gallic acid solutions were made according to the procedures mentioned previously.

Three 10 mL tubes were used as the gallic acid standards. The first standard contained 10 µl of gallic acid, the second standard contained 25 µl of gallic acid, the third 50 µl of gallic acid. The tubes were then filled to volume (10 mL) with D.I. water. These concentrations produced standards with 0.0025

mg/mL GAE, 0.00625 mg/mL GAE, and 0.0125 mg/mL GAE respectively**.** Once the standard concentrations were made, they were placed to the side until further use.

Test tubes were then prepared for the DPPH Assay. Three test tubes were labeled with an S1, S2, or S3 and were used for the gallic acid standards that were to be run in the spectrophotometer. One milliliter was removed from the test tube with 0.0025 mg/mL GAE and added to a new test tube labeled S1. This was repeated for the 0.00625 mg/mL GAE, and 0.0125 mg/mL GAE, which were labeled S2 and S3 respectively. Blank test tubes were then labeled with the sample that was being analyzed. The test tubes for the cocoa samples received 500 µl of the phenolics fraction of the cocoa samples that was being tested. Then one milliliter of DPPH was added to each test tube. The test tubes were wrapped in foil and a stir bar was placed into each. The test tubes were then placed on a stir plate and mixed at room temperature for 30 minutes. While the samples were mixing, the spectrophotometer was set to 517 nm. When the samples were done mixing, the solutions from the test tubes were poured into cuvettes and the absorbency was read at 517 nm. This procedure was repeated two more times, for an average of three values for each rep.

Calculations

DPPH values were reported as Gallic Acid Equivalents (GAE). To calculate GAE, the sample's gallic acid concentration needed to be found from the linear regression line of the standard gallic acid concentrations. The gallic acid standards and their absorbance values were plotted and a linear regression

line was found. The absorbance values of the samples were plugged into the equation and used to solve for the gallic acid. The value from the linear regression line was used in the following equation for C, the sample gallic acid concentration.

Gallic Acid = C*V*D/W

C= the sample gallic acid concentration from the linear regression analysis.

V= The final volume of the sample that was prepared for analysis (mL)

D= The dilution factor of the sample preparation.

W= Weight of the sample being analyzed (mg)

Data Analysis

The Gallic Acid Equivalent values were calculated in Microsoft Excel (Version 12.2.8). A completely randomized design, with three replicates per treatment was used to compare four roasting temperatures, with four roasting times. Gallic Acid Equivalent values were analyzed by analysis of variance, which was conducted using mixed models using SAS 9.2 (2002-2008).

Cocoa Beans From Different Countries

The second objective was to determine the antioxidant capacity of cocoa beans from different countries. Once the applied roasting time and temperature was decided, the treatment was applied to cocoa beans from five different countries. The cocoa beans were from Dominican Republic, Ecuador, Indonesia, Haiti, and Ivory Coast. The cocoa beans were then analyzed for antioxidant capacity by the ORAC assay, Total Phenolics assay, and DPPH assay.

Sample Collection

Unroasted cocoa beans were selected based on their quality. For each country, samples of beans were chosen that were free of holes and cracks in the shell, were a good color, and were not flat. Once the cocoa beans were chosen from the large sample bags they were sorted into groups for roasting. These groups were separated into reps for roasting. Each rep, for each country, contained 50 g of cocoa beans that were then roasted in the oven.

L a* b* Values*

Before the cocoa beans were roasted, L* a* b* values were measured using a Hunter Miniscan Spectrophotometer. Twenty grams of unroasted cocoa beans from each country were separated into groups and the shells were removed. The nibs from the different countries were then broken into pieces and placed into separate sample cups. The sample cups were completely filled with nib, making sure the center of the nib was placed face up for analysis.

The sample cup, with the cocoa nibs from the specific country, was placed under the Spectrophotomter and the L* a* b* values were measured. This process was repeated two more times for a total of three reps from a country and the process was repeated for each country.

Roasting

The cocoa beans were roasted in the same convection oven and on the same mesh trays as previously mentioned. Once the oven was preheated to 130°C, the beans from the different countries were placed in the oven and left to roast for 30 minutes. The oven door was only opened once during each roasting,

to remove the cocoa beans. This ensured that heat was not loss during the roasting process. The roasting time and temperature was repeated two more times for each country, for a total of three reps. The average temperature in the oven for each country can be found in figure 10 of the appendix.

When the beans were done roasting, they were removed from the oven and left to cool. Once the beans were cool, they were weighed again in order to measure the moisture loss. The average moisture loss for the cocoa beans from Dominican Republic, Ecuador, Indonesia, Haiti, and Ivory Coast roasted at different times and temperatures is seen in table 5 of the appendix. After the weight had been recorded, they were labeled with the country or origin, and rep, and placed into a freezer, kept at -2°C, until needed.

In order for the cocoa beans to be analyzed for antioxidants the shell needed to be removed from the nib. The nib was removed the same was as previously described. The nib was then crushed on the cutting board with the rolling pin. The nibs were stored in the freezer, and kept at -2°C until further analyzed.

Lipid Extraction

The lipid extraction was performed the same way as previously described.

Procyanidin Fraction

The procyanidin fraction was performed the same way as previously described. After the procyanidin fraction was collect, the vial was labeled with the country, rep and was stored in a -2°C freezer until it was analyzed.

Phenolic Fraction

The phenolic fractions were collected as previously described. After the phenolics were collected from the sample, the vial was labeled with the country, rep and was stored in a -2°C freezer until it was analyzed.

Chemical Reagents

All of the chemical reagents were made as previously described in the first material and methods section.

ORAC Assay Procedure

Procedure

Cocoa bean samples were analyzed based on hydrophilic oxygen radical absorbance capacity (H-ORAC). The procedure was performed as previously described and was based on the method of Prior and coworkers with slight modifications (Prior and others 2005).

Calculations

The calculations were performed as previously described in the first material and methods.

Data Analysis

The fluorescent-minute values and Trolox Equivalents were calculated in Microsoft Excel (Version 12.2.8). A completely randomized design, with three replicates per treatment, was used to compare five countries. Trolox Equivalent values were analyzed by analysis of variance, which was conducted using mixed models and run with SAS 9.2. (2002-2008)

Folin-Ciocalteu Assay Procedure

Procedure

The Folin-Ciocalteu procedure was performed as previously described in the first material and methods.

Calculations

The Gallic Acid Equivalence was calculated as previously described in the first material and methods.

Data Analysis

The Gallic Acid Equivalent values were calculated in Microsoft Excel (Version 12.2.8). A completely randomized design, with three replicates per treatment, was used to compare five countries. Gallic Acid Equivalent values were analyzed by analysis of variance, which was conducted using mixed models using SAS 9.2 (2002-2008).

DPPH Assay Procedure

Procedure

The DPPH assay was performed as previously described in the first material and methods section.

Calculations

The Gallic Acid Equivalent values were calculated in Microsoft Excel (Version 12.2.8). A completely randomized design, with three replicates per treatment, was used to compare five countries. Gallic Acid Equivalent values were analyzed by analysis of variance, which was conducted using mixed models using SAS 9.2 (2002-2008).

Data Analysis

The Gallic Acid Equivalent values were calculated in Microsoft Excel (Version 12.2.8). The values were then analyzed using SAS 9.2 (SAS 2002-2008).

CHAPTER III
RESULTS AND DISCUSSION

Effects of Roasting Ivory Coast Cocoa Beans at Different Times and Temperatures on the Antioxidant Capacity

ORAC

Roasted Ivory Coast cocoa beans had Trolox Equivalent (TE) values that ranged from a low of 38,402 TE µmol/g to a high of 533,349 TE µmol/g. Figure 7 and table 2 show the TE values as determined by Oxygen Radical Absorbance Capacity (ORAC). The cocoa beans roasted at 100°C for 40 minutes had the highest measured TE value of 533,349 TE µmol/g. This value was statistically the same as cocoa beans roasted at 100°C 10 minutes, 20 minutes, and 130°C 30 minutes. The cocoa beans roasted at 190°C for 40 minutes had the lowest Trolox Equivalent value of 38,402 TE µmol/g. This value was statistically the same as cocoa beans roasted at 190°C 20 minutes and 30 minutes.

Cocoa beans roasted at 130°C for 30 minutes were chosen as the roasting temperature and time to apply to cocoa beans from different countries. The cocoa beans roasted at 130°C for 30 minutes had a TE value of 522,789 TE µmol/g. Since this value was statistically the same as the highest ORAC value, the higher roasting temperature of 130°C was selected over the roasting temperature of 100°C. The lower temperature would usually not be used since it may not develop the flavor characteristics typical for cocoa powder.

R^2 values are used to indicate how well the linear regression line fits the original data points. The R^2 values from the ORAC standard curves ranged from 0.968 to 0.998 indicating that the linear regression was the best fitting model.

Different ORAC values were expected for the different heat treatments because heat can be used to create antioxidants and also destroy them if the temperature becomes too high. The ORAC values varied considerably across the different temperatures and they did not react in the same way (i.e. some started high and decreased, while others slowly increased with the time in the oven).

Overall the highest antioxidant values were reached when cocoa beans were roasted at 100°C for 40 minutes, which was the lowest temperature that cocoa beans were roasted. Previously discussed literature roasted cocoa beans at the low temperatures of 70°C, 110°C, 120°C and 182°C. So, 100°C was a low temperature, especially compared to 182°C.

