TOTAL PHENOLICS AND ANTIOXIDANT CAPACITY OF COCOA PULP:
PROCESSING AND STORAGE STUDY

By

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ABSTRACT OF THE THESIS

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Cocoa pulp, the white mucilage that surrounds cocoa beans, is the main substrate in cocoa bean fermentation and is rich in macro- and micronutrients. Yet, the presence of phenolics and their antioxidant activity in cocoa pulp has not been explored. The objectives of this study were to quantify the total phenolics of cocoa pulp by using Folin-Ciocalteu assay and antioxidant activity by using Oxygen Radical Absorption Capacity (ORAC) assay as well as Cellular Antioxidant Activity (CAA) assay. The effect of single and double pasteurization was investigated to better understand the potential health value and subsequently promote the utilization of cocoa pulp. Eight weeks storage studies at 4°C, 25°C, and 37°C were also performed to monitor the stability of phenolics, ORAC values, and color of pasteurized cocoa pulp.

Single and double pasteurization decreased phenolics content of the cocoa pulp by 25% and 38%, respectively, when compared to unpasteurized pulp (103.76±4.79 mg

GAE/100 g pulp). The ORAC values were not significantly different between unpasteurized (1871 ± 58 $\mu\text{mol TE}/100$ g pulp), single (1835 ± 41 $\mu\text{mol TE}/100$ g pulp), and double pasteurized (1681 ± 157 $\mu\text{mol TE}/100$ g pulp) pulp. There was a significant increase in the CAA values (EC_{50}) of double pasteurized pulp (59.76 ± 9.26 $\mu\text{mol QE}/100$ g pulp) as compared to unpasteurized pulp (23.71 ± 3.12 $\mu\text{mol QE}/100$ g pulp). Thus, there was a weak negative correlation between ORAC and CAA values in quantifying the antioxidant activity of cocoa pulp. Overall color differences (ΔE^*) relative to unpasteurized pulp were not significantly different between single (11.50 ± 6.64) and double pasteurized pulp (14.96 ± 8.64).

Throughout the eight week storage studies, single and double pasteurized pulp showed very little change in the phenolics content and the ORAC values at 4°C and 25°C . However, at 37°C , up to 50% relative loss in phenolics content and 40% relative loss in the ORAC values were observed in single pasteurized pulp; whereas double pasteurization resulted in lesser relative loss. Both single and double pasteurized pulp showed the lowest lightness (L^*) values when stored at 37°C , as well as the lowest hue angle and chroma values when stored at 4°C , suggesting less color changes at lower storage temperature.

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I. LITERATURE REVIEW

I.1. Cocoa pulp

Cocoa pulp, as shown in **Figure I.1.b**, is a white mucilaginous layer, which firmly envelops individual seed of the fruit of *Theobroma cacao* plant (**Figure I.1.a**). It is formed during pod development from endocarp meristem and makes up approximately 40% of fresh seed weight (Biehl and Ziegleder, 2003).

Figure I.1 a) Cocoa fruits grow on the branches and trunk of cocoa tree (Salamone, 2010); b) Inside a cocoa pod, cocoa seeds are surrounded by white pulp. A cross section of a fresh cocoa seed was shown to show its purple color and a thin outer layer of adhering cocoa pulp (<http://www.dominican-republic-live.com/dominican-republic/beauty/the-cacao.html>).

I. 1.a. Composition of cocoa pulp

The constituents of Ivorian cocoa pulp that are summarized in **Table I.1-5** are good representation of cocoa pulp composition from all over the world. Cocoa pulp mainly consists of water, sugars, acids, and pectin (**Table I.1**). Sugars in cocoa pulp are mainly sucrose, fructose, and glucose (**Table I.2**). Pectin, which gives cocoa pulp a thick consistency, presents at approximately 1% on fresh weight basis (**Table I.3**). Citrate is the major organic acids, which inversely affected the pH of cocoa pulp. Other non-volatile organic acids such as malic, tartaric, and oxalic acids are less than 0.1% in cocoa pulp (Pettipher, 1986). The most abundant mineral is potassium (**Table I.4**), whereas the most abundant vitamin is ascorbic acid, which constitutes 97% of all vitamins present (**Table I.5**). The concentrations of those nutrients will vary as influenced by different cultivars of cocoa, ripeness as well as growing regions and subsequently, their climate. Because of the composition of cocoa pulp that is rich in macro- and micronutrients, it is a favorable medium for microbial growth and thus, a suitable substrate for cocoa bean fermentation. Having pH of 3.50-3.80, fresh cocoa pulp has a combination of sweet and mildly acidic taste with a note of tropical flavor, popular in cocoa-growing regions.

Table I.1 Composition of Ivorian cocoa pulp (Pettipher, 1986).

	g/ 100 g fresh pulp
Water	82.60
Mono- and disaccharides	11.15
Plant cell walls polymers	2.81
Citrate	1.31
Protein	0.74
Fat	0.45
Metals	0.24
Vitamins	0.05
Ethanol	0

Table I.2 Concentration of mono- and disaccharides of fresh Ivorian cocoa pulp (Pettipher, 1986).

	g/ 100 g fresh pulp
Sucrose	4.35
Fructose	3.80
Glucose	3.00

Table I.3 Concentration of pectin, cellulose, hemicellulose, and lignin in freeze-dried Ivorian cocoa pulp (Pettipher, 1986).

	g/ kg dry weight
Pectin	66.1
Cellulose	51.8
Hemicellulose	28.5
Lignin	15.0

Table I.4 Concentration of metal ions in freeze dried Ivorian cocoa pulp (Pettipher, 1986).

	mg/ kg dry weight
Potassium	12,000
Magnesium	800
Calcium	730
Iron	230
Sodium	40
Zinc	15
Copper	6
Manganese	5
Nickel	2

Table I.5 Concentration of vitamins in a composite sample freeze dried Ivorian cocoa pulp (Pettipher, 1986).

	µg/ 100 g dry weight
Ascorbic acid (C)	3.0×10^5
Pyridoxin (B ₆)	4.0×10^3
Niacin	2.6×10^3
Pantothenate	2.3×10^3
Riboflavin (B ₂)	250
Thiamine (B ₁)	220
Folic acid	95
Biotin	63
Cyanocobalamin (B ₁₂)	30

I.1.b. The role of cocoa pulp in cocoa bean fermentation

Commercially available cocoa products, such as chocolate bars, cocoa drinks, and chocolate treats, are made from cocoa powder that is derived from the seeds (beans) of *Theobroma cacao* plant. One major quality measure of those products is cocoa flavor, which is developed through a microbial fermentation of cocoa beans. In the old practice, cocoa fermentation was done to aid in removing the mucilaginous pulp and facilitate in drying for subsequent processing of the beans. However, it was later realized that during fermentation, microorganisms utilize cocoa pulp to induce biochemical reactions within the beans to form color, flavor, and aroma precursors, which determine the overall quality of finished cocoa products. Although, genetic factors of cocoa variety play a great influence in the quality of chocolate produced, yet flavor potentials can only be released by a proper curing process (Schwan and Wheals, 2004). Overall, fermentation process is indispensable for flavor development and thus, the presence of cocoa pulp is crucial for fermentation to proceed.

Cocoa fermentation is a batch process and usually lasts about 5-8 days. It begins spontaneously as the cocoa seeds are removed from the pods and the pulp is accidentally inoculated with different microorganisms from the air, hands of the workers, knives, surface of the containers used to transport the beans to the fermentary, or previously used plantain leaves that have remnants of dried mucilage (Lopez and Dimick, 1991). In general, fermentation process can be done in two ways; in heaps lined with plantain leaves or in wooden boxes, an improved fermentation method (Thompson et al., 2001). Fermentation process that utilizes wooden boxes (**Figure I.2**) is usually adapted by larger estates. It requires a fixed volume of cocoa beans in a specified box size to attain

optimum mass temperature as fermentation progresses. The containers, so called sweat boxes, can be a single unit or structured from a number of smaller compartments and arranged in tiers. The box is built above ground level, over a drain which collects the pulp sweating liberated from the mucilage degradation during fermentation. The wooden floor of the box has gaps which facilitate both drainage and aeration. Mixing is done by removing the dividing wall between the compartments and shoveling the beans into the adjacent box, or in the case of tier design, into the box below. The boxes are filled within 10 cm from the top and covered with banana leaves or jute sacking to retain the heat and prevent surface drying. As a guideline, 1 m³ sweat box holds about 600-700 kg of fresh cocoa beans with 2-3 mixings required during the fermentation.

Figure I.2 Fermentation of cocoa beans done in wooden boxes (Beckett, 2008).

During fermentation, yeasts, lactic acid bacteria, and acetic acid bacteria grow in the pulp in succession. Yeasts are predominant for the first 24-48 hours of fermentation due to favorable conditions of pH of 3.5-4.0, 10-12% sugar content, and a low oxygen concentration. A typical alcoholic fermentation takes place and citric acid is used up causing an increase in pH. At this stage, yeasts also produce pectinolytic enzymes that degrade the pulp, which subsequently aids in draining. With time, depletion of sugars and oxygen, ethanol and heat buildup as well as production of acetic acid from ethanol become less suitable for yeasts and this induces the growth of lactic acid bacteria. Lactic acid bacteria further metabolize citric acid to release acetic acid, lactic acid and carbon dioxide. As the beans are mixed and the structure of the pulp has collapsed, fermentation becomes aerobic. This increased aeration leads to the decrease in the lactic acid bacteria dominance, and paving way to increase in the acetic acid bacteria population. Acetic acid production increases progressively and exothermic ethanol oxidation to acetic acid raises the temperature of the bean mass to about 50°C by the end of day 3. At this stage, only thermophilic, aerobic, spore-forming bacteria and molds thrive. When all the alcohol has been used up and acetic acid has been converted to carbon dioxide and water, the temperature of the mass decreases and thus, fermentation subsides thereafter. As the fermentation has ended, beans are then transferred for drying.

The diffusion of alcohols, acetic acid, water, as well as other metabolites produced during fermentation through the testa of cocoa seeds into the beans induces bean death, disabling the seed germination. As the beans die, biochemical barriers that segregate cellular components break down and enzymatic reactions are initiated. This event ultimately commences the enzyme activity to form flavor precursors, such as free

amino acids, reducing sugars and organic acids. In summary, complex biochemical reactions between external pulp and beans require stringent maintenance of pH and temperature of the external pulp. This favorable environment is in turn conducive for the progressive microbial and enzyme activities, which determine the end quality of cocoa beans with reduced astringency and bitterness.

I.1.c. Potential use of cocoa pulp

Along with other by-products of cocoa processing such as cocoa pod husk and unfermented cotyledons, cocoa pulp can be further processed into value-added products (Sukha, 2003). These products could become a significant source of secondary income in the cocoa growing communities especially when the demand of cocoa is low in the international market or inevitable diseases such as witches broom emerges (Dias et al., 2007).

Although cocoa pulp is an essential ingredient for cocoa bean fermentation, successful fermentation does not necessarily require all of the pulp. In fact, loss of pulp naturally occurs as seeds are spread out and subsequently pulp drains down the fermentation boxes. Up to 20% of pulp (fresh bean weight) can be removed without significant effects on the fermentation process and organoleptic quality of cocoa bean (Lopez, 1979). In certain cultivars, partial removal of cocoa pulp before fermentation has been purposely done to reduce high acidity in cocoa beans. Biehl and Ziegler (2003) found that strong acidic fermentation (pH <4.0) produced beans with lower aroma potentials compared to moderate nib acidity during fermentation (pH 5.0-5.5). This was due to the unfavorable condition for enzymes to produce free amino acids and

oligopeptides as potential flavor precursors. [Biehl et al. \(1989\)](#) also suggested that if the volume ratio of pulp to seed was reduced to below 0.6 from about 1.1-1.3, acetic acid production was significantly reduced. It was reasoned that with thinner pulp layer, anaerobic fermentation phase is shortened causing an accelerated microbial succession, temperature increase and rise in pH on the cotyledon; leading to a rapid progression of fermentation in overall. Nevertheless, the above studies provide information of potential secondary use of cocoa pulp in value-added products instead of a discarded by-product.

Several attempts to further utilize cocoa pulp, extracted before and after bean fermentation, have been made. [Dias et al. \(2007\)](#) were successful in making a fruit wine by fermenting fresh cocoa pulp using *Saccharomyces cerevisiae* strain. In Malaysia, cocoa pulp has been used to make juice and jellies ([Malaysian Cocoa Board, 2004](#)) as it contained high amount of pectin and sugar. In Ghana, unfermented pulp has been formulated into jams ([Cocoa Research Institute of Ghana, 2010](#)). On the other hand, pulp that is collected during or after fermentation, so called '*cocoa sweating*', has been shown ([Adams et al., 1982](#)) to be an ideal medium for the production of alcohol and vinegar. It contained ethanol concentration that was economically feasible to be recovered through distillation. Gin and brandy from cocoa pulp were commercially available in Ghana ([Cocoa Research Institute of Ghana, 2010](#)). Nonetheless, the versatile utilization of cocoa pulp in the aforementioned applications may offer a promising diversification opportunity for cocoa agro industry.

The commercial utilization of cocoa pulp nonetheless will require the availability of high quality and hygienic pulp through an efficient extraction process. Mechanical and enzymatical methods have been developed to hygienically extract cocoa pulp ([Schwan](#)

and Wheals, 2004). Large scale extraction of pulp has been done with commercially available depulpers (Bangerter et al., 1993). Such depulper can remove up to 80% by weight of pulp based on fresh seeds weight. In contrast, enzymatical extraction of cocoa pulp is done by inoculating yeast starter culture and fermenting under controlled conditions. Buamah et al. (1997) reported that 60% increase in sweating yield was obtained in controlled fermentation compared to spontaneous fermentation. Most importantly, the study also confirmed that there were no deleterious effects on the final quality of cocoa beans.

