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COLOR AS AN INDICATOR OF FLAVANOL CONTENT IN THE FRESH SEEDS OF *THEOBROMA CACAO L.*

A Thesis in

Food Science

by

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ABSTRACT

Chocolate has recently gained attention for its high levels of flavanol antioxidants. These compounds, implicated in protection against cardiovascular disease, inflammation, and cancer, have become the object of a large quantity of research that seeks to fully appreciate the significance of flavanols for human health. Continuing in that vain, this thesis aims to recognize the bioactive role of flavanols in chocolate, but points out that flavanols are important not only in terms of human health, but also to other issues in chocolate production. Flavanols contribute to chocolate flavor and may also offer disease resistance to cacao seeds prior to harvest.

Both flavor and disease resistance are difficult to breed for and a better understanding of their association through flavanols could greatly assist in more efficient management of the crop. The goal of this thesis is to develop a marker for economically important traits through these central flavanol compounds. Since pigments in fresh cacao seeds are flavanols themselves, the thesis proposes that fresh seed color may be a ready indicator of native flavanol content in the fresh seed.

Two hundred seeds from 14 different varieties, chosen to cover the full range of color, were studied in order to develop a marker for flavanol content. Seeds were assessed in terms of light reflectance properties and mapped on scales of observable color based on a standard human observer. The measurements were compared to a chemical characterization of color based on anthocyanin concentrations from individual seed extracts. Through HPLC-MS, four anthocyanin pigments were identified, two of which were previously unknown in cacao. A significant correlation was found between total

anthocyanin concentration and lightness of the seed. Additional variation in seed color was explained through *in vivo* effects such as copigmentation and pH of the cell environment.

In order to assess the ability of color to act as a marker for flavanols, concentrations of other flavanols (catechins, and procyanidins) were measured as well. Seeds were found to contain total extractable flavanol concentrations ranging from 1.25 to 26 μg/ml catechin equivalents per gram seed dry weight. A positive relationship was uncovered in the sample set between procyanidin and anthocyanin concentrations recorded for seeds. This trend was consistent for subsets of the data grouped by pod, with the exception of one pod whose seeds showed a negative relationship between anthocyanins and procyanidins. Apparently, all flavanol subspecies are coregulated in seeds throughout the species, although there is opportunity for exceptions or fine-tuning mechanisms.

In testing observable color as a predictor for flavanol content, a statistically significant relationship was established. Lightness of the outer surface of the seed was found to be the best measure of the flavanol content, with lighter seeds containing low levels of flavanols, and darker seeds containing higher concentrations. Colorimetric measurements of seed lightness can be used to estimate flavanol concentrations to within ± 4 μg/mL catechin equivalents per gram seed dry weight (on average).

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PREFACE

This thesis is organized into chapters written as scientific papers to be submitted for publication. As a result each contains an independent focus. However, they sometimes overlap, although in the context of this thesis they are compiled to lay out a continuous story. Chapters are placed in a specific order prefaced by an introduction and concluding with a final remarks section. In order to reduce repetition, references for all chapters may be found at the end of the thesis.

The first article is a review article in which an argument is made for the importance of flavanols in the various traits surrounding *Theobroma cacao* L. It outlines a new direction of research and therefore serves well as the literature review, introducing the hypothesis for the experimental work to follow.

The second and third articles focus on method development. In preliminary studies on seed color, novel compounds were identified. These compounds are introduced in the second article which expands the concept of cacao seed color, useful in subsequent chapters. The third article describes the separation of procyanidin compounds. Characterization of procyanidins in the past has represented a chromatographic challenge. Here, an alternative approach is introduced.

The final article concentrates on answering the primary question of the thesis. Putting aside background issues about methodology or the intricacies of color, this paper answers the question of how color may be used as a practical indicator for flavanol content.

Although the papers list co-authors, I am the primary author in each of the works. The concept for the research developed from conversations with my advisors. Collection

of samples was done through collaboration with cocoa research stations. Colorimetric analyses and sample preparation were done by me. High performance liquid chromatography and mass spectroscopy were accomplished through the Intercollegiate Mass Spectroscopy Center.

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I would like to express my sincere gratitude to my advisors for their support in the completion of this thesis. They allowed me ample room for creativity, and, as a result, I feel very proud of the confidence gained through this work. Trying to complete a thesis which is multidisciplinary in nature requires a great openness and willingness on the part of the professors involved, and I was extremely blessed to have such insightful mentors.

I'd like to thank the scientists who graciously worked with me from CATIE, Costa Rica, University of the West Indies, Trinidad, INRA, Cameroon and USDA Tropical Research Station in Mayaguez, Puerto Rico. I was shown great hospitality from these people who procured samples for me repeatedly and sent them over great distances. Studying cacao in Pennsylvania is a challenge of the imagination, and without their practical experience and advice, this research would have been impossible.

I would finally like to thank all the graduate students of the Food Science Department who were my family for the time I spent at Penn State. They made me a second home in Borland Lab, and I feel very grateful to have been able to work in such a supportive and sharing environment. Similarly, it was a real gift to be able to share lab meetings with the cacao research group in the horticulture department. I learned so much about what it means to be a scientist through interactions with them.

This thesis is dedicated to my Dad.

In following in your footsteps, I find the secrets of your heart that you left along the way.