Flavor was not evaluated during this research, but in the future, research can be conducted to determine if the characteristic flavors and aromas of chocolate are still developed during a low roasting temperature for a long time (40 minutes). If so, more antioxidants can be available to consumers.

The Trolox Equivalent values for the cocoa beans roasted at the varying temperatures and times did not have a uniform decrease or increase as shown in figure 7. The initial heating temperature was more than 20°C below the roasting temperatures due to the opening of the door to place the beans into the oven. This variation in temperature may have caused some of the variation in the ORAC values. The cocoa beans roasted at 100°C and 190°C have TE values that are statistically the same at 10 minutes at a 10% significance level.

There were many different factors that needed to be considered when running the ORAC assay. First, the instrument is known to be temperature sensitive. The temperature is automatically set in the instrument, but the machine is constantly adjusting to keep the correct temperature. The Fluorescein and AAPH solutions were made before each ORAC assay, so there was room for human error when the solutions were being diluted with phosphate buffer. Also, the blank that were used to calculate the net AUC were taken as an average and applied across the samples. For future research, the blanks should be calculated with each sample because the first readings of the phosphate buffer are dependent on the first injection of Fluorescein and APPH into the wells.

Total Phenolics

When Total Phenolics Assay was used to calculate the Gallic Acid Equivalents (GAE) for Ivory Coast cocoa beans roasted at different times and temperatures, the values ranged from 1.60 mg/L GAE up to 5.45 mg/L GAE. Cocoa beans roasted at 100°C for 40 minutes had the highest antioxidants detected, with 5.45 mg/L GAE. The Cocoa beans roasted at 190°C for 20 minutes had the lowest value with 1.60 mg/L GAE. Figure 8 and table 3 show the GAE values as determined by Total Phenolics assay.

Cocoa beans roasted at 100°C for 40 minutes were statistically the same as 100°C 10 minutes, 30 minutes, 130°C 10 minutes, 20 minutes, 160°C 20 minutes, and 190°C 10 minutes at a 10% significance level. Cocoa beans roasted at 190°C for 20 minutes were statistically the same as 100°C 10 minutes,

20 minutes, 30 minutes, 130°C 10 minutes, 30 minutes, 40 minutes, 160°C 10 minutes, 20 minutes, 30 minutes, 40 minutes, and 190°C for 20 minutes, 30 minutes, and 40 minutes at a 10% significance level.

The gallic acid equivalent of cocoa beans roasted at 130°C for 30 minutes was 2.49 mg/L GAE. These cocoa beans were statistically the same as cocoa beans roasted at 100°C 10, 20, 30 minutes, 130°C, 160°C, and 190°C for 20, 30, and 40 minutes.

This assay had data that was similar to the ORAC assay data. The Total Phenolics assay found the highest amount of antioxidants in the same sample (100°C for 40 minutes) and the lowest amount of antioxidants in the same sample (190°C for 20 minutes). The data that was statistically similar did vary though. This could be explained by the same values that were used for the Total Phenolics assay, so more differences were found versus the large values from the ORAC assay.

This assay measures the amount of substance being tested that is needed to inhibit the oxidation of the reagent. The Folin-Ciocalteu reagent that was used measures the total reducing capacity of a sample. In addition to polyphenols, the Total Phenolic assay also determines other reducing compounds. These other compounds react to determine the overall antioxidant capacity. So, overall the Total Phenolic assay is able to detect more antioxidants then the DPPH assay, which could account for the higher gallic acid equivalents values. This assay is not a complete assessment of the quantity of antioxidants, which is why other assays, such as ORAC, are used.

DPPH

When DPPH Assay was used to calculate the Gallic Acid Equivalents (GAE) for Ivory Coast cocoa beans roasted at different times and temperatures, the values ranged from 0.520 mg/L GAE up to 1.50 mg/L GAE. Figure 9 and table 4 show the GAE values as determined by DPPH assay.

Cocoa beans roasted at 100°C for 30 minutes had the highest antioxidant concentration with 1.50 mg/L GAE and cocoa beans roasted at 190°C for 20 minutes had the lowest antioxidant concentration with 0.502 mg/L GAE. Statistically, cocoa beans roasted at 100°C, 130°C, and 160°C for 10 minutes, or 20 minutes and cocoa beans roasted at 190°C for 10 minutes and 30 minutes were all statistically the same at a 10% significance level.

For the lowest antioxidant concentration (0.520 mg/L GAE), cocoa beans roasted at 160°C for 30 minutes and 40 minutes, and cocoa beans roasted at 190°C for 20 minutes and 40 minutes were all statistically the same at a 10% significance level.

The gallic acid equivalent of cocoa beans roasted at 130°C for 30 minutes was 1.48 mg/L GAE. These cocoa beans were statistically the same as cocoa beans roasted at 100°C, 130°C, and 160°C for 10 minutes, 20 minutes, 30 minutes, and 190°C for 10 minutes and 30 minutes.

The DPPH assay had data that was similar to the ORAC assay data, but did not find the same high concentrations of antioxidants. The assay found the lowest concentration of antioxidants in the same sample (190°C for 20 minutes), but DPPH found 100°C for 30 minutes had the highest concentration of

antioxidants. This data was statistically the same at a 10% significance level as 100°C 40 minutes, which is what the ORAC assay determined was the highest concentration of antioxidants.

The DPPH assay showed lower gallic acid equivalent values then the Total Phenolics assay. The cocoa beans roasted at the lowest temperatures, 100°C and 130°C had the highest gallic acid equivalent values and were statistically the same at a 10% significance level. A study by Lee et al. found that DPPH might under estimate antioxidant capacity as compared to other assays. These low antioxidant estimates are thought be from interruptions in the absorbance by other compounds.

Effects of Roasting Time and Temperature on Cocoa Beans from Different Countries

L a* b* Values*

When L* was measured the values ranged from a low of 17.66 (Ivory Coast) to a high of 21.69 (Dominican Republic). Cocoa beans from Dominican Republic had statistically different L* values then cocoa beans from Ecuador, Indonesia, Haiti, and Ivory Coast. This statistically different L*, which was higher than the other countries, means that the cocoa beans from the Dominican Republic were closer to white on the L* a* b* scale

The a* values ranged from a low of 4.80 (Haiti) to a high of 7.36 (Indonesia). When a* was measured, cocoa beans from Dominican Republic and Ivory Coast were statistically the same as every other country. Cocoa beans from Indonesia were statistically the same as Ecuador, Dominican Republic and

Ivory Coast. Haiti was statistically the same as Ivory Coast and Dominican Republic. All of the a* values were positive which meant the cocoa beans had more of a red/magenta color to them. This red/magenta could have been caused by some of the purple beans, which were less fermented then others. Since the highest a* value was from Indonesia, the Indonesia cocoa nibs had the most magenta color to them according to the Hunter Spectrophotometer.

The b* values ranged from a low of 3.54 (Haiti) to a high of 7.27 (Indonesia). When b* was measured, cocoa beans from Haiti, Dominican Republic, and Ecuador were statistically the same at a 10% significance level. For the highest value, Indonesia, Ecuador, and Ivory Coast were statistically the same at a 10% significance level. All of the b* values were positive which meant they were more of a yellow color instead of blue. Indonesia had the highest b* value, so it had the most yellow color detected out of all of the countries. Haiti had the lowest b* value, so it had the least amount of yellow color when compared to the countries.

The a* and b* values were multiplied together to get an a*b* value that represented how brown the sample was. The values ranged from a low of 17.35 (Haiti) to a high of 53.80 (Indonesia). The cocoa beans from Haiti had the lowest value and were therefore the lightest brown. Cocoa beans from Indonesia had the highest value and were the darkest brown. At a 10% significance level, cocoa beans from Ecuador, Ivory Coast, and Indonesia were statistically the same. Cocoa beans from Dominican Republic, Ecuador, Haiti, and Ivory Coast

were also statistically the same at a 10% significance level. The average L* a* b* values can be found in table 6 of the appendix

ORAC

When Oxygen Radical Absorbance Capacity Assay was used to calculate the Trolox Equivalents for cocoa bean from different countries roasted at 130°C for 30 minute, the ORAC assay found that Trolox Equivalent values ranged from 190,423 TE μmol/g to 487,913 TE μmol/g. Cocoa beans from Dominican Republic had the highest Trolox Equivalent values reported and the data showed that cocoa beans from Dominican Republic were statistically the same as Ecuador. Ivory Coast had the lowest Trolox Equivalent value and was statistically different from all other countries. As previously mentioned, the ORAC assay is a measurement of total antioxidant capacity and it measures the antioxidant reactions to completion, which gives a better overall assessment of the quantity of antioxidants. Figure 11 and table 7 show the TE values as determined by Oxygen Radical Absorbance Capacity assay.

Total Phenolics

When Total Phenolics assay was used to calculate the Gallic Acid Equivalents for cocoa beans from different countries roasted at 130°C for 30 minutes, the values ranged from a low of 0.783 mg/L GAE to a high of 3.264 mg/L GAE. The cocoa beans from Haiti had the highest value of 3.264 mg/L GAE and the cocoa beans from Ivory Coast had the lowest value of 0.783 mg/L GAE.

Haiti, which had the highest value, was statistically different from all other countries. Cocoa beans from Dominican Republic and Indonesia were statistically the same at a 10% significance level. Ivory Coast and Ecuador were statistically the same at a 10% significance level.