Unfortunately, in the current trend of food industry that is mostly influenced by health and wellness, information regarding the potential health benefits of cocoa pulp is still very limited. This may hinder the value-added utilization of cocoa pulp in the midst of competition with other known antioxidant-rich commodities. In contrast, many studies have suggested the advantageous health benefits of the counterpart of cocoa pulp, which is cocoa bean, due to the presence of epicatechin, catechin, and procyanidins (Keen et al., 2005). This flavanoid in cocoa bean has been further explored to possess antioxidant activity as measured using chemical (Adamson et al., 1999) and biological assays (Noé et al., 2004). Hence, with a similar approach, cocoa pulp will be investigated for its chemical and cellular antioxidant activity using commonly used assays in food applications as described in the following section.

I.2. Antioxidant measurement assay

Antioxidants are compounds that are able to provide protection against free radicals damage harmful to cellular structures and macromolecules. Due to the inefficiency of our endogenous defense system, dietary antioxidants are needed to overcome oxidative stresses such as pollution, exercise, smoke exposure, and radiation (Shahidi and Ho, 2007). Carotenoids and phenolic compounds are the most abundant dietary antioxidants, which are mainly obtained from fruits and vegetables. Owing to the importance of the antioxidant potentials from foods as well as dietary supplements, the need for reliable and physiologically relevant antioxidant measurement methods are necessary. Prior et al. (2005) suggested that standardized antioxidant capacity methods may provide guidance for: 1) suitable assay for specific applications, 2) meaningful comparison of foods or other commercial products, and 3) offering a provision of quality standards for regulatory issues and health claims. Among numerous antioxidant methods, chemical and cell culture assay provide the ability to monitor antioxidants in inhibiting oxidation in a model system, by assessing the physical, chemical, or biological changes by instrumental means.

I.2.a. Chemical antioxidant assay

In general, chemical antioxidant assay are divided into two categories, Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET) mechanisms. While HAT-based system is based on the ability of antioxidant to quench free radicals by hydrogen donation, SET-based assay detect the ability of antioxidant to transfer one electron to reduce other compounds including radicals, carbonyls and metals. Out of many HAT-

based assays, Oxygen Radical Absorbance Capacity (ORAC) and Total Radical-trapping Antioxidant Parameter (TRAP) meet more of the *ideal* requirements of a standardized assay, such as: 1) the ability to measure the chemistry occurring in potential applications; 2) utilizes a physiologically relevant radical source; 3) simple but repeatable and reproducible; 4) has a defined chemical mechanism; 5) adaptable for a high-throughput analysis (Prior et al., 2005). Both methods have been used in a large number of studies to measure the antioxidant capacity of foods and phytochemical compounds (USDA ARS, 2007; Bank and Schauss, 2004). On the other hand, SET-based assays are well represented by Ferric Reducing Antioxidant Power (FRAP) and Copper Reduction Assay (CUPRAC). Combinations of HAT and SET assays have also been developed, such as Trolox Equivalent Antioxidant Capacity (TEAC) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH).

Despite the absence of a standard method that is suitable for a variety of target applications, ORAC assay has the capability of measuring both hydrophilic and lipophilic antioxidant capacity in complex biological samples. ORAC assay was developed by Cao and Prior (1999). The assay measures the ability of antioxidant to scavenge peroxyl radical induced by 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37°C. β -phycoerythrin (β -PE) was initially used as the fluorescence probe in this assay. However, due to its susceptibility of photo-bleaching, it was replaced by a more stable probe, fluorescein (Ou et al., 2001). The loss of fluorescence from the probe is used as an index of oxidative damage from its reaction with peroxyl radicals. The protective effect of the antioxidant was quantified by measuring the area under the curve (AUC) of the samples as compared to known concentrations of Trolox, a water soluble vitamin E analog, after

subtraction of blank, in which no antioxidant is present. ORAC assay provides a complete assessment of antioxidant capacity based on inhibition time and inhibition degree as the reaction is driven to completion. Hence, ORAC assay is able to provide better information compared to methods based on fixed time point and especially suitable to assess different antioxidants or mixture systems that have different reaction kinetics. Additionally, ORAC assay has been automated for high throughput applications (Prior et al., 2003). Nonetheless, the assay is highly temperature sensitive in which reproducibility of the assay might be decreased unless it is well-controlled (Huang et al., 2002).

I.2.b. Cellular Antioxidant Assay (CAA)

Cellular Antioxidant Assay (CAA) was developed due to the inability of the ‘test tube’ chemical antioxidant assays to reflect the physiological conditions as well as bioavailability and metabolism issues (Wolfe and Liu 2007). This disparity may be due to variability in absorption, distribution, metabolism, and excretion (ADME) of bioactive components that are neglected in many *in vitro* methods (Shahidi and Ho, 2007). Other discrepancies between *in vivo* and *in vitro* have also been observed through studies which suggested that the original bioactive compounds may not exhibit antioxidant activity *in vivo* but rather their metabolites (Scalbert and Williamson, 2000). Animal and human studies, on the other hand, are oftentimes time consuming and financially limiting to understand the complexity of polyphenols metabolism chemistry. With the need to bridge the gap between *in vivo* and *in vitro* antioxidant assay, CAA would allow simple and rapid method to understand phytochemicals absorption in relation to food matrix,

processing, structures of bioactive compounds as well as interactions with other components.

CAA shares a similar principle with ORAC. It measures the ability of samples to scavenge free radicals, as shown in **Figure I.3**. In this method, antioxidant compounds, radical generators, and dichlorofluorescein diacetate (DCFH-DA or $C_{24}H_{16}Cl_2O_7$), which is a fluorescent probe, is taken up by HepG2 human hepatocarcinoma cells. As DCFH-DA enters into the cells, cellular esterases cleave the diacetate moiety to form the more polar DCFH, which is trapped within the cell. Peroxyl radicals that are generated from the decomposition of peroxyl generators ABAP (2,2'-azobis-2-amidinopropane) lead to the oxidation of DCFH to DCF ($C_{20}H_{10}Cl_2O_5$), which emits fluorescence. Hence, the fluorescence level is proportional to the degree of oxidation. The presence of phytochemicals in foods will quench peroxyl radicals and inhibit the generation of DCF, which subsequently reduces fluorescence intensity. Hence, CAA assay utilizes the ability of reactive peroxyl radicals to induce the formation of a fluorescent response as a result of oxidative stress in cell culture, and measures the ability of antioxidants to prevent oxidation.

In CAA assay, appropriate selection of each component is necessary to closely mimic biological systems. HepG2 cells are used since they are a better model to simulate and address metabolism issues. In comparison to other cell lines, including Caco-2 cells and RAW 264.7, lower coefficient of variation can be obtained using HepG2 cells (Wolfe et al., 2008). DCFH-DA is used on this assay due to its rapid uptake and stable final concentrations (Royall and Ischiropoulos, 1993). Furthermore, the wide array of ROS (Reactive Oxygen Species) that are able to oxidize DCFH to its fluorescent DCF, offers it

CAA have been reported to assess antioxidant activity of fruits (Wolfe et al., 2008), vegetables (Song et al., 2010) common in American diet, processed soy milk (Xu et al., 2010), and broccoli (Eberhardt et al., 2005). In conjunction to cellular antioxidant assay, other study also utilized Caco-2 cells monolayers to assess differences in bioavailability between foods (Boyer et al., 2004). The study presented a useful model to compare the uptake of quercetin aglycon and quercetin-3-glucosides when applied as pure compounds in comparison to extracts obtained from onion and apple. Whether or not CAA is better than chemical antioxidant assays in predicting the biological activity of antioxidants remains to be proven by *in vivo* antioxidant status after consumption of antioxidant containing samples (Wolfe and Liu, 2007).

I.3. Antioxidant activity as affected by thermal processing

In fruit-based applications, one factor that largely affects antioxidant activity is the thermal processing step. Processing is done to ensure the safe consumption of the food and extend its shelf life to satisfy distribution requirements. However, thermal treatment may also lead to the deterioration of antioxidant activity due to the adverse heat exposure. Conversely, in some cases thermal processing may increase antioxidant activity due to the release of compounds that possess antioxidant activity from the cell matrices. For the purpose of this thesis, only common thermal processing suitable for unclarified, pulpy fruit-based products will be reviewed to represent many processing and preservation methods available to extend the shelf life of cocoa pulp, possibly without the use of additives.

I.3.a. Thermal processing of fruit-based products

The interest to utilize cocoa pulp as value-added product at large scale requires it to be handled immediately to avoid its short ambient shelf life due to spontaneous alcoholic fermentation by microorganisms. Economically feasible thermal processing that ensures safety but also maintains nutritional as well as sensory characteristics, is desired. Hence, the design of any type of thermal processing should meet the following three basic criteria (Fellows, 2000):

1. Reduce the number of spoilage and pathogenic microorganisms to an acceptable level that they are no longer a concern of public health hazard,
2. To create an internal package environment that suppresses the growth and activity of spoilage microorganisms,
3. To prevent recontamination post-processing and during storage.

It is important to understand that thermal processing is intended to attain shelf-stability but not sterility of food products. In fact, producing a safe product at the expense of nutritional and eating quality is never an option.

One of the most common methods of thermal processing of fruit-based products, such as juices, is pasteurization. It is a mild heat treatment in which juices are heated to target temperature range of 60-90°C. In the traditional batch pasteurization technique, called the Holder process, juices are held at about 60°C for a relatively long period of time (~30 minutes) in an open pan and then hot-filled into containers, sealed and inverted, thus sterilizing the upper part of the containers and lids (Lewis, 2006). This type of hot-fill process is simple and suitable for fruit-based products with pH below 4, since the high acidity precludes the growth of *Clostridium botulinum*, a pathogen which

may produce the deadly botulinum toxin. It also has the additional advantages of creating a partial vacuum in the sealed container as vapor condenses upon cooling. Unfortunately, rapid cooling cannot be obtained with this method, and thus scorching occurs due to the extended holding period before and after filling (Bates et al., 2001).

HTST (High Temperature Short Time) pasteurization was then introduced to improve finished product quality. Run in a continuous manner, this type of processing operation offer a number of advantages, such as faster heating and cooling rates, shorter holding times and regeneration, which saves both heating and cooling costs and contributes to the low processing costs incurred in thermal processing. In a large scale pasteurization of low viscosity liquids, such as fruit juices, plate heat exchangers are usually employed. Plate heat exchangers comprise of a series of thin vertical stainless steel plates that are held tightly together in a metal frame. The plates create parallel channels in which juices and heating medium, steam or hot water, are pumped through alternate channels in a counter-current flow manner. Mixing of the product and the heating or cooling media are prevented since the synthetic rubber gaskets are installed so that each plate is watertight sealed. The plates are corrugated to induce turbulence in the liquids as well as to reduce fouling due to the velocity induced by pumping. In practice, juice is first pumped to a regeneration section, where it is pre-heated by the juice that has been just pasteurized. It is then heated to the intended pasteurization temperature and holding period in the holding tube. The pasteurized product is then cooled gradually, first through the regeneration section and then further cooled by chilled water in the cooling section. For more viscous juices, the use of concentric tube heat exchanger is suggested. The system consists of a number of concentric stainless steel coils that are made from

double- or triple-walled tube. Juice passes through the tube, and the heating or cooling media is re-circulated through the tube walls. After pasteurization, juice is immediately filled into containers, sealed, and refrigerated until consumption (Fellows, 2000).

To reduce the cost of refrigeration during handling and storage, heat sterilization process was developed. Sterilized juices, as a result, have a shelf life of more than 6 months at ambient temperatures. In-container sterilization is commonly done using a retort process. With the heating medium of saturated steam, steam-air mixtures, or hot water (with overriding pressure), the process could be a batch or continuous, coupled with static or agitating mode. Thorough investigation of the heat resistance of the target microorganisms, size and type of packaging, heat transfer of the food should be done to shorten the processing time. Those critical factors will increase process efficiency as well as the safety, nutritional and sensory quality in the finished product (Ramaswamy et al., 2004).

For fruit beverages, purees, and juices containing discrete particles, aseptic or ultra-high temperature (UHT) processing has become a viable option to improve product quality of heat processed, shelf-stable foods. Briefly, sterilization of the product is achieved by rapid heating to temperatures about 140°C, holding for several seconds, followed by rapid cooling. In this process, juices and packaging materials are sterilized separately and then assembled under sterile conditions (Lewis 2006). Although direct heating methods are available, indirect method of heating juices using plate or tubular heat exchangers are used extensively. Nonetheless, aseptic processing is cost intensive that requires sophisticated operation system, maintenance, and trained personnel.

I.3.b. Effect of thermal processing on sensory and nutritional quality of fruit-based products

Although thermal treatment remains the most important methods of food processing, heat destroys some components in fruit-based products that are responsible for color, aroma, flavor, texture, and nutritional values. Color deterioration in pasteurized fruit juices is mainly caused by enzymatic browning. Inactivation of enzyme activity may be achieved through thermal processing, and oftentimes supplemented with deaeration prior to pasteurization to minimize enzyme activity during processing (Fellows, 2000). Browning due to non-enzymatic pathway was also observed in peach puree during heating (Garza et al., 1999). Oftentimes due to heat treatment, chlorophyll is converted to pheophytin when heated above 60°C (Weemaes et al., 1999), carotenoids are isomerized from 5,6-epoxides to less intensely colored 5,8-epoxides (Lee and Coates, 2003), and anthocyanins are degraded to form brown pigments through polymerization (Boyles and Wrolstad, 1993).