The data from Total Phenolics assay showed the same results for the highest antioxidant capacity as the ORAC assay. The data found that cocoa beans from Haiti had the highest antioxidant capacity and the data was statistically different from all of the other countries. The Total Phenolics assay the found that Ivory Coast cocoa beans have the lowest antioxidant capacity. This data is different from the ORAC assay because the ORAC assay found that Ivory Coast cocoa beans were statistically the same as the highest antioxidant capacity found in Haiti and Dominican Republic. Figure 12 and table 8 show the GAE values as determined by Total Phenolics assay.

DPPH

When DPPH assay was used to calculate the Gallic Acid Equivalents for cocoa beans from different countries roasted at 130°C for 30 minutes, the values ranged from a low of 0.600 mg/L GAE to a high of 0.623 mg/L GAE. The cocoa beans from Indonesia had the lowest value of 0.600 mg/L GAE and the cocoa beans from Ivory Coast had the highest value of 0.623 mg/L GAE. Cocoa beans from Indonesia, Haiti, Ecuador, and Dominican Republic were statistically the same at a 10% significance level. Cocoa beans from Ivory Coast and Dominican Republic were statistically the same at 10% significance level.

The data from DPPH assay was slightly different from the Total Phenolics assay and ORAC assay. DPPH assay found Ivory Coast had the highest antioxidant capacity. For ORAC, the Ivory Coast cocoa beans reported the lowest antioxidant capacity, and were statistically different from all other countries. The Total Phenolics assay also found Ivory Coast to have the lowest antioxidant capacity. Figure 13 and table 9 show the GAE values as determined by DPPH assay.

CHAPTER IV
CONCLUSIONS AND RECOMMENDATIONS

The results and conclusions from this study can only hold true for the cocoa beans that were used for the experiment. Different growing conditions, fermentation practices, and storage conditions all affect the cocoa beans. Each of the cocoa beans from their respective countries were grown, cultivated, fermented, dried, and stored independently. Also, literature has shown that some countries such as Dominican Republic are known to under ferment their cocoa products. The cocoa beans were stored and roasted under similar conditions in the UT Food Science pilot plant. These different variables can all contribute to the total antioxidant capacity.

The research found that Ivory Coast cocoa beans roasted at 100°C for 40 minutes had the highest concentration of antioxidants when measured by the ORAC assay and Total Phenolics assay and cocoa beans roasted at 100°C for 20 minutes had the highest concentration of antioxidants when measured with DPPH assay. When cocoa beans from the different countries were measured, for antioxidants, the ORAC and Total Phenolics assay found that cocoa beans from Haiti had the highest concentration. The DPPH assay found that Indonesia had the highest antioxidant concentration. The differences of antioxidant capacity shown in the different countries can be results of many different factors.

Cocoa beans are plant products and contain natural polyphenol compounds, and specifically flavonoids, so antioxidants are present before any processing occurs. Cocoa beans are expected to show antioxidant capacity and

roasting may increase the antioxidant capacity through the process of Maillard reaction.

The antioxidants detected by the ORAC assay, Total Phenolic Assay, and DPPH assay, are thought to be in part from the Maillard reaction. The Maillard reaction occurs during high temperatures such as roasting and baking. During the Maillard reaction, aldoses and ketoses are heated along with amines, which cause reactions to occur. These reactions produce compounds, which are responsible for flavor, aroma, and color of cocoa. These compounds have been shown to possess antioxidant activity.

A possible reason for the increase in gallic acid equivalents for the Total Phenolics assay may be explained by a study conducted by Ikawa and coworkers (2003). This study showed that the Folin-Ciocalteu reagent reacts with some nitrogen compounds and can give higher antioxidant readings. The Maillard reaction requires amines to continue its process, so amines are present in roasted cocoa beans. These amines may add to the overall reducing capacity of the test.

Overall, the ORAC assay and Folin-Ciocalteu assay produced the most similar results for the antioxidant capacity of the different countries. The ORAC assay and the Folin-Ciocalteu assay are best used for predicting the overall antioxidant capacity of a sample. This could help explain why similar results were seen. The Folin-Ciocalteu assay is easier to run, takes less time, and requires fewer chemicals, then the ORAC assay, but the ORAC assay is more comprehensive and takes into account antioxidant reactions as they come to a

completion, instead of measuring them at a specific time in the spectrophotometer.

Future Research

For future research, it is suggested that more control be put into the cocoa bean process. The cocoa beans were given for the research experiment, but no history is known, except for the country of origin. More research can also be conducted that would look at the sensory properties of the different roasted cocoa beans. If research shows that the characteristic chocolate flavor and aroma is present in the cocoa beans roasted at low temperatures then greater antioxidant contents may be available in cocoa products.

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APPENDIX

Table 1: Average Moisture Loss[1] of Cocoa Beans from the Ivory Coast Roasted at Different Times and Temperatures

	Roasting Time (Minutes)			
Temperature	10	20	30	40
100°C	0.17	0.23	0.50	0.43
130°C	0.33	0.57	0.77	0.83
160°C	0.83	1.03	1.17	1.13
190°C	1.17	1.40	1.43	1.53

[1]Average Moisture Loss in grams

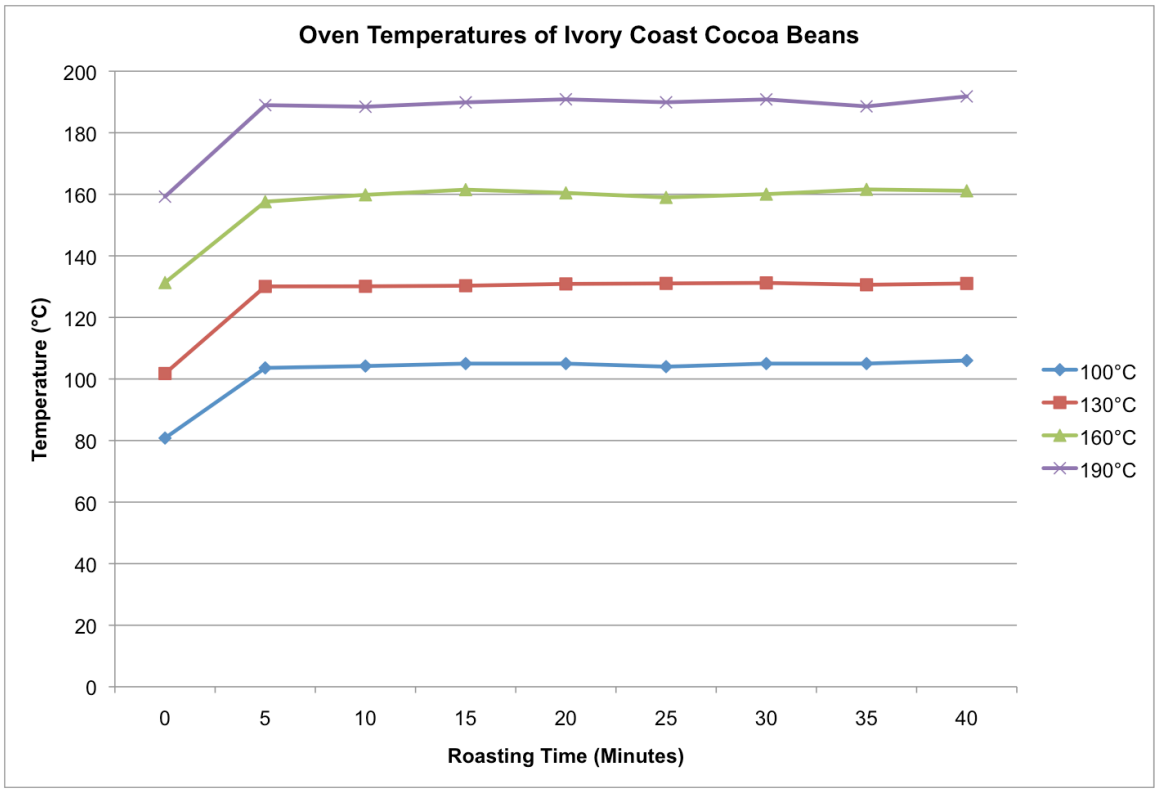

Figure 1: Convection Oven Temperatures of Ivory Coast Cocoa Beans Roasted at Different Times and Temperatures

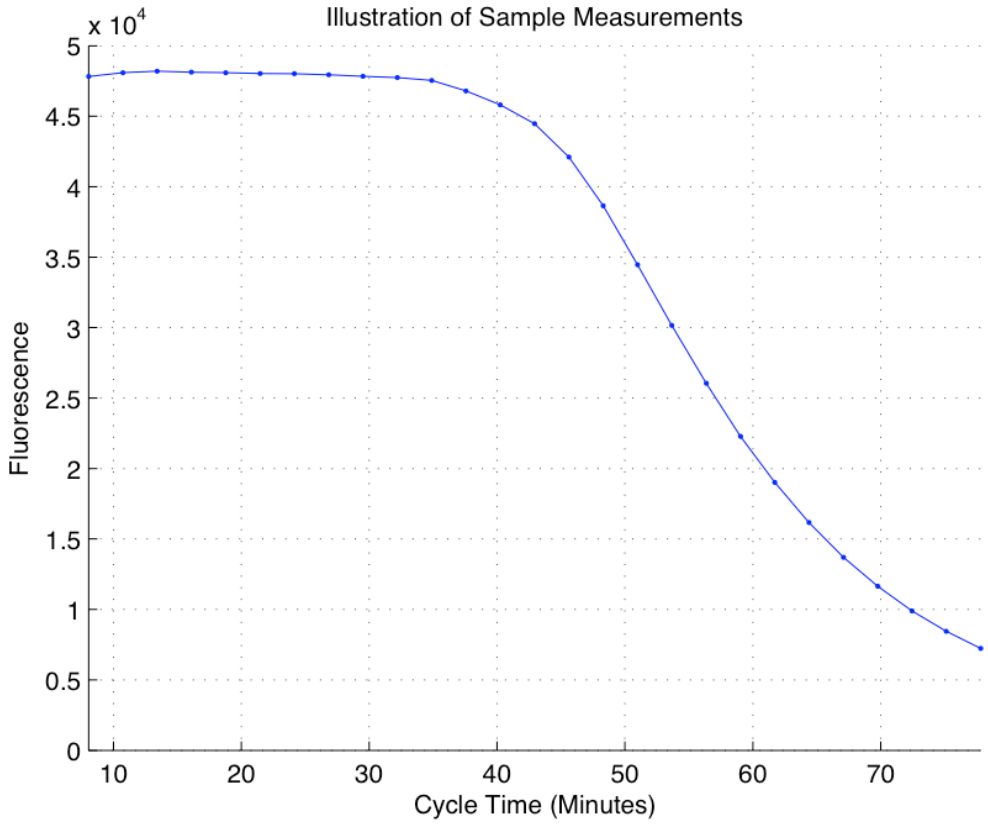

Figure 2: Example of Sample Measurements from ORAC Assay

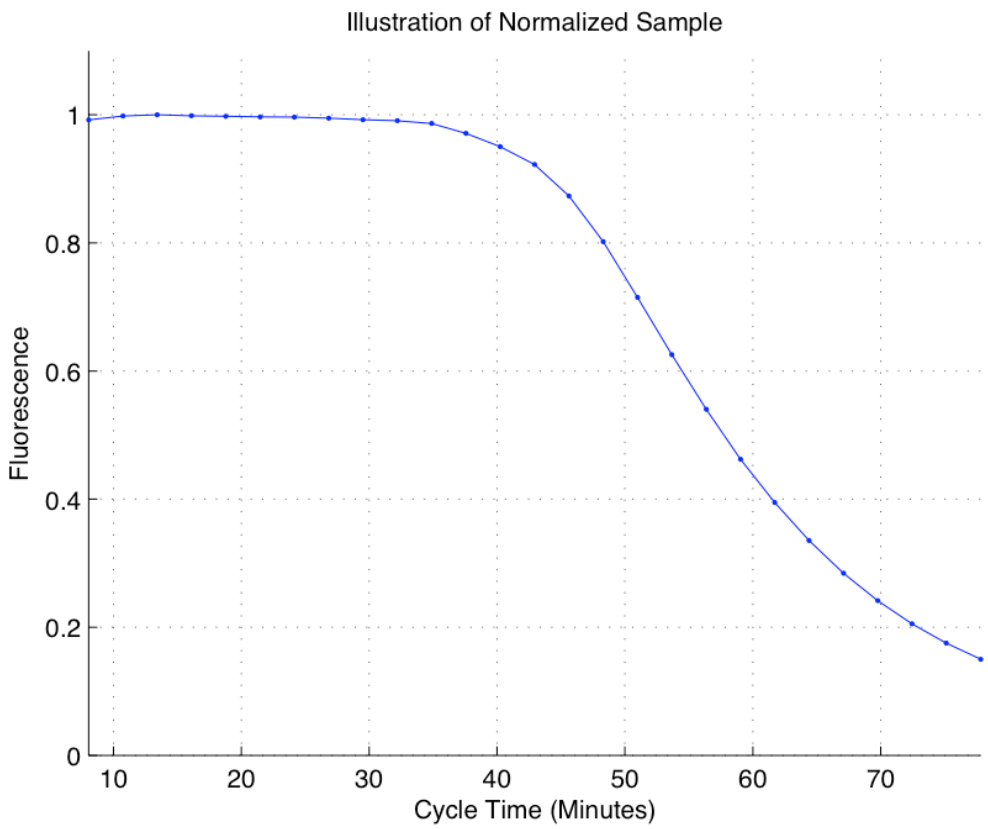

Figure 3: Example of Normalized Sample Measurements from ORAC assay

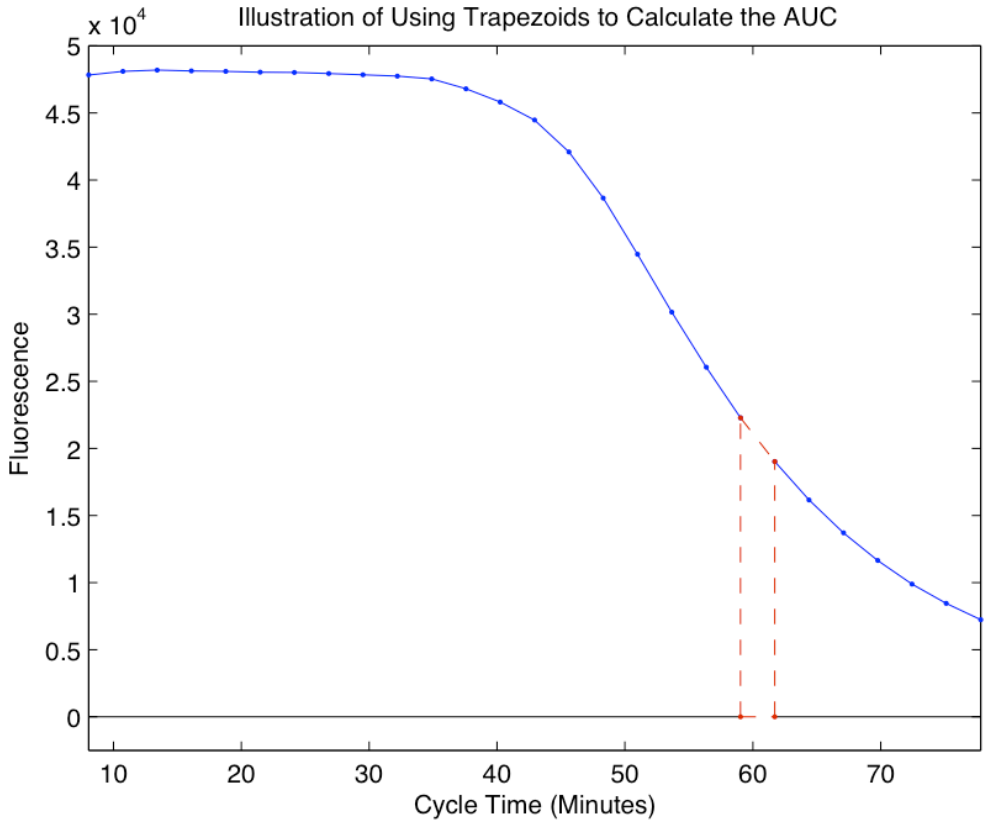

Figure 4: Example of a Trapezoid Used to Calculate AUC From ORAC Assay

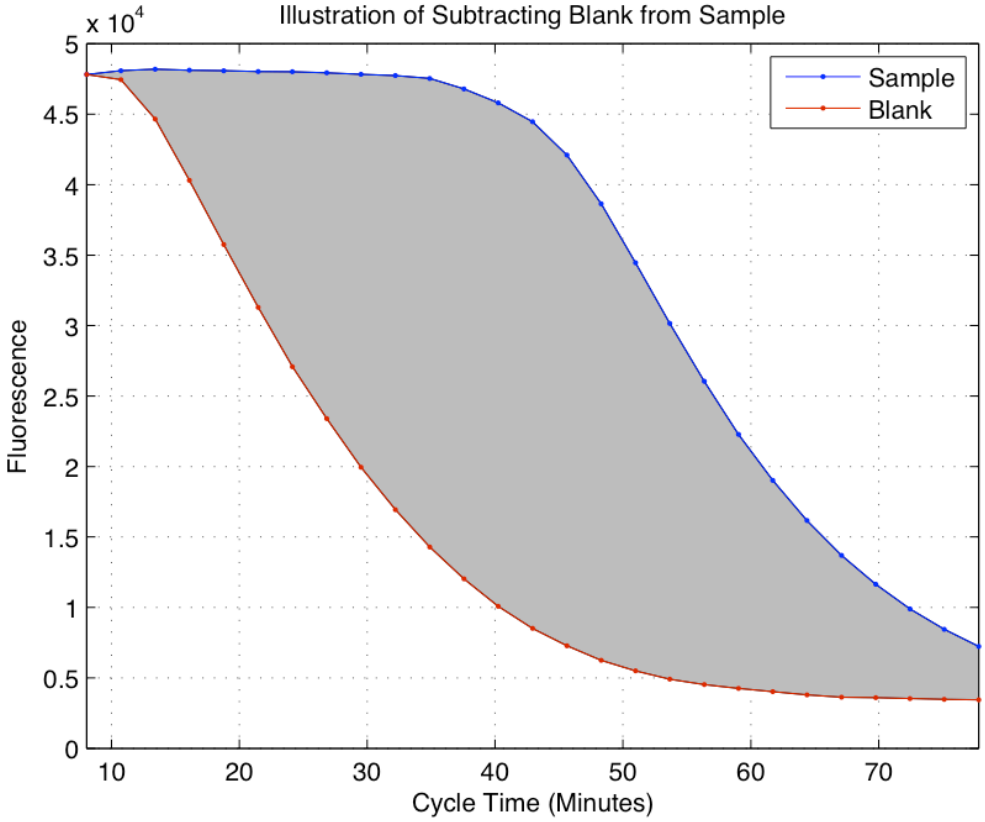

Figure 5: Example of Sample Measurements and Blank Measurements from ORAC Assay

Equation 1: Equation to Calculate AUC from ORAC Assay

$$AUC = [(f1/f1 + f2/f1)/2)*CT] + [(f2/f1 + f3/f1)/2)*CT] + [(f3/f1 + f4/f1)/2*CT] + \ldots [(fn-1/f1 + fn/f1)*CT$$

f = raw data of the fluorescent reading at the given time

CT = the cycle time

n = last data point.