A loss of volatile had also been observed in juices, leading to the unmasking of cooked flavors. Yen and Lin (1999) showed that heating at 95°C for 5 min caused the loss of most flavors in guava juice. These volatile compounds that are lost during thermal treatment or evaporation can be recovered through distillation, partial condensation, and pervaporation (Pereira et al., 2006) to be added back to retain the sensorial quality of juices. Due to its high cost, however this practice is not widely adopted.

The delicate fresh flavor juices are easily altered by heat treatment. The difference in the profile of fresh-squeezed orange juices and pasteurized reconstituted juices from

concentrate has been compared quantitatively by [Nisperos-Carriedo and Shaw \(1990\)](#). The study showed that pasteurized single-strength juices (non-concentrate) did not show differences in the flavor profile when compared to fresh juice. In contrast, pasteurized reconstituted juices from concentrate showed decreases in acetaldehyde, methyl acetate, methyl butyrate, and ethyl butyrate, prominent contributors to ‘top-note’ fruit flavors especially in fresh citrus.

The influence of thermal processes on the viscosity of fruit juices, thinning and thickening, is very much dependent on the composition ([Heldman and Hartel, 1997](#)) such as soluble solids content. [Yen and Lin \(1998\)](#) found out that pasteurized guava juice showed a significant decrease in viscosity and increased in turbidity. Those observations were due to the degradation of pectic substance that aggregated and led to the increase in the cloud content. Similar result was also obtained by [Farnworth et al. \(2001\)](#) in orange juices.

Aside from maturity stage and cultivar types of fruits, bioactive components in fruit juices are heavily influenced by post-harvest processing and storage. In thermally processed fruits juices, significant losses may occur in heat-labile, water soluble vitamins, particularly ascorbic acid. Inevitable loss of ascorbic acid of 2-6% was observed in black currant nectars ([Iversen, 1999](#)) and up to 25% in yellow passion fruit ([Talcott et al., 2003](#)) post pasteurization. Thus, ascorbic acid is oftentimes used as a marker of quality changes in fruits and vegetables due to the severity of food processing. Other study ([Gil-Izquierdo et al., 2002](#)) however found that there was no significant decrease of ascorbic acid in citrus juice after processing. The low pH of fruits provided the protective effect for the stability of ascorbic acid during thermal processing.

Beneficial fruit polyphenolic compounds that inhibit oxidative reactions (Chun et al., 2005) were also affected by thermal processing in combination with light and oxygen (Rickman et al., 2007). Studies in clingstone peach by Asami et al. (2003) showed that processing above 220°F for 10 minutes showed a decreased in total phenolics of up to 21%. The study also revealed that a 30–43% loss in phenolics content occurred during the first 3 months in storage after canning. The same trend was also observed in clarified blackberry juice (Hager et al., 2008). Pasteurization at 90°C for 3 minutes caused 67% decreased of total monomeric anthocyanin as well as 55% decreased in antioxidant activity measured using ORAC assay. In contrast, heat treated sweet corn (115°C, 25 minutes) and tomatoes (88°C, 15 minutes) were found to have 44% and 34% higher antioxidant activity, respectively (Dewanto et al., 2002a; Dewanto et al., 2002b). Thermal treatment was suggested to disrupt cell membranes and cell walls, thus releasing phytochemicals, which in turn increases their bio-accessibility and absorption.

II. OBJECTIVES AND RATIONALE

II.1. Objectives

The objectives of the study are the following:

- To quantify the total phenolic content of unprocessed and pasteurized cocoa pulp,
- To quantify the chemical antioxidant capacity of unprocessed and pasteurized cocoa pulp using Oxygen Radical Absorbance Capacity (ORAC) assay,
- To quantify the cellular antioxidant capacity of unprocessed and pasteurized cocoa pulp by using Cellular Antioxidant Assay (CAA) method,
- To study the stability of the phenolic, antioxidant capacity, and color of cocoa pulp after pasteurization and during storage in comparison to unprocessed pulp.

II.2. Rationale

The results of this study provide beneficial information regarding the potential health benefits of cocoa pulp that will support its utilization in value-added products. The success in the commercialization and marketability of cocoa pulp, as incorporated in arrays of value-added product, will provide a diversification opportunity for the cocoa industry. Furthermore, understanding the effect of thermal processing on the nutraceuticals profile of cocoa pulp will help extend its shelf life and maintain its key nutritional and sensorial attributes. The results of this investigation will be useful for further utilization of cocoa pulp in other consumer products, which can increase its value and marketability.

III. MATERIALS AND METHODS

III.1. Schematic approach of the study

The overall strategy of the study is shown in **Figure II.1** below. Unpasteurized and pasteurized pulp was obtained from supplier. Double pasteurization was done by pasteurizing the already pasteurized cocoa pulp. All cocoa pulp samples, unprocessed, single and double pasteurized pulp, were subjected to total phenolics, chemical and cellular antioxidant capacity, and color determination. Eight weeks stability studies at 4°C, 25°C, and 37°C were done to monitor the stability of total phenolics, chemical antioxidant values and color of cocoa pulp.

Figure III.1 Schematic approach of the overall study.

III.2. List of chemicals used

The chemicals listed were used in the following procedures:

a. Extraction:

- Acetone (Sigma-Aldrich, St. Louis, MO)
- Acetic acid (Fisher Scientific, Pittsburgh, PA)

b. Folin-Ciocalteu assay:

- Gallic acid (Sigma-Aldrich, St. Louis, MO)
- Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO)
- Sodium carbonate (Na_2CO_3) (Sigma-Aldrich, St. Louis, MO)
- polyvinylpyrrolidone (PVPP) (Sigma-Aldrich, St. Louis, MO)

c. Oxygen Radical Absorbance Capacity (ORAC) assay:

- 2,2'-azobis-2-amidinopropane dihydrochloride (AAPH or ABAP) (Sigma-Aldrich, St. Louis, MO)
- Fluorescein (Sigma-Aldrich, St. Louis, MO)
- Sodium phosphate dibasic (Sigma-Aldrich, St. Louis, MO)
- Sodium phosphate monobasic (Sigma-Aldrich, St. Louis, MO)
- 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (Sigma-Aldrich, St. Louis, MO)

d. Cellular Antioxidant Activity (CAA) assay:

- 2',7'-Dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO)
- L-glutamine (Sigma-Aldrich, St. Louis, MO)
- Hepes (Sigma-Aldrich, St. Louis, MO)
- Hydrocortisone (Sigma-Aldrich, St. Louis, MO)
- Insulin (Sigma-Aldrich, St. Louis, MO)
- Phosphate buffer saline (PBS) (Sigma-Aldrich, St. Louis, MO)
- Quercetin (Sigma-Aldrich, St. Louis, MO)
- 0.25% Trypsin-EDTA (Fisher Scientific, Pittsburgh, PA)
- Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, CA)
- Hanks' Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA)
- Penicillium-streptomycin (Invitrogen, Carlsbad, CA)
- Williams' Medium E (WME) (Invitrogen, Carlsbad, CA)
- CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI)
- HepG2 cells (American Type Culture Collection (ATCC), Rockville, MD)

III.3. Cocoa pulp samples

Cocoa pulp, unpasteurized and pasteurized, was obtained from Quicornac (Guayaquil, Ecuador) through iTi Tropicals, Inc. (Lawrenceville, NJ).

III.4. Thermal processing of cocoa pulp

Single pasteurized cocoa pulp that we received from Quicornac was already processed at 85°C at 60 s. To simulate the practice in the industry, secondary thermal processing of cocoa pulp was done by packing the already pasteurized pulp in pouches and immersing them in hot water (85°C) contained in a steam-jacketed kettle as illustrated in **Figure III.2**. For a faster heat transfer rate, every 30 g of cocoa pulp were vacuum packed in a Meals Ready to Eat (MRE) pouch having dimensions of 10 x 12 cm using a table-top ULTRAVAC vacuum sealer (Koch Equipment LLC, Kansas City, MO). MRE pouch is a flexible and retortable packaging of individual field ration that consists of polyester (0.030 mm), aluminum foil (0.045 mm), biaxially oriented polyamide (0.015 mm), and unoriented polypropylene (0.075 mm) successively from the external to internal food contact surface layer. The average thickness of filled pouches was approximately 5 mm.

Approximately 100 pouches were prepared and placed in between the spirals of a stretched slinky, which was attached in a metal wire basket. A pouch located in the geometric center of the basket, which is located at the point of slowest heating rate, had a C-4 flexible Ecklund thermocouple attached to it. This type of thermocouple is designed for conducting heat penetration tests of food in cans, jars, and flexible plastic packages. The thermocouple was then connected to a data acquisition system, which consisted of a high speed USB career NI USB9162 (National Instruments, Austin, TX) connected to a laptop computer. Labview 7[®] (National Instruments, Austin, TX) was used to record real time, temperature data and calculate F-values. Based on the pH of cocoa pulp (~pH 3.80), the reference temperature used for this thermal processing was 200°F (93.33°C)

and z-value of 16°F (8.8°C) (GMA Science and Education Foundation, 2007). Hence, the filled basket was immersed into the hot water to reach 85°C, and the thermal processing was stopped when the calculated F-value, displayed on the screen, reached 6 s. The cooling process was initiated by discharging the hot water and pouring in a mixture of cold water and ice into the kettle at the same time. The basket was removed from the kettle when the temperature reached near room temperature (22°C).

Figure III.2 Thermal processing set up for MRE pouches containing cocoa pulp.

III.5. Storage study

Three storage temperatures, 4°C, 25°C, and 37°C, were selected to study the stability of phenolics, antioxidant activity and color of cocoa pulp. Temperature controlled chambers were used in this storage study to store pouches of pasteurized pulp. Analyses were done bi-weekly and samples were monitored for up to 8 weeks.

III.6. Pulp extractions

Extracts were obtained from the pulp using an extracting solvent of acetone/water/acetic acid (70:29.5:0.5, v/v), as described by [Prior et al. \(2003\)](#). Briefly, in triplicate, cocoa pulp was subjected to two steps extraction procedure using 1:3 (w/v) and 1:1.5 (w/v) pulp to solvent ratios, respectively. In both extraction steps, samples were stirred continuously at room temperature for 10 min. Homogenates were then centrifuged at 5000 g for 10 min. The supernatant obtained from the two extraction steps were combined, and subjected for total phenolics content as well as chemical and cellular antioxidant capacity measurement.

III.7. Determination of total phenolic content

Total phenolic contents were determined using Folin-Ciocalteu method with gallic acid as standard ([Singleton et al., 1999](#); [Dewanto et al., 2002b](#)). Several gallic acid samples with concentrations range of 50-600 µg gallic acid/ ml were prepared to develop the standard curve and the absorption reading of the undiluted pulp extracts fell within that standard curve concentration range. For each analysis, 125 µl of gallic acid solution

or pulp extract was added to 3.25 ml de-ionized water. Then, 125 μ L of Folin-Ciocalteu reagent was added, and the sample was vortexed. Following that, 1 ml of 7% sodium carbonate solution was added. After 30 min at room temperature, the intensity of the blue color developed was measured at 745 nm against the prepared blank using a Cary 50 UV-Vis spectrophotometer (Varian, Palo Alto, CA). The absorbance readings from samples obtained were then interpolated back to the standard curves and expressed as mg gallic acid equivalents (GAE) per 100 g of pulp $\pm$ standard error (SE) for three replicates.

Due to the presence of other interfering compounds, such as sugars and ascorbic acid, which could overestimate the measurement of the total phenolics content, correction was done ([Makkar et al., 1993](#)) by binding extracts to polyvinylpyrrolidone (PVPP). PVPP is an adsorbent and precipitant of phenolics compounds, thereby leaving interfering compounds in the unadsorbed or unprecipitated portions. Thus, extracts post-PVPP binding accounts for the absorbance of the interfering compounds. The procedure for PVPP binding is the following: pooled extract were subjected to 4% (w/v) PVPP binding for 1 h at 22°C and centrifugation for 10 min. The corrected total phenolics values were obtained by subtraction of total phenolics values post-PVPP binding from the initial total phenolics values (pre-PVPP binding).

III.8. Chemical Antioxidant Capacity Assay

Antioxidant capacity measurements for unpasteurized and pasteurized pulps were done using a modified Oxygen Radical Absorbance Capacity (ORAC) assay according to ([Ou et al., 2001](#)). Briefly, solutions and extracts were made and further diluted appropriately using 75 mM phosphate buffer at pH 7.4, which was also used as blank.

400 μL of extracts or blank or Trolox solution (as a standard) with a concentration range of 6.25-50 μM , were mixed with 2.4 ml of 1:1000 0.08 μM fluorescein stock solution in a silica cuvette. The mixture was mixed with the aid of micro stirrer bar, incubated and equilibrated to 37°C for 5 min. The reactions then were initiated by the addition of 400 μL of 153 mM AAPH to the individual cuvette. The fluorescence was monitored kinetically using a Cary Eclipse Fluorescence spectrophotometer (Varian, Palo Alto, CA) every 30 seconds with excitation wavelength of 485 nm, excitation slit width of 20 nm, and emission wavelength of 540 nm, emission slit width of 20 nm. The reaction was stopped when the fluorescence intensity was below 2% of the initial fluorescence signal. ORAC values were then obtained by interpolating net Area Under the Curve (AUC) of extracts, against a standard curve of net AUC of different Trolox concentrations. Trapezoidal method was used to derive AUC. The formula for AUC and net AUC is the following:

$$\text{AUC} = (0.5 + f_2/f_1 + f_3/f_1 + f_4/f_1 + f_5/f_1 + \dots + f_n/f_1) \times \text{RC}$$

$$\text{Net AUC} = \text{AUC}_{\text{extracts}} - \text{AUC}_{\text{blank}}$$

where f_1 is the fluorescence intensity at the initiation of the reaction, f_n is the fluorescence signal measured at the last cycle, and RC is the period of reading cycle of fluorescence intensity measured in minutes. ORAC values were calculated as μmol Trolox equivalent/ 100 g of pulp ($\mu\text{mol TE/g}$) $\pm$ standard error (SE) for three replicates.