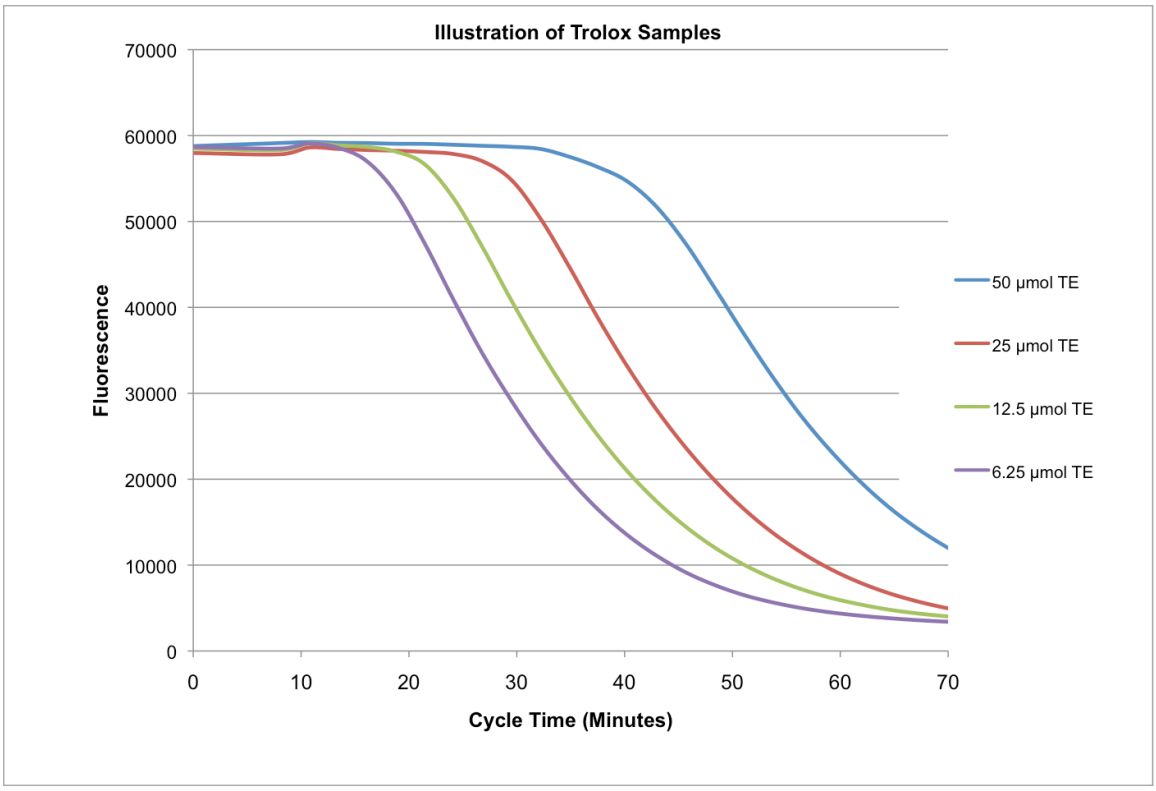

Figure 6: Example of Trolox Measurements from ORAC Assay

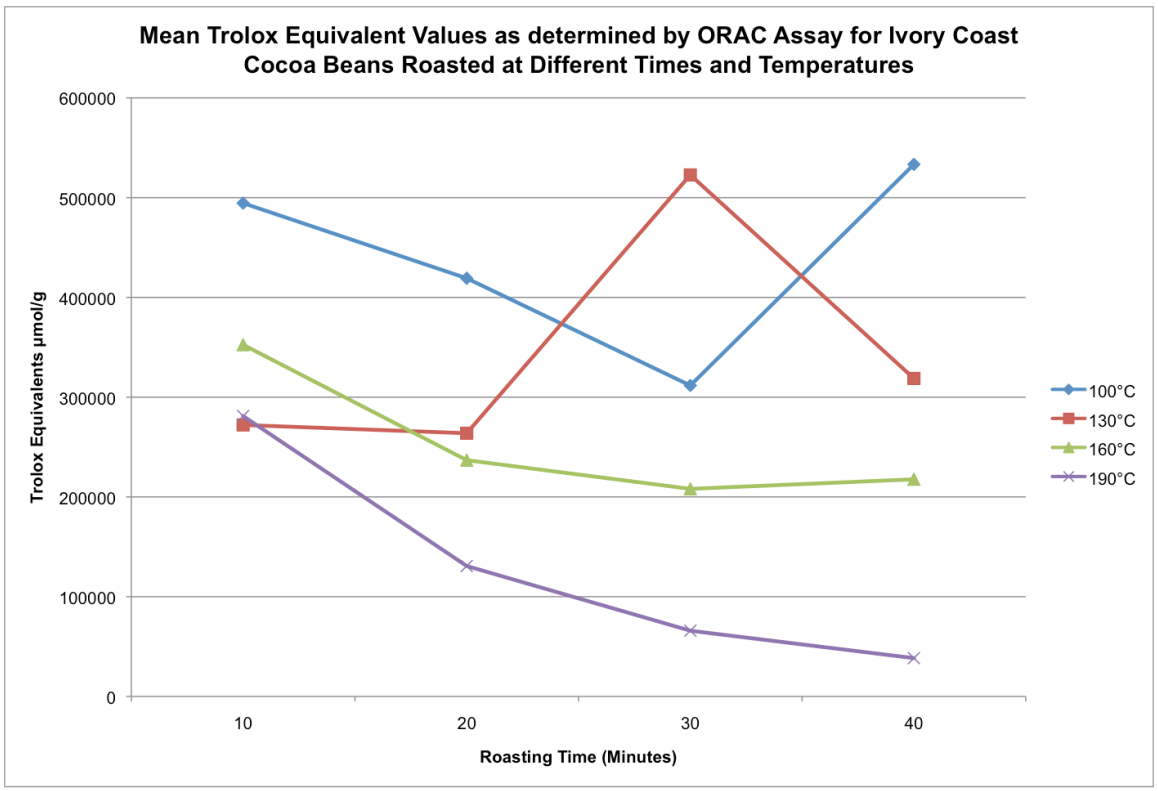

Figure 7: Mean Trolox Equivalent Values as determined by ORAC Assay for Ivory Coast Cocoa Beans Roasted at Different Times and Temperatures

Table 2: Mean[1] Trolox Equivalent[2] Values as determined by ORAC Assay[3] for Ivory Coast Cocoa Beans Roasted at Different Times and Temperatures

	Roasting Time (Minutes)			
Temperature	10	20	30	40
100°C	494516_{a}	419194_{ab}	311701_{bcd}	533349_{a}
130°C	272150_{cd}	263857_{cde}	522789_{a}	318775_{bcd}
160°C	352547_{bc}	236852_{cde}	208072_{de}	217694_{de}
190°C	281073_{cd}	130750_{ef}	65896_{f}	38402_{f}

[1] Means followed by the same letter are not significantly different from one another at $\alpha = 0.10$.
[2] Trolox Equivalents = µmol/g of defatted cocoa nib
[3] Oxygen Radical Absorbance Capacity
n= 3 replications, each measured 4 times.

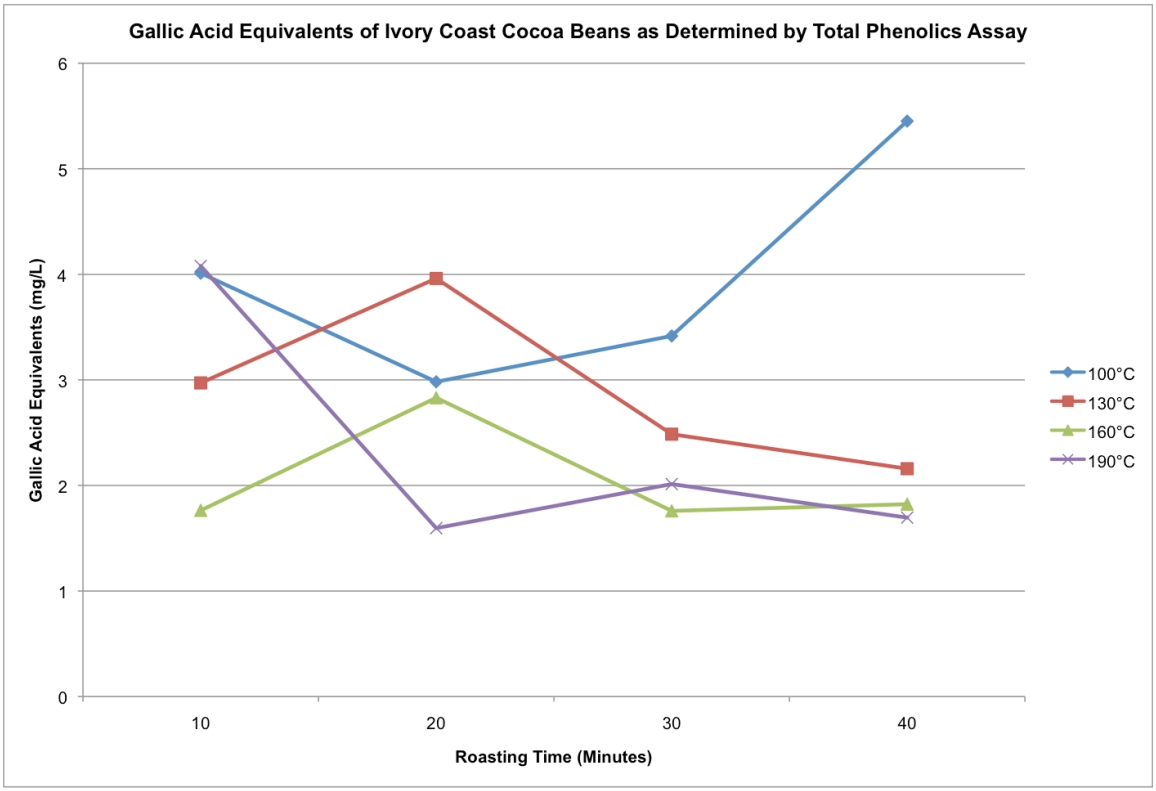

Figure 8: Mean Gallic Acid Equivalent[1] Values as determined by Total Phenolics Assay[2] for Ivory Coast Cocoa Beans Roasted at Different Times and Temperatures

[1] Gallic Acid Equivalents = mg/L
[2] Assay used to measure the total reducing capacity of a sample

Table 3: Mean[1] Gallic Acid Equivalent[2] Values as determined by Total Phenolics[3] Assay for Ivory Coast Cocoa Beans Roasted at Different Times and Temperatures

	Roasting Time (Minutes)			
Temperature	10	20	30	40
100°C	4.01_{abcd}	2.98_{bcd}	3.42_{abcd}	5.45_{a}
130°C	2.97_{abcd}	3.96_{abc}	2.49_{cd}	2.16_{bcd}
160°C	1.76_{d}	2.83_{abcd}	1.76_{d}	1.82_{d}
190°C	4.08_{ab}	1.60_{d}	2.01_{bcd}	1.70_{d}

[1] Means followed by the same letter are not significantly different from one another at α = 0.10.
[2] Gallic Acid Equivalents = mg/L
[3] Assay used to measure the total reducing capacity of a sample
n= 3 replications

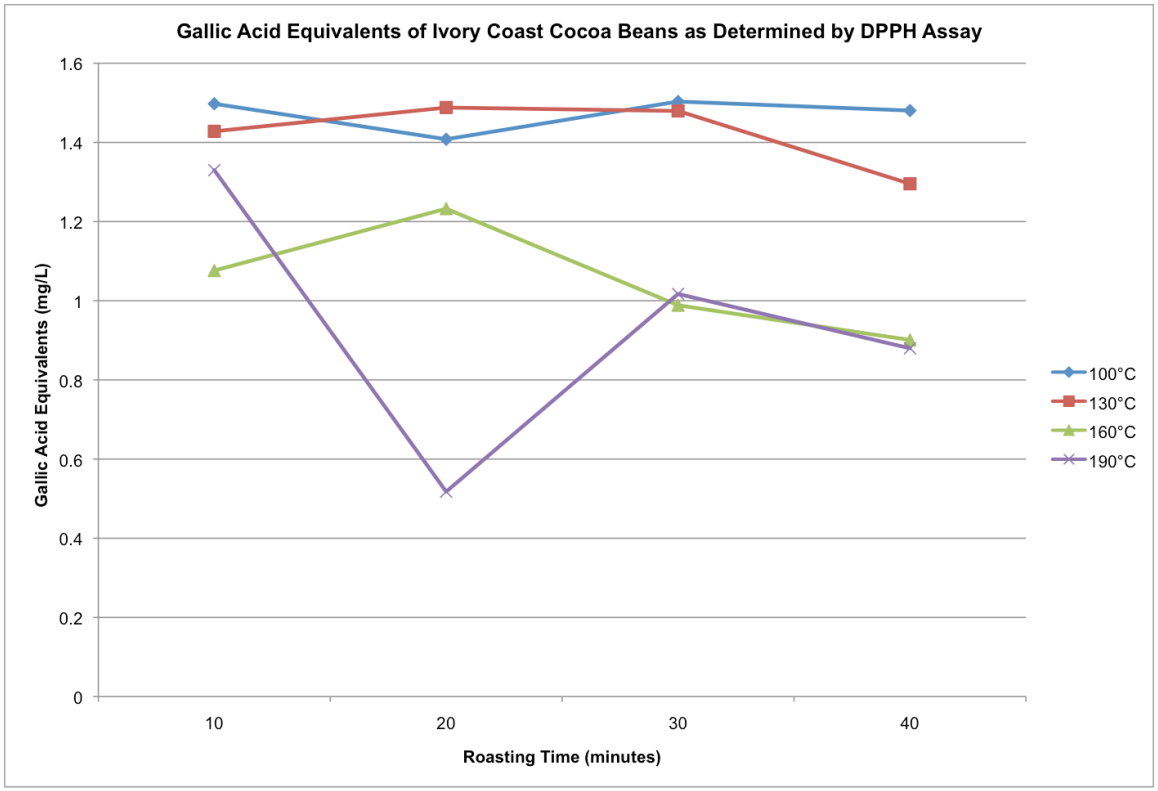

Figure 9: Mean Gallic Acid Equivalent Values[1] as determined by DPPH[2] Assay for Ivory Coast Cocoa Beans Roasted at Different Times and Temperatures

[1] Gallic Acid Equivalents = mg/L
[2] Assay used to measure the activity of antioxidants

Table 4: Mean[1] Gallic Acid Equivalent[2] Values as determined by DPPH[3] Assay for Ivory Coast Cocoa Beans Roasted at Different Times and Temperatures

	Roasting Time (Minutes)			
Temperature	10	20	30	40
100°C	1.50_{a}	1.41_{ab}	1.50_{a}	1.48_{ab}
130°C	1.43_{ab}	1.49_{a}	1.48_{ab}	1.30_{abc}
160°C	1.08_{abc}	1.23_{abc}	0.99_{bcd}	0.90_{cd}
190°C	1.33_{abc}	0.52_{d}	1.02_{abc}	0.88_{cd}

[1] Means followed by the same letter are not significantly different from one another at α = 0.10.
[2] Gallic Acid Equivalent = mg/L
[3] Assay used to measure the activity of antioxidants
n= 3 replications

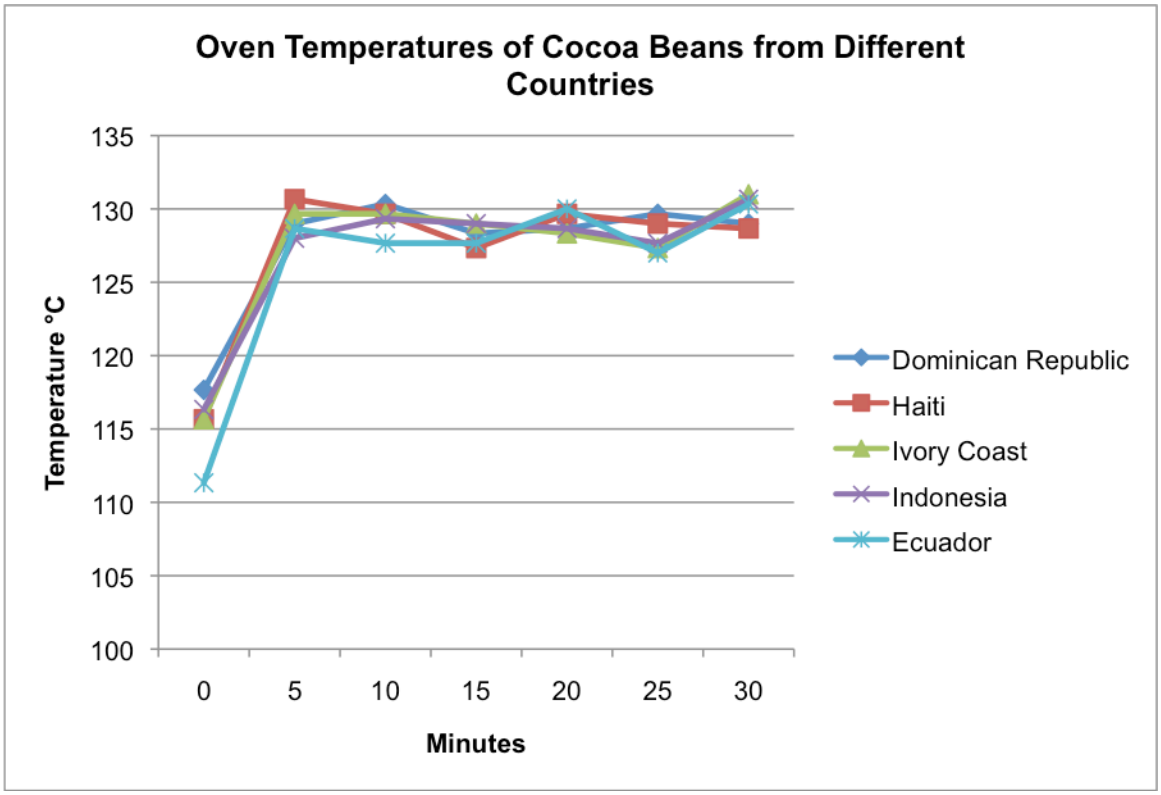

Figure 10: Average Convection Oven Temperatures of Cocoa Beans from Different Countries

Table 5: Average Moisture Loss[1] of Cocoa Beans From Dominican Republic, Ecuador, Indonesia, Haiti, and Ivory Coast roasted at 130°C for 30 minutes.