III.9. Color analysis

Color measurement of cocoa pulp were done using a Konica Minolta CR-400 chroma meter based on CIE L a*b* (CIELAB) color parameters adopted by the International Commission on Illumination (usually abbreviated to CIE for its French name, Commission Internationale de l'éclairage). The colorimeter was standardized using a white calibration plate (CR-A43). Color was expressed as lightness, hue angle, and saturation/ chroma (Wrolstad et al., 2005) depicted in Hue-Saturation-Lightness (HSL) color space shown in **Figure III.3.a**. L* values represent the lightness of the pulp, in which 100 means absolute white and 0 indicates absolute black. Hue angle was derived from a* and b* values, calculated as $\arctan(b^*/a^*)$. The hue angle was expressed on a 360° grid (**Figure III.3.b**), where 0° = bluish-red, 90° = yellow, 180° = green, and 270° = blue. Saturation was calculated as $(a^* + b^*)^{1/2}$. Total color difference, ΔE^* , obtained from Hunter-Scotfield equation: $[(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2}$ was calculated to obtain overall color difference before and after pasteurization.

Figure III.3 a) HSL color space; **b)** Hue color wheel
(<http://www.diaginc.com/iq/colorspace.html>).

III.10. Cellular Antioxidant Assay (CAA)

All steps of the CAA assay performed were according to Wolfe and Liu (2007), with the exception of the cytotoxicity assay. The details are as follows:

III.10.a. Preparations of pure phytochemicals and cocoa pulp extracts

A 10 mM stock solution of quercetin dihydrate, as a standard of CAA, was freshly made in DMSO prior to use. Stock solution of 20 mM DCFH-DA was prepared in methanol, aliquoted, and stored at -20°C. A 200mM stock solution of ABAP in water was aliquoted and stored in -40°C. Pulp extracts obtained from section II.6 were evaporated to dryness under vacuum at 50°C. The residues were then reconstituted in DMSO and stored at -40°C. Further dilution of pure phytochemicals and reconstituted pulp extract were done in treatment medium (WME supplemented with 2mM L-glutamine and 10 mM Hepes). Final treatment solutions contain less than 2% of solvent, which did not show cytotoxic effect to HepG2 cells.

III.10.b. Cell culture

HepG2 cells were grown in growth medium that consisted of WME that was supplemented with 5% FBS, 10mM Hepes, 2 mM L-glutamine, 5 µg/ml insulin, 0.05 µg/ml hydrocortisone, 50 units/ml penicillin, 50 µg/ml streptomycin. HepG2 cells were maintained at 37°C and 5% CO₂. Cells used in the study were between passages 12 and 24.

III.10.c. Cytotoxicity

Cytotoxicity was done using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), which is composed of solutions of tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate) PMS. MTS is bio-reduced into a formazan product that is soluble in tissue culture medium due to the dehydrogenase enzyme activity produced by metabolically active cells. The quantity of formazan formed as measured through its absorbance at 490 nm is directly proportional to the number of living cells in culture. Briefly, HepG2 cells were seeded at 6×10^4 /well on a 96 well-plates in 100 μ l of growth medium and incubated for 24 h at 37°C. The growth medium was removed and the cells were washed with PBS. 100 μ l of treatment medium (WME with 2mM L-glutamine and 10 mM Hepes), which contains pure phytochemicals or pulp extracts, were then applied to the cells followed by incubation at 37°C and 5% CO₂ for 1 h. After incubation, 20 μ l of CellTiter 96® AQueous One Solution Reagent was added into each well of the 96-well assay plate without removing the treatment medium. The plate was incubated for 90 min at 37°C and 5% CO₂; and measured for its absorbance at 490 nm. Concentrations of pure phytochemicals or pulp extracts that decreased the absorbance by 10% compared to the control wells (contains only treatment medium) were considered to be cytotoxic.

III.10.d. Cellular Antioxidant Activity (CAA) assay of pure phytochemicals and cocoa pulp extracts

HepG2 cells were seeded into 96-well plate at a density of 6×10^4 / well in 100 μ l of growth medium/ well. The outer wells were not used for the assay since there was much more temperature fluctuations and thus, signal variations compared to the inner wells. Instead, they were filled with PBS to create a thermal mass. Twenty four hours after seeding, the growth medium was removed and the cells were washed with PBS. Triplicate wells were treated for 1 h with 100 μ l of pure phytochemicals or pulp extracts in treatment medium containing 25 μ M DCFH-DA. The cells were then washed with 100 μ l PBS. Following that, 100 μ l of HBSS containing 600 μ M ABAP was applied to each well. The plate was immediately placed in a pre-heated (37°C) Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT) for kinetic fluorescence measurement. The emission filter used was 485 nm, 20 nm bandpass, and excitation filter of 528 nm, 20 nm bandpass. The plate reader was controlled by KC4™ version 3.4 (revision 10) with a sensitivity setting of 50. The fluorescence reading was taken every 5 min for 1 h. In each plate, triplicate wells of control and blank were also included. Control wells contained treatment medium with DCFH-DA and HBSS with oxidant. Blank wells contained treatment medium with DCFH-DA and HBSS without oxidant.

III.10.e. Quantification of CAA

After subtracting blank from all fluorescence readings, the net area under curve was calculated from the fluorescence versus time curve of each concentration of pure phytochemical and pulp extracts as well as control wells. The area under the curve (AUC) was calculated using a trapezoidal method, as follows:

$$\text{AUC} = (0.5 + f_2/f_1 + f_3/f_1 + f_4/f_1 + f_5/f_1 + \dots + f_n/f_1) \times \text{RC}$$

where f_1 is the fluorescence intensity at the initiation of the reaction, f_n is the fluorescence signal measured at the last cycle, and RC is the period of reading cycle of fluorescence intensity measured in minutes.

Then, the CAA unit was calculated using the formula as follow:

$$\text{CAA unit} = 100 - [(\text{AUC of samples} \div \text{AUC of control}) \times 100]$$

As the CAA units of different concentrations of each pure phytochemical or pulp extracts were obtained, the dose-response curve were generated. The dose response-curve was then converted to median effect plot of $\log (f_a/f_u)$ versus $\log (\text{concentration or dose})$ to determine the median effective dose (EC_{50}) of pure phytochemical and pulp extracts. f_a is the fraction affected, which is equal to CAA unit, and f_u is the fraction unaffected, calculated as: $100 - \text{CAA unit}$. EC_{50} , which is determined from the median effect plot, is the concentration needed to induce halfway reduction of AUC in a given exposure time. In other words, EC_{50} value is the concentration at which $f_a/f_u = 1$ ($\log f_a/f_u = 0$) or $\text{CAA} = 50$ as calculated from the linear regression of the median effective plot. EC_{50} values were converted to CAA unit of $\mu\text{moles of quercetin equivalents (QE)}/100 \text{ g pulp}$, derived

by normalizing the EC₅₀ of the pulp samples to the EC₅₀ of quercetin as a reference. EC₅₀ were stated as mean $\pm$ standard error (SE).

III.11. Statistical Analyses

All results are presented as mean $\pm$ SE. Statistical analysis were performed using MATLAB 7.9.0.529 (R2009b) (The MathWorks, Inc., Natick, MA). Differences between means were detected by ANOVA, which is followed by multiple comparisons using Tukey significant difference test. The determination of the differences of total phenolic content, before and after correction using PVPP, was evaluated using a two-tailed paired Student's *t*-test for each treatment. Results were considered to be significant when the *p* value was <0.05 .

IV. RESULTS AND DISCUSSION

The results from analyses performed on unpasteurized, single pasteurized and double pasteurized cocoa pulp are presented and discussed in this chapter. Changes of total phenolics content, ORAC values, and color of cocoa pulp after processing and during storage were evaluated and compared to unprocessed control. Differences in CAA values before and after pasteurization were also assessed.

IV.1. Secondary thermal processing of cocoa pulp

In this study, secondary thermal processing of cocoa pulp was done to mimic the double processing scenario that commercialized fruit-based products undergo in the industry setting. Those products were thermally processed by the supplier, which is followed by the secondary processing that leads to the production of shelf stable ready-to-eat/ drink products. **Figure IV.1** shows the variation of temperature and calculated F-value with time of the secondary thermal processing, which was acquired through the data acquisition system.

Figure IV.1 Variation of temperature and calculated F-value with time during secondary thermal treatment of cocoa pulp.

As shown in **Figure IV.1**, thermal processing using hot water immersion experienced a slow heating rate. It was difficult to rapidly reach the intended final temperature of 85°C due to: 1) low volume ratio of hot water to samples given the size of the kettle; 2) the absence of mixing element to achieve a uniform convection heat transfer from the steam on the jacket layer of the kettle to the surrounding water; and 3) the thick and pulpy consistency of the pulp. Furthermore, **Figure IV.1** also showed that by the end of this process, F-value of 7 s was obtained instead of 6 s. It was due to the manual action of opening a valve to discharge hot water and pouring in ice-water mixture to start the cooling process. The cooling rate did not significantly contributed to the F-value.

IV.2. Total phenolics content of cocoa pulp

Total phenolics content of cocoa pulp was determined using Folin-Ciocalteu assay, with gallic acid as a standard compound. Folin-Ciocalteu assay is a spectrophotometric method and thus, the absorbance of several gallic acid concentrations were measured and shown in **Figure IV.2**.

Figure IV.2 Gallic acid standard curve used in Folin-Ciocalteu total phenolics determination (absorbance at 745 nm $\pm$ SE, n=3).

As the absorbance of the unpasteurized, single, and double pasteurized pulp extracts were interpolated into the standard curve in **Figure IV.2**, the average total phenolics content before PVPP binding obtained were 137.10 ± 4.69 , 111.04 ± 0.56 , and 97.58 ± 0.37 mg GAE/ 100 g pulp for unpasteurized, single, and double pasteurized pulp, respectively. However, total phenolics content of unpasteurized, single, and double pasteurized pulp after PVPP binding were 103.76 ± 4.79 , 77.88 ± 0.65 , and 64.21 ± 0.32 mg GAE/ 100 g pulp, respectively. This showed that by binding extracts to PVPP, a significant overestimation of total phenolics, due to interfering compounds, by 24%, 30%, and 34%, for unpasteurized, pasteurized and double pasteurized pulp, respectively, could be avoided. Uncorrected and corrected values of total phenolics content cocoa pulp samples were summarized in **Figure IV.3**. Nevertheless, unpasteurized cocoa pulp (corrected values) contained a measurable amount of total phenolics very similar to green grapes, which was reported to have total phenolics content of 145 mg GAE/ 100 g grapes (Wu et al., 2004).

PVPP binding is a differential method; one of the most commonly used methods to account for interfering compounds such as ascorbic acid, sugars, and protein. Ascorbic acid and sugars are additive interfering compounds since they reduce Folin-Ciocalteu reagent and thus, their presence increases total phenolics value. On the other hand, protein is an inhibitory interfering compound since it can bind to phenolic compounds through hydrophobic interactions and hydrogen bonding; hence, causing the active site of phenolic compounds to be unavailable to reduce Folin-Ciocalteu reagent (Singleton et al., 1999). However, possible inhibitory effects by protein were neglected in this study since the protein content of cocoa pulp is very low. This PVPP binding method had been

adopted in determining corrected total phenolics content of oak acorns as influenced by thermal processing (Rakić et al., 2007).

PVPP adsorption to phenolics lacks sharp cutoff points because PVPP binds to different sizes and types of polyphenols. PVPP has a greater affinity for the larger phenolic compounds and do not readily adsorb non-tannins such as gallic acid and (+)-catechin, unless sufficient condensed tannin or other less soluble phenols are available for cross-linking (Singleton et al., 1999). Other factors such as surface area of the adsorbent and level of hydroxylation of the phenol compounds as binding sites to bind adsorbent may be manipulated to increase PVPP binding capacity to phenolic compounds; however complete removal of phenolics is not be possible. Approximately 80-90% removal of flavanols from flavanols-spiked beer by PVPP binding had been observed (McMurrough et al., 1995). Due to the significant presence of interfering compounds detected by the PVPP differential method in this study, only the corrected total phenolics values were used to express total phenolics content hereafter.

Additionally, as observed in **Figure IV.3**, single and double pasteurization of cocoa pulp led to, correspondingly, 25% and 38% reduction of phenolics ($p < 0.05$) compared to unpasteurized pulp. This observation may be due to the adverse effect of heat, other chemicals present in the pulp matrix, light or oxygen that leads to oxidation and subsequent polymerization and degradation of phenolics compounds (Nicoli et al., 1999).

Figure IV.3 Total phenolics content of unpasteurized and pasteurized pulp (mean $\pm$ SE, $n=3$). Statistically significant differences ($p < 0.05$) in the average values are indicated by different letter. Asterisks (*) indicate significant difference ($p < 0.05$) between total phenolic and corrected total phenolics content based on paired Student t -test in each treatment.

IV.3. Chemical antioxidant capacity of cocoa pulp

Chemical antioxidant capacity of cocoa pulp was measured using ORAC assay, with Trolox as a standard compound and fluorescein as a fluorescence probe. The fluorescence decay curve of the fluorescein, due to the oxidation of radicals, given different concentrations of Trolox is shown in **Figure IV.4**. After the AUC of each decay curve was calculated using a trapezoidal method, a standard curve was set up as shown in **Figure IV.5**.

Figure IV.4 Fluorescence decay curve of Trolox at different concentrations.

Figure IV.5 Trolox standard curve used in ORAC assay (area under the curve $\pm$ SE, $n=3$).