Countries				
Dominican Republic	Ecuador	Indonesia	Haiti	Ivory Coast
1.57	1.73	1.76	2.03	1.76

[1] Average Moisture Loss in grams

Table 6: Mean[1] L*, a*, b* Values of Unroasted Cocoa Beans from Dominican Republic, Ecuador, Indonesia, Haiti, and Ivory Coast.

Country	L*	a*	b*	ab*
Dominican Republic	21.69_{a}	5.98_{ab}	4.83_{bc}	29.80_{b}
Ecuador	18.96_{b}	6.65_{a}	5.57_{abc}	38.12_{ab}
Haiti	17.84_{b}	4.80_{b}	3.54_{c}	17.35_{b}
Indonesia	19.53_{b}	7.36_{a}	7.27_{a}	53.80_{a}
Ivory Coast	17.66_{b}	5.84_{ab}	5.81_{ab}	34.23_{ab}

[1]Means in the same column, followed by the same letters do not significantly differ at a 5% level of probability.
n=3 replications

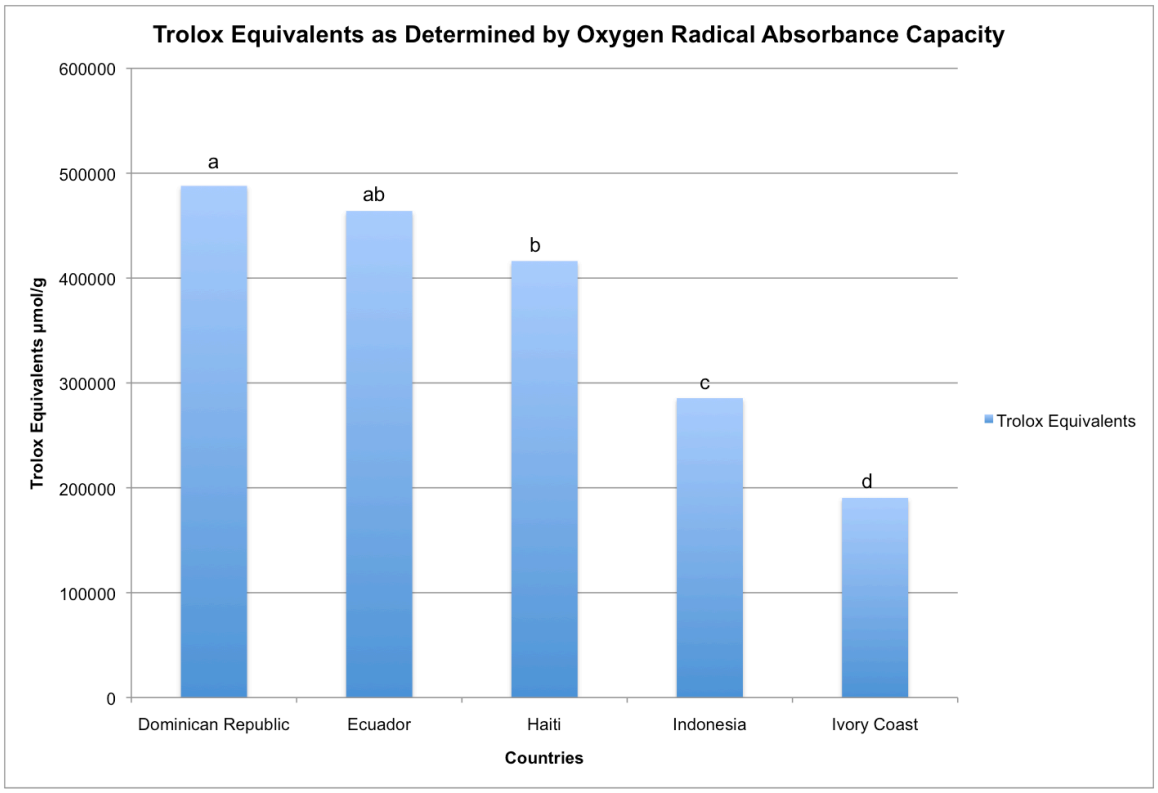

Figure 11: Mean[1] Trolox Equivalents[2] Values as Determined by ORAC Assay for Cocoa Beans Roasted at 130°C for 30 Minutes

[1]Means followed by the same letters do not significantly differ at a 10% level of probability.
[2] Trolox Equivalents = µmol TE/g of defatted cocoa nib

Table 7: Mean[1] Trolox Equivalents Values[2] of Cocoa Beans from Different Countries as Determined by ORAC Assay.

Countries	Dominican Republic	Ecuador	Haiti	Indonesia	Ivory Coast
µmol TE/g	487913_{a}	463958_{ab}	416251_{b}	285499_{c}	190423_{d}

[1] Means followed by the same letter are not significantly different from one another at α = 0.10.
[2] Trolox Equivalents = µmol TE/g of defatted cocoa nib
n= 3 replications measured 4 times each

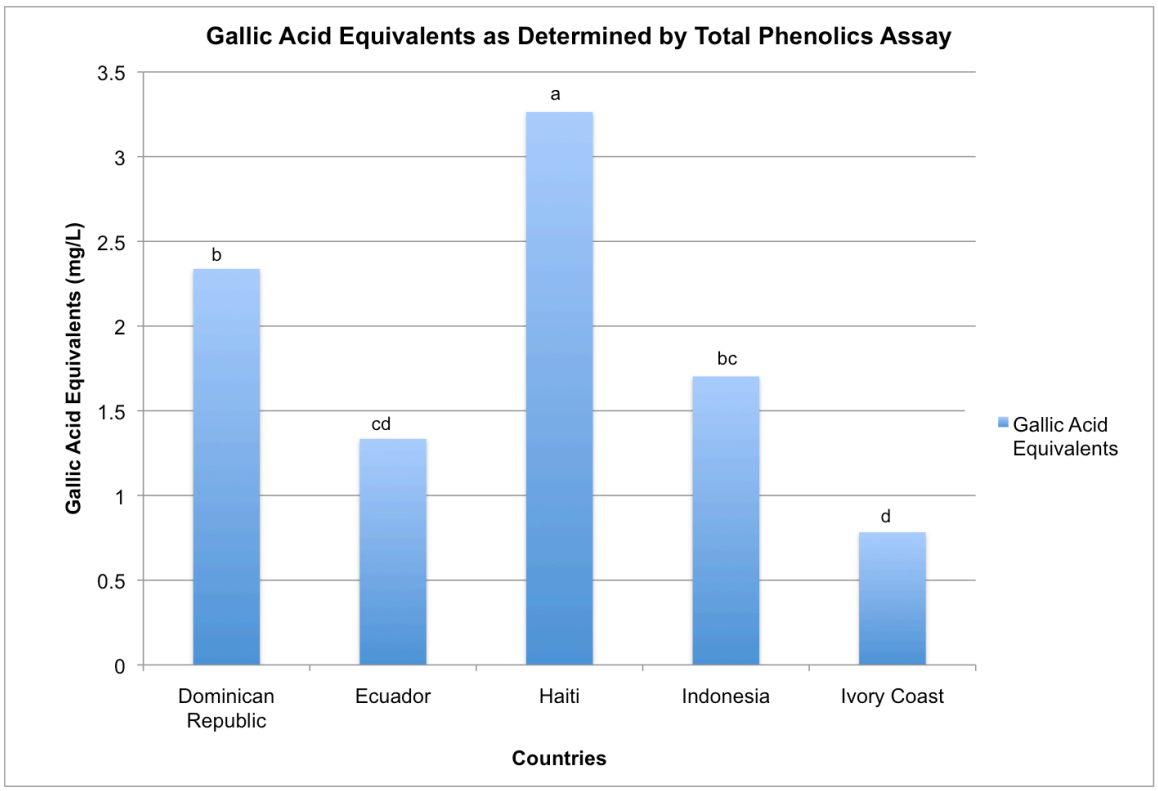

Figure 12: Mean[1] Gallic Acid Equivalent Values as determined by Total Phenolics Assay for Roasted Cocoa Beans From Dominican Republic, Ecuador, Haiti, Indonesia, and Ivory Coast.

[1] Means labeled as the same letter are not significantly different from one another at α = 0.10.

Table 8: Mean[1] Gallic Acid Equivalents[2] Values of Cocoa Beans From Different Countries as Determined by Total Phenolic[3] Assay

Countries				
Dominican Republic	Ecuador	Haiti	Indonesia	Ivory Coast
2.338_{b}	1.335_{cd}	3.264_{a}	1.703_{d}	0.783_{bc}

[1] Means followed by the same letter are not significantly different from one another at α = 0.10.

[1]Gallic Acid Equivalents = mg/L
[2] Assay used to measure the total reducing capacity of a sample
n= 3 replications

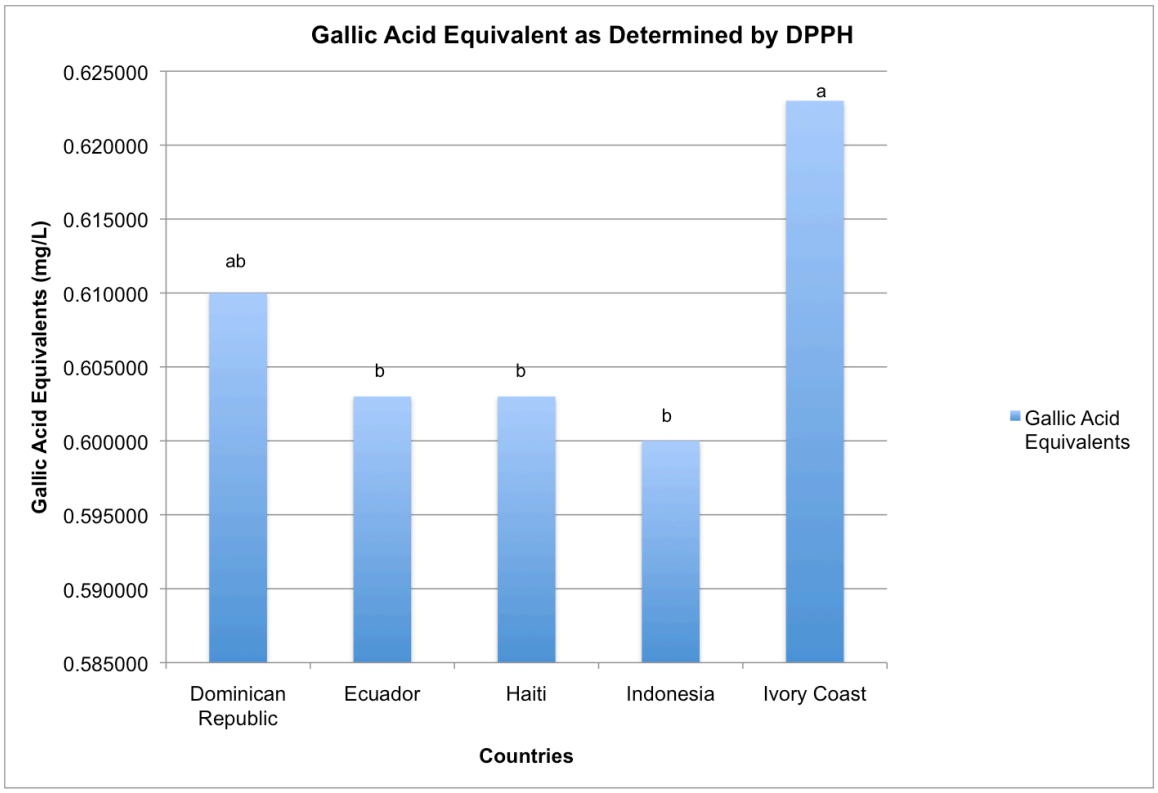

Figure 13: Mean[1] Gallic Acid Equivalent Values as determined by DPPH Assay for Roasted Cocoa Beans From Dominican Republic, Ecuador, Haiti, Indonesia, and Ivory Coast.

[1] Means labeled as the same letter are not significantly different from one another at α = 0.10.

Table 9: Mean[1] Gallic Acid Equivalent[2] Values of Cocoa Beans From Different Countries as Determined by DPPH[3] Assay

Countries				
Dominican Republic	Ecuador	Haiti	Indonesia	Ivory Coast
0.610_{ab}	0.603_{b}	0.603_{b}	0.600_{b}	0.623_{a}

[1] Means followed by the same letter are not significantly different from one another at α = 0.10.

[2] Gallic Acid Equivalents = mg/L
[3] Assay used to measure the activity of antioxidants
n= 3 replications

P	B	B	B	B	B	B	B
B	S1	X1	X5	X9	X13	S4	B
B	S2	X2	X6	X10	X14	S3	B
B	S3	X3	X7	X11	X15	S2	B
B	S4	X4	X8	X12	X16	S1	B
B	B	B	B	B	B	B	B

Figure 14: Micro-plate Layout for ORAC

P= Fluorescein Working Solution
B= Buffer
S1= 50 μm Trolox
S2= 25 μm Trolox
S3= 12.5 μm Trolox
S4= 6.25 μm Trolox

X1 to X4 = Sample 1
X5 to X8 = Sample 2
X9 to X12 = Sample 3
X13 to X16 = Sample 4

Phosphate Buffer Solution

Solution #1

1) Weigh out 10.21 g of Monopotassium Phosphate
2) Put into a 1000 mL flask
3) Add 700 mL of DI water and magnetic stir bar. Stir on a stir-plate until the phosphate is dissolved
4) Remove the stir bar and fill the flask with 300mL of DI water

Solution #2

1) Weigh out 13.06 g of Dipotassium Phosphate
2) Put into a 1000 mL flask
3) Add 700 mL of DI water and magnetic stir bar and stir on a stir plate until the phosphate is dissolved
4) Remove the stir bar and fill the flask with 300 mL of DI water

Buffer Solution

1) Pour 800 mL of Solution #2 in a 1000mL beaker
2) Put stir bar into the beaker and place on a stir plate
3) Start reading pH
4) Slowly pour about 200mL of Solution #1 into the beaker

Slowly add more solution #1 until the pH reads 7.4

Fluorescein Solution

1) Weigh out 0.0225g of Fluorescein
2) Pour 50 mL of Phosphate buffer into a 150mL beaker
3) Mix Fluorescein and phosphate buffer on a stir plate
4) Remove 50 μl (0.05 mL) a put in a centrifuge tube
5) Add 10 mL of Phosphate buffer to centrifuge tube and vortex
6) Aliquot 1.5 mL into Eppendorf tubes
7) Store Eppendorf tubes in -20°C until needed

Fluorescein Working Solution

1) Remove tubes from the freezer the night before
2) Turn on the water bath and heat to 37°C
3) Pipette 0.8 mL of Fluorescein in to 50 mL of phosphate buffer into a centrifuge tube
4) Keep the Fluorescein working solution warm until ready for use
5) Pipette 200 μl into first well before warming the plate

Trolox Standard

1) Weigh out 0.025 g of Trolox
2) Put Trolox in a 250 mL beaker
3) Pour 100 mL of Phosphate Buffer pH 7.4 in the beaker
4) Mix with a stir bar until Trolox is dissolved (1 mM Trolox)
5) Dilute with 100 mL of Phosphate Buffer (0.05 mM Trolox)
6) Aliquot 1.5 mL in 1.5 mL Eppendorf tubes
7) Store in the freezer chest (-85°C)

Trolox Working Solution:

1) Remove one tube of Trolox the night before
2) Take 1 mL of Trolox and add it to 9 mL of phosphate buffer = 50 μm (S1)
3) Remove 20 μl and aliquot into S1 position

4) Remove 2 mL from S1 and add 2 mL of phosphate buffer = 25 μm (S2)
5) Remove 20 μl and aliquot into S2 position

6) Remove 2 mL from S2 and add 2 mL of phosphate buffer = 12. 5μm (S3)
7) Remove 20 μl and aliquot into S3 position

8) Remove 2 mL from S3 and add 2mL of phosphate buffer = 6.25 μm (S4)
9) Remove 20 μl and aliquot into S4 position

AAPH Solution

Store AAPH in the refrigerator (4°C) until ready to use

1. Bring 5 mL of phosphate buffer to room temperature
2. Dissolve 0.108 g of AAPH into phosphate buffer and put into a centrifuge tube
3. Use the solution to prime pump two of the Fluostar Optima

Gallic Acid Write-Up

Store in the refrigerator for up to nine days

1. Measure out 0.05 g Gallic acid and put into a 100 mL volumetric flask
2. Add 3 mL of ethanol to dissolve the gallic acid
3. Fill the flask to volume with D.I water (100 mL)

Folin-Ciocalteu Write-Up

Will stay fresh for a few months

1. Pour 12.5 mL of 2N Folin-Ciocalteau into a 100 mL volumetric flask
2. Fill the flask to volume (100 mL) with DI water
3. Store the solution in an amber bottle.

Sodium Carbonate Write-Up

Will stay fresh for a few months

1. Dissolve 12.4 g of Na_2CO_3 in 100 mL of DI Water
2. Mix on a stir plate until everything is dissolved
3. Store in an amber bottle

DPPH Write-Up

Dissolve 0.004 g of DPPH in 100 mL of methanol

Cover the flask with foil and stir until dissolved

Keep the solution covered with foil to prevent light from getting to it

Definitions of Faults in cocoa beans detected by cut-test

Moldy Bean- A cocoa bean on the internal parts of which mold is visible to the naked.

Slaty Bean- A cocoa bean which shows a slaty color over half or more of the surface.

Insect Damaged Bean- A cocoa bean the internal parts of which contain insects at any stage of development, or have been attacked by insects which have caused damage visible to the naked eye.

Germinated Bean- A cocoa bean the shell of which has been pierced, slit, or broken by the growth of the seed germ.

Flat Bean- A cocoa bean of which two cotyledons are so thin that it is not possible to obtain a cotyledon surface by cutting.

VITA

Whitney Leigh Harrington was born in Roanoke Virginia on March 20th and is the daughter of Terry and Marcia. She grew up in Roanoke Virginia and attended Northside High School. After graduation, she attended Virginia Polytechnic Institute and State University (Virginia Tech) where she received her B.S. degree in Human Nutrition, Food, and Exercise in May 2009. In 2011, she graduated from University of Tennessee with a M.S. degree in Food Science and Technology.