Cocoa pulp, unpasteurized and pasteurized, contained a measurable amount of antioxidant activity that is shown in **Figure IV.6**. Antioxidant capacity values of cocoa pulp were obtained through extrapolation of area under the curve of pulp samples to the standard curve (**Figure IV.5**). The antioxidant capacity of unpasteurized pulp was 1871 ± 58 $\mu\text{mol TE/ 100 g pulp}$. This value was comparable with green grapes of 1118 ± 96 $\mu\text{mol TE/ 100 g raw fruit}$ (Wu et al., 2004). The antioxidant capacity of single and double pasteurized pulp were not significantly different from unpasteurized pulp ($p>0.05$). The ORAC values of single and double pasteurized pulp were 1835 ± 41 $\mu\text{mol TE/ 100 g pulp}$ and 1681 ± 157 $\mu\text{mol TE/ 100 g pulp}$, respectively.

The stability of the antioxidant capacity values of pasteurized pulp in comparison to unpasteurized pulp (**Figure IV.6**) could be due to the degradation of hydrolysable tannins, a larger polyphenolics compound, which result in the formation of smaller degradation products, such as gallic acid, that maintains antioxidant activity (Rakić et al., 2007). Thus, thermally treated pulps possessed equal antioxidant capacity compared to the unprocessed pulp. It is also possible that during processing, improvement of antioxidant properties occurred due to the release of phytochemicals from cell matrix, which has been proven effective for lycopene in tomatoes (Dewanto et al., 2002b). At the same time, Maillard Reaction Products (MRPs), which can be formed through intense heat treatment, could also contribute to the higher antioxidant properties (Nicoli et al., 1997).

Figure IV.6 Antioxidant capacity of unpasteurized and pasteurized pulp (mean $\pm$ SE, $n=3$). Statistically significant differences ($p < 0.05$) in the average values are indicated by different letter.

IV.4. Color of cocoa pulp

CIELAB colorimetric space, which consists of L^* , a^* , and b^* coordinates, was used to characterize the changes in color due to thermal treatment. The color characteristics of cocoa pulp are summarized in **Table IV.1**.

Table IV.1 Color characteristics of cocoa pulp (mean $\pm$ SE, n=3).

Color characteristics	Cocoa pulp		
	Unpasteurized	Single pasteurized	Double pasteurized
L^*	61.17 ± 0.29^a	55.86 ± 0.20^b	46.68 ± 0.19^c
a^*	6.25 ± 0.29^a	10.09 ± 0.12^b	7.97 ± 0.07^c
b^*	6.71 ± 0.25^a	16.12 ± 0.08^b	9.99 ± 0.04^c
Hue angle ($^\circ$)	47.07 ± 0.24^a	57.96 ± 0.26^b	51.40 ± 0.12^b
Chroma	9.17 ± 0.38^a	19.02 ± 0.12^b	12.78 ± 0.07^c
ΔE^*	n/a	11.50 ± 6.64^a	14.96 ± 8.64^a

Different superscript letters across different thermal treatment indicate significant difference ($p < 0.05$).

A decreased of L^* values were observed in both single and double pasteurized pulp, which may be due to browning reactions that are accelerated by thermal processing. Similar observation was also reported when heating pear puree between 80-98°C at different processing time (Garza et al., 1999) and orange juice 94.6°C for 30 s in tubular heat exchanger (Yeom et al., 2000). There were an overall increased of a^* and b^* values of thermally processed pulp which indicated a direction toward more yellow and red in pasteurized pulp. The hue angle of both single and pasteurized pulp were significantly higher ($p < 0.05$) than unpasteurized pulp. Furthermore, higher chroma values of thermally processed pulp were consistent with the visual differences perceived as intense and dark cream color-like (RGB: 255, 253, 208). It was suggested that the severity of thermal

treatments affected the color of juices and fruit purees through the formation of 5-hydroxymethylfurfural (HMF) which were present in more abundance at higher temperature and longer duration of processing (Garza et al., 1999).

The overall metric difference of color, expressed in ΔE^* , were not significant ($p>0.05$) between single and double pasteurized pulp in comparison to unpasteurized pulp, which indicated that only the first pasteurization step led to significant color changes in cocoa pulp. Based on the interpretation of numerical color difference, Francis and Claydesdale (1979) indicated that ΔE^* of 2 is indicative of noticeable differences, while ΔE^* of 3 would represent unacceptable color changes in many products. Unfortunately, both single and double pasteurized pulp had ΔE^* greater than 3, a sign of undesirable color differences. Nevertheless, thermal processing is necessary to extend the shelf life of cocoa pulp. Hence, exploration of non-thermal processes that lead to better color retention might be beneficial.

IV.5. Stability study of single and double pasteurized cocoa pulp

The influences of storage time and temperature on total phenolics content (corrected values), ORAC values, and color of cocoa pulp after pasteurization were monitored and determined bi-weekly for a period of 8 weeks. Both single and double pasteurized cocoa pulp was stored in darkness under 3 different storage conditions, 4°C, 25°C, and 37°C.

IV.5.a. Stability study of single pasteurized pulp

Changes in total phenolics throughout a stability study of 8 weeks were summarized in **Table IV.2** for single pasteurized pulp. The influence of storage temperature and storage time as well as their interaction had significant influence ($p < 0.05$) on total phenolics of cocoa pulp (**Table IV.3**). **Figure IV.7** showed that there were differences in the stability of total phenolics content in different storage conditions. The rate of phenolics degradation was significantly lower ($p < 0.05$) when single pasteurized pulp was stored at 4°C, followed by 25°C and 37°C. By the end of the eight weeks, there was a decrease of 14%, 26%, and 50% in total phenolics content at storage temperature of 4°C, 25°C, and 37°C, respectively. Furthermore, when comparing the total phenolics values that were taken at the same storage period but stored at different temperature, total phenolics contents of single pasteurized pulp stored at 37°C were consistently and significantly ($p < 0.05$) the lowest. Those observations were consistent with the stability study of hawthorn fruit and drink stored at 4°C in comparison to 23°C and 40°C done by [Chang et al. \(2006\)](#). Possible degradation pathways of the phenolics may be related to oxidation ([de Gaulejac et al., 2001](#)), hydrolysis ([Yamada et al., 1997](#)), or isomerization ([Wang and Helliwell, 2000](#)), which were suggested from previous studies but not investigated in the present study. Furthermore, certain enzymes may be still active in the fruits and contribute to the degradation of the active components ([Tomás-Barberán and Espín, 2001](#)).

Table IV.2 The stability of total phenolics in single pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
4°C	77.88 $\pm$ 0.65 ^a _a	73.09 $\pm$ 0.33 ^b _a	77.90 $\pm$ 0.54 ^a _a	74.30 $\pm$ 0.59 ^b _a	66.94 $\pm$ 1.04 ^c _a
25°C	77.88 $\pm$ 0.65 ^a _a	68.79 $\pm$ 0.09 ^b _b	65.95 $\pm$ 0.48 ^b _b	72.91 $\pm$ 0.91 ^c _a	57.62 $\pm$ 0.91 ^d _b
37°C	77.88 $\pm$ 0.65 ^a _a	61.13 $\pm$ 0.21 ^b _c	44.28 $\pm$ 0.50 ^c _c	50.14 $\pm$ 0.39 ^d _b	38.97 $\pm$ 0.23 ^c _c

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Table IV.3 Two-way ANOVA of the stability of total phenolics in single pasteurized pulp.

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	3055.83	2	1527.91	1391.15	0
Time	2577.71	4	644.43	586.74	0
Temperature*time	1228.27	8	153.53	139.79	0
Error	32.95	30	1.1		
Total	6894.76	44

Figure IV.7 The stability of total phenolics in single pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

The influence of storage temperature and storage time as well as their interaction were significant ($p<0.05$) on the ORAC values of single pasteurized cocoa pulp (**Table IV.5**). The antioxidant capacity of single pasteurized cocoa pulp, as shown in **Figure IV.8**, was most stable when stored at 4°C. Based on the similarity of the stability trend between total phenolics content and ORAC values of single pasteurized pulp, it was possible that the antioxidant activity was mostly contributed by phenolics. 95%, 85%, and 60% retention (**Table IV.4**) were obtained from single pasteurized pulp by the end of the shelf life study for 4°C, 25°C, and 37°C storage conditions, respectively.

Table IV.4 The stability of antioxidant activity in single pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
4°C	1835 $\pm$ 41 ^a _a	1623 $\pm$ 52 ^a _{a,b}	1697 $\pm$ 70 ^a _a	1699 $\pm$ 94 ^a _a	1738 $\pm$ 32 ^a _a
2°C	1835 $\pm$ 41 ^a _a	1632 $\pm$ 21 ^{a,b} _a	1525 $\pm$ 22 ^b _{a,b}	1678 $\pm$ 58 ^{a,b} _a	1565 $\pm$ 89 ^b _a
37°C	1835 $\pm$ 41 ^a _a	1411 $\pm$ 65 ^b _b	1302 $\pm$ 66 ^{b,c} _b	1219 $\pm$ 63 ^{b,c} _b	1097 $\pm$ 58 ^c _b

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).
 Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Table IV.5 Two-way ANOVA of the stability of antioxidant activity in single pasteurized pulp.

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	997701.2	2	498850.6	49.17	0
Time	771577.4	4	192894.3	19.01	0
Temperature*time	431536.3	8	53942	5.32	0.0003
Error	304357.8	30	10145.3		
Total	2505172.6	44

Figure IV.8 The stability of antioxidant activity in single pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Color characteristics, L^* , a^* , b^* , of single pasteurized cocoa pulp, as summarized in **Table IV.6.**, were not affected by the interaction of storage temperature and storage time based on two-way ANOVA statistical method ($p < 0.05$) followed by Tukey significant difference test. However, the effect of individual variables of storage temperature and storage time is significant. By the end of the storage study, L^* values were statistically different when stored at 4°C and 25°C, but lowest at 37°C. The a^* and b^* values remained stable when stored at 4°C and 25°C. However, there was a significant decrease in a^* values and increase in b^* values at 37°C, which respectively corresponded to a decrease in redness and an increase in yellowness.

By the end of the storage study, hue angle of single pasteurized pulp (**Table IV.7**) increased when stored at 4°C and 25°C. On the other hand, at 37°C storage temperature, hue angle showed some fluctuations, increasing up to week 6 and then decreasing on week 8. As a result, the hue angle of pulp stored at 4°C and 37°C were not significantly different ($p > 0.05$). This observation may be due to misplacement of some pouches which laid flat and stacked to each other. Thus, temperature variation inside pouches may occur during storage. Chroma values of single pasteurized pulp (**Table IV.8**) remained stable when stored at 4°C; however there were slight increases when stored at 25°C and 37°C. Throughout the storage study, the stability of chroma at 25°C and 37°C were very similar. The observed color of the pulp was more intense when stored at higher temperature. It was suggested that elevated temperature enabled and accelerated Maillard reactions to proceed (BeMiller and Whistler, 1996). Furthermore, polyphenol-oxidases, which have not yet been inactivated, may cause darkening on the sample through enzymatic browning pathway (Haard and Chism, 1996; Whitaker, 1996).

Table IV.6 The stability of L*, a*, and b* in single pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
L*					
4°C	55.86 $\pm$ 0.20 ^a _a	53.69 $\pm$ 0.04 ^b _a	53.73 $\pm$ 0.11 ^b _{a,b}	53.72 $\pm$ 0.13 ^b _{a,b}	54.83 $\pm$ 0.05 ^c _a
25°C	55.86 $\pm$ 0.20 ^a _a	54.72 $\pm$ 0.07 ^b _b	55.69 $\pm$ 0.17 ^a _a	55.07 $\pm$ 0.04 ^b _a	53.71 $\pm$ 0.10 ^c _a
37°C	55.86 $\pm$ 0.20 ^a _a	53.90 $\pm$ 0.05 ^{a,b} _a	52.82 $\pm$ 0.86 ^b _b	53.42 $\pm$ 0.59 ^b _b	47.46 $\pm$ 0.44 ^c _b
a*					
4°C	10.09 $\pm$ 0.12 ^a _a	10.69 $\pm$ 0.05 ^b _a	9.66 $\pm$ 0.10 ^a _a	9.88 $\pm$ 0.19 ^a _a	9.02 $\pm$ 0.07 ^c _a
25°C	10.09 $\pm$ 0.12 ^{a,b} _a	10.20 $\pm$ 0.04 ^{a,b} _b	9.01 $\pm$ 0.57 ^b _a	9.46 $\pm$ 0.10 ^b _{a,b}	8.52 $\pm$ 0.36 ^{b,c} _a
37°C	10.09 $\pm$ 0.12 ^a _a	10.22 $\pm$ 0.03 ^a _b	9.27 $\pm$ 0.19 ^b _a	8.94 $\pm$ 0.12 ^b _b	10.53 $\pm$ 0.23 ^a _b
b*					
4°C	16.12 $\pm$ 0.08 ^a _a	14.84 $\pm$ 0.05 ^b _a	14.42 $\pm$ 0.37 ^b _a	14.58 $\pm$ 0.15 ^b _a	16.23 $\pm$ 0.07 ^a _a
25°C	16.12 $\pm$ 0.08 ^a _a	16.88 $\pm$ 0.01 ^{a,b} _b	17.83 $\pm$ 0.41 ^b _b	18.14 $\pm$ 0.08 ^b _b	19.47 $\pm$ 0.24 ^c _b
37°C	16.12 $\pm$ 0.08 ^a _a	18.39 $\pm$ 0.02 ^b _c	17.95 $\pm$ 0.55 ^{b,c} _b	19.22 $\pm$ 0.59 ^{b,c} _b	17.73 $\pm$ 0.26 ^c _c

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Table IV.7 The stability of hue angle (°) in single pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
4°C	57.96 $\pm$ 0.26 ^a _a	54.22 $\pm$ 0.03 ^b _a	55.76 $\pm$ 0.78 ^b _a	55.90 $\pm$ 0.26 ^b _a	60.93 $\pm$ 0.12 ^c _a
25°C	57.96 $\pm$ 0.26 ^a _a	58.86 $\pm$ 0.12 ^a _b	63.24 $\pm$ 0.91 ^b _b	62.46 $\pm$ 0.27 ^b _b	66.37 $\pm$ 0.97 ^c _b
37°C	57.96 $\pm$ 0.26 ^a _a	60.95 $\pm$ 0.04 ^a _c	62.25 $\pm$ 1.07 ^{a,b} _b	65.02 $\pm$ 0.95 ^b _b	59.28 $\pm$ 0.87 ^a _a

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Table IV.8 The stability of chroma in single pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
4°C	19.02 $\pm$ 0.12 ^a _a	18.29 $\pm$ 0.06 ^{a,b} _a	17.41 $\pm$ 0.32 ^{a,b} _a	17.61 $\pm$ 0.23 ^{a,b} _a	18.57 $\pm$ 0.09 ^a _a
25°C	19.02 $\pm$ 0.12 ^a _a	19.73 $\pm$ 0.02 ^a _b	19.99 $\pm$ 0.63 ^{a,b} _b	20.46 $\pm$ 0.09 ^b _b	21.26 $\pm$ 0.25 ^b _b
37°C	19.02 $\pm$ 0.12 ^a _a	21.03 $\pm$ 0.03 ^b _c	20.21 $\pm$ 0.40 ^b _b	21.21 $\pm$ 0.49 ^b _b	20.63 $\pm$ 0.16 ^b _b

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

To account the overall changes in L^* , a^* , and b^* values of single pasteurized pulp throughout its stability study, overall color difference (ΔE^*) of single pasteurized pulp was derived using Hunter-Scotfield equation (section III.9) and shown in **Figure IV.9**. The largest overall color difference of 8.5 was observed when single pasteurized pulp was stored at 37°C. Furthermore, at the end of the storage study, overall color changes of approximately 4 and 1.5 were observed for samples that were stored at 25°C and 4°C, respectively. All ΔE^* values of single pasteurized pulp were visually perceptible and undesirable.

Figure IV.9 Overall color difference of single pasteurized pulp during storage study

IV.5.b. Stability study of double pasteurized pulp

Total phenolics content of double pasteurized pulp was not affected by storage temperature, storage time, and the interaction between storage temperature and storage time (**Table IV.10**). At 4°C storage temperature, there was a significant increase ($p < 0.05$) in total phenolics content of double pasteurized pulp, which can be observed starting at week two and remained stable until week eight (**Figure IV.10**). The observed slight increase of total phenolics content however, might not pose any practical importance. In comparison, total phenolics content of double pasteurized pulp were very stable when stored at 25°C. However, at 37°C storage temperature, total phenolics of double pasteurized pulp appeared to be the least stable as shown by a steeper decrease of phenolics retention by the end of the storage study when compared to those stored at 4°C and 25°C. On the 8th week, the retention of phenolics was only 70% compared to 99-100% when stored at 4°C and 25°C (**Table IV.9**). These observations were in agreement with previous studies which also reported a slight increase in phenols content in orange juice (Gil-Izquierdo et al., 2002) and the stability of phenolics content in kiwifruit during storage at refrigeration temperature for 2 months (Tavarini et al., 2008). Furthermore, by the end of the stability study, total phenolics values of double pasteurized pulp were slightly higher compared to those in single pasteurized pulp in each respective storage temperature. This suggested that degradation occurred faster during the storage of single pasteurized pulp.

Table IV.9 The stability of total phenolics in double pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
4°C	63.78 $\pm$ 0.32 ^a _a	72.21 $\pm$ 0.95 ^b _a	71.71 $\pm$ 0.59 ^b _a	72.93 $\pm$ 1.23 ^b _a	74.17 $\pm$ 1.42 ^b _a
25°C	63.78 $\pm$ 0.32 ^a _a	62.00 $\pm$ 1.48 ^a _b	64.05 $\pm$ 1.00 ^a _b	62.54 $\pm$ 1.16 ^a _b	63.06 $\pm$ 1.13 ^a _b
37°C	63.78 $\pm$ 0.32 ^a _a	58.33 $\pm$ 0.21 ^b _b	55.20 $\pm$ 2.37 ^b _c	44.25 $\pm$ 0.46 ^c _c	44.82 $\pm$ 0.68 ^c _c

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Table IV.10 Two-way ANOVA of the stability of total phenolics in double pasteurized pulp.

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	2354.9	2	1177.29	341.59	0
Time	142.21	4	35.55	10.32	0
Temperature*time	947.63	8	118.45	34.37	0
Error	103.39	30	3.45		
Total	3547.82	44

Figure IV.10 The stability of total phenolics in double pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Chemical antioxidant capacity of double pasteurized pulp was not affected by storage time and the interaction between storage temperature and period; however the influence of storage temperature was significant (**Table IV.12**). Based on the analysis of the stability of antioxidant capacity of double pasteurized pulp (**Figure IV.11**), the trend was very similar to its phenolics content (**Figure IV.10**). Antioxidant capacity of double pasteurized pulp remained stable and was very similar between 4°C and 25°C storage conditions. The result was in accordance with [Miller et al. \(1995\)](#), in which the antioxidant activity in apple juice remained stable in a 10 days storage study on apple juice at the 4°C and room temperature. Respectively, at 4°C, 25°C, and 37°C storage conditions, the retention of ORAC values by the end of the shelf life study were 100%, 95%, and 77%.

Table IV.11 The stability of antioxidant activity in double pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
4 °C	1681 $\pm$ 157 ^a _a	1828 $\pm$ 47 ^a _a	1917 $\pm$ 52 ^a _a	1917 $\pm$ 120 ^a _a	1726 $\pm$ 78 ^a _a
25 °C	1681 $\pm$ 157 ^a _a	1764 $\pm$ 43 ^a _a	1730 $\pm$ 34 ^a _{a,b}	1637 $\pm$ 49 ^a _a	1593 $\pm$ 48 ^a _a
37 °C	1681 $\pm$ 157 ^a _a	1591 $\pm$ 73 ^{a,b} _a	1468 $\pm$ 59 ^{a,b} _b	1270 $\pm$ 27 ^b _b	1294 $\pm$ 35 ^{a,b} _b

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Table IV.12 Two-way ANOVA of the stability of antioxidant activity in double pasteurized pulp.

Source	Sum Sq.	d.f.	Mean Sq.	F	Prob>F
Temperature	953144.7	2	476572.4	20.23	0
Time	219151.5	4	54787.6	2.33	0.0792
Temperature*time	366864.2	8	45858	1.95	0.0893
Error	706853.8	30	23561.8		
Total	2246013.1	44

Figure IV.11 The stability of antioxidant activity in double pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Color characteristics, L^* , a^* , b^* , of double pasteurized cocoa pulp, were summarized in **Table IV.13**. Those parameters were not affected by the interaction of storage temperature and time based on two-way ANOVA statistical method ($p < 0.05$) followed by Tukey significant difference test. However, the effect of individual variables of storage temperature and time is significant. Lightness (L^*) values of double pasteurized pulp decreased with storage time. L^* values were highest when stored at 25°C, which was slightly higher compared to 4°C and 37°C storage conditions. However, by the end of the storage study of double pasteurized pulp the lightness values were higher ($p < 0.05$) than single pasteurized pulp at the same storage time. This indicated that there might be a bleaching action from trace metals, oxygen and lights (Bohart and Carson, 1955). a^* and b^* values of double pasteurized, which were very similar to single pasteurized pulp, also increased with storage period in all three different storage temperatures. This is consistent with the visual appearance of the pulp that showed an increase in yellowness and redness, respectively.

Hue angle (**Table IV.14**) also increased with storage time with the highest value obtained when stored at 4°C, and followed by 37°C and 25°C storage conditions. By the end of the stability study, the values of hue angle were very similar to single pasteurized pulp. As expected, the trend of chroma (**Table IV.15**) values were consistent with single pasteurized pulp, highest when double pasteurized pulp were stored at 37°C, followed by 25°C and 4°C storage conditions.

Table IV.13 The stability of L*, a*, and b* in double pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
L*					
4 °C	46.68 $\pm$ 0.19 ^a _a	53.06 $\pm$ 0.06 ^b _a	52.84 $\pm$ 0.11 ^{b,c} _a	52.67 $\pm$ 0.09 ^{b,c} _a	53.46 $\pm$ 0.08 ^b _a
25 °C	46.68 $\pm$ 0.19 ^a _a	53.53 $\pm$ 0.53 ^b _a	54.35 $\pm$ 0.18 ^{b,c} _a	54.70 $\pm$ 0.05 ^{b,c} _b	55.39 $\pm$ 0.25 ^c _b
37 °C	46.68 $\pm$ 0.19 ^a _a	54.46 $\pm$ 0.19 ^b _b	52.16 $\pm$ 1.67 ^b _a	53.55 $\pm$ 0.09 ^b _c	53.26 $\pm$ 0.15 ^b _a
a*					
4 °C	7.97 $\pm$ 0.07 ^a _a	9.51 $\pm$ 0.07 ^b _a	9.47 $\pm$ 0.04 ^b _a	9.43 $\pm$ 0.05 ^b _a	8.97 $\pm$ 0.06 ^c _a
25 °C	7.97 $\pm$ 0.07 ^a _a	9.29 $\pm$ 0.09 ^b _a	8.84 $\pm$ 0.14 ^b _b	8.64 $\pm$ 0.07 ^b _b	8.37 $\pm$ 0.12 ^{a,b} _b
37 °C	7.97 $\pm$ 0.07 ^a _a	8.50 $\pm$ 0.18 ^{a,b} _b	8.35 $\pm$ 0.16 ^{a,b} _b	8.59 $\pm$ 0.09 ^b _b	8.14 $\pm$ 0.03 ^{a,b} _b
b*					
4 °C	9.99 $\pm$ 0.04 ^a _a	14.15 $\pm$ 0.10 ^b _a	14.16 $\pm$ 0.05 ^b _a	14.27 $\pm$ 0.04 ^b _a	14.04 $\pm$ 0.08 ^b _a
25 °C	9.99 $\pm$ 0.04 ^a _a	15.51 $\pm$ 0.46 ^b _b	16.40 $\pm$ 0.16 ^{b,c} _b	17.24 $\pm$ 0.14 ^c _b	17.65 $\pm$ 0.04 ^c _b
37 °C	9.99 $\pm$ 0.04 ^a _a	17.65 $\pm$ 0.09 ^b _c	18.36 $\pm$ 0.12 ^b _c	19.46 $\pm$ 0.17 ^c _c	19.01 $\pm$ 0.07 ^c _c

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Table IV.14 The stability of hue angle (°) in double pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
4 °C	51.40 $\pm$ 0.12 ^a _a	56.10 $\pm$ 0.07 ^b _a	56.22 $\pm$ 0.03 ^b _a	56.54 $\pm$ 0.10 ^b _a	57.42 $\pm$ 0.17 ^c _a
25 °C	51.40 $\pm$ 0.12 ^a _a	59.05 $\pm$ 0.51 ^b _b	61.67 $\pm$ 0.15 ^c _b	63.37 $\pm$ 0.23 ^d _b	64.62 $\pm$ 0.37 ^d _b
37 °C	51.40 $\pm$ 0.12 ^a _a	64.30 $\pm$ 0.37 ^b _c	65.38 $\pm$ 0.32 ^c _c	66.18 $\pm$ 0.17 ^{c,d} _c	66.81 $\pm$ 0.15 ^d _c

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Table IV.15 The stability of chroma in double pasteurized pulp at different storage conditions (mean $\pm$ SE, n=3).

Storage Temperature	Time (week)				
	0	2	4	6	8
4 °C	12.78 $\pm$ 0.07 ^a _a	17.04 $\pm$ 0.12 ^b _a	17.04 $\pm$ 0.06 ^b _a	17.10 $\pm$ 0.06 ^b _a	16.66 $\pm$ 0.09 ^c _a
25 °C	12.78 $\pm$ 0.07 ^a _a	18.08 $\pm$ 0.44 ^b _a	18.63 $\pm$ 0.20 ^{b,c} _b	19.28 $\pm$ 0.13 ^c _b	19.53 $\pm$ 0.02 ^c _b
37 °C	12.78 $\pm$ 0.07 ^a _a	19.59 $\pm$ 0.16 ^b _b	20.17 $\pm$ 0.17 ^{b,c} _c	21.27 $\pm$ 0.18 ^c _c	20.68 $\pm$ 0.05 ^c _c

Different superscript letters in the same storage temperature indicate significant difference ($p < 0.05$).

Different subscript letters in the same storage period indicate significant difference ($p < 0.05$).

Overall color difference (ΔE^*) of double pasteurized pulp during the stability study is shown in **Figure IV.12**. The largest overall color difference was observed on week 2 in all storage conditions. After week 2, overall color difference at 4°C storage condition remained stable with ΔE^* value of 8 by the end of the storage study. On the other hand, ΔE^* values of samples stored at 25°C and 37°C increased at similar rate until week 8. By the end of the storage study, double pasteurized pulp stored at 25°C and 37°C had ΔE^* values of approximately 12. Since the ΔE^* values of double pasteurized pulp were larger than 3, the changes in color were visually perceptible and undesirable.

Figure IV.12 Overall color difference of double pasteurized pulp during storage study.

IV.6. Cellular antioxidant activity of cocoa pulp

Cellular antioxidant activities of unpasteurized and pasteurized cocoa pulp were measured using CAA. The kinetics of fluorescence generation in HepG2 cells by ABAP are shown in **Figure IV.13-16**. The increase in fluorescence through DCF formation was inhibited by quercetin (**Figure IV.13.A-C**), unpasteurized (**Figure IV.14.A-C**), single pasteurized (**Figure IV.15.A-C**), and double pasteurized (**Figure IV.16.A-C**) cocoa pulp, in a dose dependent manner. To further calculate the EC₅₀ of quercetin and cocoa pulp, the dose-response curve (**Figure IV.17**) and median effect plots (**Figure IV.18**) were plotted for each sample, which were derived from the data presented in **Figure IV.13-16**.

The EC₅₀ values and median cytotoxicity doses of quercetin and cocoa pulp, as well as the CAA values of pulp expressed as μmol of QE/100g pulp, were listed in **Table IV.16**. The values summarized were inter-experimental triplicates. The EC₅₀ of quercetin standard was slightly higher than previously reported by [Wolfe and Liu \(2007\)](#), which was $5.92 \pm 0.07 \mu\text{M}$. The CAA values of unpasteurized and pasteurized cocoa pulp were expressed as micromoles of QE per 100 g pulp to compare their antioxidant quality with quercetin. Double pasteurized pulp was the most effective at inhibiting peroxyl radical induced DCFH oxidation, followed by single pasteurized and unpasteurized pulp, respectively. Nevertheless, unpasteurized pulp shared a similar CAA values with apple ($28.1 \pm 4.1 \mu\text{mol}$ of QE/100g) and red grape ($24.1 \pm 1.7 \mu\text{mol}$ of QE/100g) ([Wolfe and Liu, 2007](#)).

The trend of the cellular antioxidant activity of cocoa pulp indicated that pasteurization increased the CAA values. [Bravo \(1998\)](#) suggested that the absorption and

metabolism of phenolics were influenced by: 1) degree of glycoylation/ acylation, 2) basic structure, 3) conjugation with other phenolics, 4) molecular size, 5) degree of polymerization, and 6) solubility. Thus, considering the presence of pectin, cellulose, hemicellulose, and lignin in cocoa pulp, it was possible that the heat during pasteurization liberated phenolics from these insoluble matrices as well as broke down the larger hydrolyzable polyphenolics compounds, producing smaller compounds that exhibited higher response in CAA. Furthermore, cocoa pulp may contain phenolics in the stable glycosides forms which may be deglycosylated by heat into aglycones, which enhanced its bioavailability. [Williamson \(2004\)](#) proposed that glycosylated flavonoids must be deglycosylated before absorption as an absorption pathway of flavonoids. Aglycones that are released, diffuse into the epithelial cells in the small intestine, and may increase phenolics bioavailability through increased solubility/ coefficients of partition. Nevertheless, further investigations are needed to support the above propositions relevant in this study.

Figure IV.13 Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by quercetin in CAA assay. Graphs A, B, and C are generated from separate experiments (mean $\pm$ SD, $n=3$).

Figure IV.14 Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by unpasteurized cocoa pulp in CAA assay. Graphs A, B, and C are generated from separate experiments (mean $\pm$ SD, $n=3$).

Figure IV.15 Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by single pasteurized pulp in CAA assay. Graphs A, B, and C are generated from separate experiments (mean $\pm$ SD, n=3).

Figure IV.16 Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by double pasteurized pulp in CAA assay. Graphs A, B, and C are generated from separate experiments (mean $\pm$ SD, $n=3$).

Figure IV.17 Dose-response curves for inhibition of peroxyl radical-induced DCFH oxidation by quercetin (A) and cocoa pulp extracts: unpasteurized (B), single pasteurized (C), and double pasteurized (D) in CAA assay. The curves shown in each graph are from inter-experimental data (mean $\pm$ SE, $n=3$).

Figure IV.18 Median effect plots for inhibition of peroxyl radical-induced DCFH oxidation by quercetin (A) and cocoa pulp extracts: unpasteurized (B), single pasteurized (C), and double pasteurized (D) in CAA assay. The curves shown in each graph are from inter-experimental data (mean $\pm$ SE, n=3).

Table IV.16 Cellular antioxidant activities of quercetin and cocoa pulp expressed in EC₅₀ and CAA (mean $\pm$ SE, n=3).

	EC ₅₀	CAA μ mol of QE/ 100g pulp	Cytotoxicity mg/ml
Quercetin	7.00 $\pm$ 0.23 μ M	-	> 30
Unpasteurized pulp	30.45 $\pm$ 3.56 mg/ml ^a	23.71 $\pm$ 3.12 ^a	> 50
Single pasteurized pulp	21.41 $\pm$ 2.89 mg/ml ^b	34.13 $\pm$ 5.31 ^b	> 50
Double pasteurized pulp	12.41 $\pm$ 2.26 mg/ml ^c	59.76 $\pm$ 9.26 ^c	> 50

Different superscript letters within a column indicate significant difference ($p < 0.05$).

IV.7. Preliminary results on correlation analyses between chemical and cellular antioxidant assay

The correlation between ORAC and CAA of unpasteurized and pasteurized pulp were plotted in **Figure IV.19**. There was a negative correlation between ORAC and CAA values despite the similar underlying basis of ORAC and CAA. Both ORAC and CAA were based on the measurement of peroxy radical scavenging ability of samples. Furthermore, the high value of the correlation coefficient (r), which subsequently contributed to the high coefficient of determination (r^2) value, may not be used as a true measure of the strength of the correlation since only three data points were used. Additional data points are necessary to confirm the strength and direction of this preliminary correlation analysis. This preliminary correlation analysis was in agreement with studies done by [Wolfe et al. \(2008\)](#) in 25 fruits commonly consumed in United States and [Eberhardt et al. \(2005\)](#) in broccoli. Both of the studies found out a weak negative correlation between ORAC and CAA. All in all, cellular antioxidant measurement may provide information that is more relevant to physiological conditions in comparison to chemical antioxidant assay. However, further testing is needed to confirm the relationship between the CAA values of cocoa pulp and their modulation to oxidation stresses *in vivo*.

Figure IV.19 Correlation between ORAC and CAA of cocoa pulp extracts.

V. CONCLUSIONS

The conclusions of the study are summarized as below:

- The total phenolics content of cocoa pulp as quantified using Folin-Ciocalteu assay was 103.76 ± 4.79 mg GAE/100 g pulp. This value was very similar to the reported total phenolics of green grapes, which contain relatively low total phenolics content. Hence, cocoa pulp is not a great source of phenolics. Furthermore, the stability of phenolics in cocoa pulp was adversely affected by pasteurization. Single and double pasteurization decreased total phenolics content by 25% and 38%, respectively. Therefore, cocoa pulp contained heat-labile phenolics compounds.
- The ORAC value of unpasteurized cocoa pulp was 1871 ± 58 μ mol TE/ 100 g pulp, which was also similar to the reported ORAC values of green grapes. ORAC values, as a measure of chemical antioxidant activity of cocoa pulp, were not affected by pasteurization. The ORAC values of single and double pasteurized pulp were 1835 ± 41 μ mol TE/ 100 g pulp and 1681 ± 157 μ mol TE/ 100 g pulp, respectively. These values were not statistically different ($p>0.05$) if compared to unpasteurized pulp. Thermal treatment might release phenolics that possessed antioxidant activity from cell matrices or induces phenolics polymerization, which maintained ORAC values post-pasteurization.

- The cellular antioxidant activity of unpasteurized cocoa pulp (EC_{50}) was 23.71 ± 3.12 μ mol of QE/100g pulp. This value is very similar to the reported CAA values of apples and red grapes. Pasteurization enhanced cellular antioxidant activity of cocoa pulp. Single and double pasteurization significantly increased ($p < 0.05$) CAA values by 44% and 152%, respectively. Pasteurization increased the pool of bio-accessible antioxidant compounds, which subsequently led to their increased cell uptake, by releasing phytochemicals from cell matrices or inducing phenolics polymerization.
- There was a negative correlation between ORAC and CAA values in quantifying the antioxidant activity of cocoa pulp. The trend difference between chemical and cellular antioxidant activity occurred since ORAC assay neglect the uptake and metabolism of phenolics compounds. Further investigation *in vivo* is needed to better understand the relevancy of chemical and cellular antioxidant activity in physiological conditions.
- Overall color differences (ΔE^*) of single and double pasteurized pulp, relative to unpasteurized pulp, were 11.50 ± 6.64 and 14.96 ± 8.64 , respectively. Pasteurization had induced undesirable visual color differences possibly through Maillard browning reactions since darkening of single and double pasteurized pulp was perceived.

- Throughout an eight week storage study, single and double pasteurized pulps showed very little changes in phenolics and ORAC values when stored at 4°C and 25°C. However, at 37°C storage temperature, up to 50% relative loss in phenolics and 40% relative loss in ORAC values were observed in single pasteurized pulp; whereas double pasteurization resulted in lesser relative loss. It was likely that single pasteurization did not completely inactivate inherent enzymes or microorganisms that produce enzymes, which are capable of oxidizing phenolics compounds. Thereby, the activity of the enzymes together with higher storage temperature augmented the rate of phenolics degradation in the stability study of single pasteurized pulp in comparison to double pasteurized pulp.
- Both single and double pasteurized pulp showed the highest overall color differences (ΔE^*) values when stored at 37°C in comparison to 4°C and 25°C storage temperature. Higher storage temperature was more conducive for possible Maillard browning reactions to proceed progressively.

VI. FUTURE WORK

Based on the results obtained in the study, further investigations, as suggested below, will be necessary to complement this study:

- Identification of biologically active phenolics compounds, both free and bound, in cocoa pulp.
- Development of process parameters that optimize safety and nutraceutical profiles of cocoa pulp.
- Non-thermal processing of cocoa pulp that may increase the retention of phenolics and antioxidant activity.

REFERENCES

- Adamson, G.E.; Lazarus, S.A.; Mitchell, A.E.; Prior, R.L.; Cao, G.; Jacobs, P.H., Kremers, B.G.; Hammerstone, J.F.; Rucker, R.B.; Ritter, K.A.; Schmitz, H.H. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem.* **1999**, *47*, 4184-4188.
- Adams, M.R.; Dougan, J.; Glossop, E. J.; Twiddy, D.R. Cocoa sweatings – An effluent of potential value. *Agricultural Wastes* **1982**, *4*, 225-229.
- Asami, D.K.; Hong, Y.J.; Barrett, D.M.; Mitchell, A.E. Processing-induced changes in total phenolics and procyanidins in clingstone peaches. *J Sci Food Agric.* **2003**, *83*, 56-63.
- Bangerter, U.; Beh, B.H.; Callis, A.B.; Pilkington, I.J. Treatment of cocoa beans for improving fermentation. U.S Patent 5,342,632, Aug 30, 1994.
- Bank, G.; Schauss, A. Antioxidant Testing: An ORAC Update, 2004. Nutraceuticals World. <http://nutraceuticalsworld.com/contents/view/13273> (accessed Nov 11, 2010).
- Bates, R.P.; Morris, J.R.; Crandall, P.G. *Principles and practices of small- and medium-scale fruit juice processing*; FAO Agricultural Services Bulletin 146; FAO: Rome, 2001.
- Beckett, S.T. Chocolate Ingredients. *The Science of Chocolate* 2nd ed.; Royal Society of Chemistry: Cambridge, UK; pp 11-38.
- BeMiller, J. N.; Whistler, R.L. Carbohydrates. In: *Food Chemistry*, 3rd ed.; Fennema, O. R. Ed. CRC Press: Boca Raton, FL, 1996; pp 157-223.
- Biehl, B.; Meyes, B.; Crone, G.; Pollmann, L. Chemical and physical changes in the pulp during ripening and post-harvest storage of cocoa pods. *J. Sci. Food Agric.* **1989**, *48*, 189-208.
- Biehl, B.; Ziegler, G. Cocoa: Chemistry of processing. In: *Encyclopedia of Food Sciences and Nutrition*, 2nd ed.; Caballero, B; Trugo, L.C.; Finglas, P.M., Eds. Academic Press: Oxford, 2003; pp 1436-1448.
- Bohart, G.S.; Carson, J.F. Effects of trace metals, oxygen, and light on the glucose-glycine browning reaction. *Nature* **1955**, *175*, 470-471.
- Boyer, J.; Brown, D.; Liu, R.L. Uptake of quercetin and quercetin-3-glucoside from whole onion and apple peel extracts by Caco-2 cell monolayers. *J. Agric. Food Chem.* **2004**, *58*, 7172-7179.

- Boyles, M.J.; Wrolstad, R.E. Anthocyanin composition of red raspberry juice: influences of cultivar, processing, and environmental factors. *J Food Sci.* **1993**, *58*, 1135-1141.
- Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews* **1998**, *56*, 317-333.
- Buamah, R.; Dzogbefia, V.P.; Oldham, J.H. Pure yeast culture fermentation of cocoa (*Theobroma Cacao L.*): effect of sweating yield and cocoa bean quality. *World J. Microbiol. Biotech.* **1997**, *13*, 457-462.
- Cao, G.; Prior, R.L. Measurement of oxygen radical absorbance capacity in biological samples. *Methods in Enzymology* **1999**, *299*, 50-62.
- Chang, Qi; Zuo, Zhang; Chow, M.S.S; Ho, Walter, K.K. Effect of storage temperature on phenolics stability in hawthorn (*Crataegus pinnatifida* var. *major*) fruits and a hawthorn drink. *Food Chemistry* **2006**, *98*, 426-430.
- Chun, O.K.; Kim, D.A.; Smith, N.; Schroeder, D.; Han, J.T.; Lee, C.Y. Daily consumption of phenolics and total antioxidant capacity from fruit and vegetables in the American diet. *J Sci Food Agric.* **2005**, *85*, 1715-1724.
- Cocoa Research Institute of Ghana. Cocoa alcohol.
http://www.crig.org/by_products_subcat_list.php?id=18 (accessed Oct 16, 2010).
- Cocoa Research Institute of Ghana. Cocoa pulp juice (sweatings) products.
http://www.crig.org/by_products_subcat_list.php?id=2 (accessed Oct 16, 2010).
- Color Space in Microscopy Imaging. SPOT Imaging Solutions.
<http://www.diaginc.com/iq/colorspace.html> (accessed May 12, 2010).
- Dewanto, V.; Wu, X.; Liu, R.L. Processed sweet corn has higher antioxidant activity. *J. Agric. Food Chem.* **2002a**, *17*, 4959-4964.
- Dewanto, V.; Wu, X.; Adom, K.K.; Liu, R.L. Thermal Processing Enhances the Nutritional Value of Tomatoes by Increasing Total Antioxidant Activity. *J. Agric. Food Chem.* **2002b**, *50*, 3010-3014.
- Dias, D.R.; Schwan, R.F.; Freire, E.S.; Santos Serôdio, R. Elaboration of a fruit wine from cocoa (*Theobroma cacao L.*) pulp. *International Journal of Food Sci. and Tech.* **2007**, *42*, 319-329.
- Dominican Republic Live. The cacao: All about cacao. <http://www.dominican-republic-live.com/dominican-republic/beauty/the-cacao.html> (accessed Mar 16, 2011).
- Eberhardt, M.V.; Lee, C.Y.; Liu, R.H. Nutrition – Antioxidant activity of fresh apple. *Nature.* **2000**, *405*, 903-904.

- Farnworth, E.R.; Lagacé, M.; Couture, R.; Yaylayan, V.; Stewart, B. Thermal processing, storage conditions, and the composition and physical properties of orange juice. *Food Research International*. **2001**, *34*, 25-30.
- Fellows, P.J. *Food processing technology: Principles and practice*, 2nd ed.; CRC Press: Boca Raton, FL, 2000.
- Francis, F.J.; Clydesdale, F.M. *Food colorimetry: Theory and applications*. The AVI Publishing Co.: Westport, CT, 1975.
- Frankel, E. N.; Meyer, A. S. The problems of using one dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925–1941.
- Garza, S.; Ibarz, A. ; Pagán, Giner, J. Non-enzymatic browning in peach puree during heating. *Food Research International*. **1999**, *32*, 335-343.
- Gil-Izquierdo, A.; Gil, M.I.; Ferreres, F. Effect of processing techniques at industrial scale on orange juice antioxidant and beneficial health compounds. *J. Agric. Food Chem.* **2002**, *50*, 5107-5114.
- Haard, N. F.; Chism, G.D. Characteristics of Edible Plant Tissues. In: *Food Chemistry*, 3rd ed.; Fennema, O. R. Ed. CRC Press: Boca Raton, FL, 1996; pp 943-1011.
- Hager, T.J.; Howard, L.R.; Prior, R.L. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blackberry products. *J. Agric. Food Chem.* **2008**, *56*, 689-695.
- Heldman, D.R.; Hartel, R.W. *Principles of Food Processing*; Aspen Publishers: Maryland, 1997.
- Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J.A.; Prior, R.L. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* **2002**, *50*, 4437-4444.
- Iversen, C.K. Black currant nectar: Effect of processing and storage on anthocyanin and ascorbic acid content. *J Food Sci.* **1999**, *64*, 37-41.
- Keen, C.L; Holt, R.R; Oteiza, P.I; Fraga, C.G; Schmitz, H.H. Cocoa antioxidants and cardiovascular health. *Am J Clin Nutr.* **2005**, *81*, 298S–303S.
- Lee, H.S; Coates, G.A. Effect of thermal pasteurization on Valencia orange juice color. *Lebensmittel-Wissenschaft und-Technologie*. **2003**, *36*, 153-156.
- Lewis, M.J. Thermal processing. In: *Food Processing Handbook*; Brenna; J.G., Ed. Wiley-VCH Verlag GmbH & Co: KGaA, Weinheim, Germany, 2006, pp 33-70.

- Lopez, A.S. Fermentation and organoleptic quality of cacao as affected by partial removal of pulp juices from the beans prior to curing. *Rev. Theobroma*. **1979**, 9, 25-37.
- Lopez, A.S.; Dimick, P.S. Enzymes involved in cocoa curing. In: *Food Enzymology*, Fox, P.F., Ed. Elsevier: Amsterdam; 1991; pp.211-236.
- Makkar, H.P.S.; Bluemmel, M.; Borowy, N.K.; Becker, K. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. *Journal of the Science of Food and Agriculture*. **1993**, 61, 161–165.
- Malaysian Cocoa Board. Products: cocoa pulp jelly and cocoa pulp juice. <http://www.koko.gov.my/lkm/> (accessed Oct 16, 2010).
- McMurrough, I.; Madigan, D.; Smyth, M.R. Adsorption by polyvinylpolypyrrolidone of catechins and proanthocyanidins from beer. *J.Agric. Food Chem.* **1995**, 43, 2687-2691.
- Miller, N.J.; Diplock, A.T.; Rice-Evans, C.A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J.Agric. Food Chem.* **1995**, 43, 1794-1801.
- Nicoli, M.C.; Anese, M.; Parpinel, M.T.; Franceschi, S.; Lericci, C.R. Study on Loss and/or Formation of Antioxidants during Processing and Storage. *Cancer Letters*. **1997**, 114, 71-74
- Nicoli, M.C.; Anese, M.; Parpinel, M.T. Influence of processing on the antioxidant properties of fruit and vegetables. *Trends in Food Science & Technology*. **1999**, 10, 94-100.
- Nisperos-Carriedo, M.O.; Shaw, P.E. Comparison of volatile flavor components in fresh and processed orange juices. *J. Agric. Food Chem.* **1990**, 36, 1048-1052.
- Noé, V; Peñuelas, S; Lamuela-Raventós, R.M; Permanyer, J; Ciudad, C.J; Izquierdo-Pulid, M. Epicatechin and a Cocoa Polyphenolic Extract Modulate Gene Expression in Human Caco-2 Cells. *J. Nutr.* **2004**, 134, 2509-2516.
- Ou, B.; Hampsch-Woodill, M.; Prior, R.L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, 49, 4619-4626.
- Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods – 2007. USDA ARS.
- Pereira, C.C.; Ribeiro Jr., C.P.; Nobrega, R.; Borges, C.P. Pervaporative recovery of volatile aroma compounds from fruit juices. *Journal of Membrane Science*. **2006**, 274, 1-23.
- Pettipher, G.L. Analysis of cocoa pulp and the formulation of a standardized artificial cocoa pulp medium. *J. Sci. Food Agric.* **1986**, 37, 297-309.

- Prior, R.L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsh-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for Hydrophilic and Lipophilic Antioxidant Capacity (oxygen radical absorbance capacity (ORAC_{FL})) of Plasma and Other Biological and Food Samples. *J. Agric. Food Chem.* **2003**, *51*, 3273-3279.
- Prior R.L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290-4302.
- Rakić, S.; Petrović, S.; Kukić, J.; Jadranin, M.; Tešević, V.; Povrenović, D.; Šiler-Marinković, S. Influence of thermal treatment on phenolic compounds and antioxidant properties of oak acorns from Serbia. *Food Chemistry* **2007**, *104*, 830-834.
- Ramaswamy, H.S.; Chen, C.; Marcotte, M. Novel Processing Technologies for Food Preservation. In: *Processing Fruits: Science and Technology*, 2nd ed.; Barrett, D.M.; Somogyi, L.; Ramaswamy, H., Eds. CRC Press: Boca Raton, FL, 2004; pp 201-219.
- Rickman, J.C.; Barrett, D.M.; Bruhn, C.M. Nutritional comparison of fresh, frozen, and canned fruits and vegetables. Part 1. Vitamins C and B and phenolic compounds. *J Sci Food Agric.* **2007**, *87*, 930-944.
- Royall, J. A.; Ischiropoulos, H. Evaluation of 2',7'-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H₂O₂ in cultured endothelial cells. *Arch. Biochem. Biophys.* **1993**, *302*, 348-355.
- Salamone, S. Computing a Sweeter Chocolate Future, 2010. Smarter Technology. <http://www.smartertechnology.com/c/a/Technology-For-Change/Computing-a-Sweeter-Chocolate-Future/> (accessed Oct 16, 2010).
- Scalbert, A.; Williamson, G. Dietary Intake and Bioavailability of Polyphenols. *J. Nutr.* **2000**, *130*, 2073S-2085S.
- Schwan, R.F.; Wheals, A.E. The microbiology of cocoa fermentation and its role in chocolate quality. *Crit. Rev in Food Sci. and Nut.* **2004**, *44*, 205-221.
- Shahidi, F. and Ho, C.T. Antioxidant measurement and applications: An Overview. In: *Antioxidant measurement and applications*, ACS Symposium Series 956; Shahidi, F. and Ho, C.T., Eds. American Chemical Society: Washington, DC, 2007: pp 2-7.
- Singleton, V.; Orthofer, R.; Lamuela-Raventos, R. Analysis of total phenolics and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods. Enzymology.* **1999**, *299*, 152-178.

- Song, W.; Derito, C.M.; Liu, K.; He, X.; Dong, M.; Liu, R.L. Cellular antioxidant activity of common vegetables. *J. Agric. Food Chem.* **2010**, *58*, 6621-6629.
- Sukha, D. A. Potential value added products from Trinidad and Tobago cocoa. <http://sta.uwi.edu/cru/Potential-value-added-prod4cocoa-inTnT.pdf> (accessed June 29, 2009)
- Talcott, S.T.; Percival, S.S.; Pittet-Moore, J.; Celoria, C. Phytochemical composition and antioxidant stability of fortified yellow passion fruit (*Passiflora edulis*). *J. Agric. Food Chem.* **2003**, *51*, 935-941.
- Tavarini, S.; Degl'Innocenti, E.; Remorini, D.; Massai, R.; Guidi, L. Antioxidant capacity, ascorbic acid, total phenols, and carotenoids changes during harvest and after storage of Hayward kiwifruit. *Food Chemistry*. **2008**, *107*, 282-288.
- Tomás-Barberán, F.A.; Espín, J.C. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agric.* **2001**, *81*, 853-876.
- Thompson, S.S.; Miller, K.B.; Lopez, A.S. Cocoa and Coffee. In: *Food microbiology: Fundamentals and Frontiers*, 2nd ed.; Doyle, M.P; Beuchat, L.R.; Montville, T.J., Eds. American Society of Microbiology Press: Washington DC, 2001; pp 722-33.
- Wang, H.; Helliwell, K. Epimerisation of catechins in green infusions. *Food Chemistry* **2000**, *70*, 337-344.
- Wang, H.; Joseph, J. A. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biol. Med.* **1999**, *27*, 612-616.
- Weemaes, C.A.; Ooms, V.; Van Loey, A.M; Hendrickx, M.E. Kinetics of chlorophyll degradation and color loss in heated broccoli juice. *J. Agric. Food Chem.* **1999**, *47*, 2404-2409.
- Whitaker, J. R. Enzymes. Characteristics of Edible Plant Tissues. In: *Food Chemistry*, 3rd ed.; Fennema, O. R. Ed. CRC Press: Boca Raton, FL, 1996; pp 431-530.
- Williamson, G. Common features in the pathways of absorption and metabolism of flavonoids. In: *Phytochemicals – Mechanism of action*; Meskin, M.S.; Bidlack, W.R.; Davies, A.J.; Lewis, D.S.; Randolph, R.K., Eds. CRC Press: Boca Raton, FL, 2004; pp 21-33.
- Wolfe, K.L.; Kang, X.; He, X.; Dong, M.; Zhang, Q.; Liu, R.L. Cellular antioxidant activity of common fruits. *J. Agric. Food Chem.* **2008**, *56*, 8418-8426.
- Wolfe, K.L.; Liu, R.H. Cellular Antioxidant Activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J. Agric. Food Chem.* **2007**, *55*, 8896-8907.

- Wrolstad, R.E.; Durst, R.W.; Lee, J. Tracking color and pigment changes in anthocyanin products. *Trends in Food Science and Technology*. **2005**, *16*, 423-428.
- Wu, X.; Beecher, G.R.; Holden J.M.; Haytowitz, D.B.; Gebhardt, S.E.; and Prior, R.L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026-4037.
- Xu, B.; Chang, S.K.C.; Liu, Z.; Yuan, S.; Zou, Y.; Tan, Y. Comparative studies on the chemical and cell-based antioxidant activities and antitumor cell proliferation properties of soy milk manufactured by conventional and commercial UHT methods. *J. Agric. Food Chem.* **2010**, *58*, 3558-3566.
- Yamada, M.; Komatsu, S.; Shirasu, Y. Changes in components of canned coffee beverage stored at high temperature. *Colloque Scientifique International sur le Café* **1997**, *17*, 205-210.
- Yen, G.C.; Lin, H.T. Effects of high pressure and heat treatment on pectic substances and related characteristics in guava juice. *J Food Sci.* **1998**, *63*, 684-687.
- Yen, G.C; Lin, H.T. Changes in volatile flavor components of guava juice with high-pressure treatment and heat processing and during storage. *J. Agric. Food Chem.* **1999**, *47*, 2082-2087.
- Yeom, H.W.; Streaker, C.B.; Zhang, Q.H.; Min, D.B. Effects of Pulsed Electric Fields on the Quality of Orange Juice and Comparison with Heat Pasteurization. *J. Agric. Food Chem.* **2000**, *48*, 4597-4605.