CHAPTER 1

INTRODUCTION

Chocolate is an ancient medicine. Man has been making and eating it for over 3000 years (Coe and Coe, 1996), and even today chocolate is still being rediscovered for its health benefits. Most recently, chocolate has gained attention for its antioxidant properties which are thought to reduce cardiovascular disease and protect against cancer (Wollgast and Anklam, 2000b). The claim is irresistible to chocolate consumers, and while scientists work hard to test the validity of the claim (Zhu et al., 2002; Kris-Etherton and Keen, 2002; Rein et al., 2000; Wan et al., 2000) methods for capitalizing on the antioxidant properties of chocolate are currently underway (Kealey et al., 2000). At the center of all this attention is a group of chemical compounds called flavanols.

Flavanols are polyphenolic compounds whose structure offers free radical scavenging ability. They are present in unusually high amounts in many foods like tea (McKay and Blumberg, 2002), red wine, and chocolate (Hammerstone et al., 2000). Aside from their bioactive properties, they also play important roles in chocolate flavor development.

Chocolate comes from seeds of the tree *Theobroma cacao* L. Before they become chocolate, these seeds undergo a long post-harvest process which includes fermentation and roasting. The plant and all of its products before processing are properly termed "cacao" (Coe and Coe, 1996). In all of the stages, from the raw seed to the final chocolate product, flavanols are present. Initially, concentrations are determined by the genetic identity of the tree, as flavanols are produced to offer disease resistance to growing seeds. As seeds are harvested for making chocolate, these compounds continue to play a role in flavor development.

Because flavanols are involved in the manifestation of important characteristics of the cacao tree and the chocolate product, there is reasonable incentive to learn more about their existence and how to manage their concentrations, especially in the tree itself. However, crop management of *T. cacao* is complicated by a lack of connection between genetic identity and expressed phenotypes. For example, it is not known exactly how flavanols are regulated by the cacao tree, nor is it known which varieties in the species synthesize the greatest amounts.

While it is not easy to distinguish between cacao varieties containing high levels of flavanols and those containing low levels, it is easy to distinguish cacao varieties by seed color. Fresh cacao seeds are brilliantly colored in shades of purple, pink and ivory, and are easy to distinguish prior to processing. These colors are closely linked to genetic identity and are widely used to distinguish the major cultivars. Moreover, the chemical compounds responsible for color are flavanols themselves, which provides ample reason to believe that seed color may be indicative of the total concentration of flavanols in the raw seed.

This thesis investigates the relationship between flavanol content and fresh seed color throughout the *Theobroma cacao* L. species. Defining the connection between the two will result in a practical marker for flavanols in the field where selection and breeding takes place. In addition, looking at flavanol profiles across the species will provide clues as to how flavanols are genetically regulated. The ultimate goal of the thesis is to create a basis for more informed and effective crop management of *T. cacao* and for bean blending for chocolate flavor or antioxidant properties.

CHAPTER 2

FLAVANOLS AS A GUIDE TO EXPRESSED TRAITS IN *THEOBROMA CACAO* L.

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2.1 ABSTRACT

Chocolate has gained much attention recently for its potential as a functional food. This is primarily due to a class of polyphenolic compounds called flavanols, widely recognized for their antioxidant capacity. Chocolate, a product of the seeds of the cacao tree (*Theobroma cacao* L.), contains naturally high levels of flavanols which have been extensively studied for their effect on human health, but quite possibly provide important functions to the raw seed as well. This review aims to combine the literature on the functionality of flavanols by showing how flavanols confer diesase resistance to the plant, affect flavor development, and contribute to sensory characteristics in the final chocolate product. Integration of what is known about flavanols in these different contexts leads to a more informed management of the cacao crop. The relevance of flavanols in crop improvement is discussed. Furthermore, a method is delineated in which the unique characteristics of flavanols may be used in the development of a field marker for cacao.

KEYWORDS: cacao, chocolate, *Theobroma cacao* L., flavanols, procyanidins, anthocyanins, disease resistance, flavor development, antioxidants, seed color.

2.2 INTRODUCTION

Flavanols are polyphenolic compounds whose structure offers free radical scavenging ability. Studies have suggested that they have certain preventive effects against cancer, heart disease and inflammation (Borchers et al., 2000). Following the discovery of unusually high amounts in commonly consumed foods like tea (McKay and Blumberg, 2002), red wine, and chocolate (Hammerstone et al., 2000), studies investigating the extent of their functionality in foods have been ubiquitous (Borchers et al., 2000). Chocolate, in particular, has been the subject of numerous papers investigating the effects of flavanols on human health (Borchers et al., 2000) and in the past three years over 16 US patents have been granted involving flavanol enrichment in chocolate and administration of the food as a health product (U.S. Patent & Trademark Office, 2003). Although much of the work done thus far has focused on flavanols for direct application in chocolate food products, this review broadens the perspective to consider the role of these compounds in the fresh seeds of the raw material, *Theobroma cacao* L.

In all stages of chocolate production, from the raw seed to the final chocolate product, flavanols are present. Initially, concentrations are determined by the genetic identity of the tree and environmental conditions, as flavanols are synthesized to offer disease resistance to growing seeds. As seeds are harvested for making chocolate, these compounds continue to play a role in flavor development, contributing to the final sensory profile of chocolate.

Because flavanols are involved in the manifestation of important characteristics of the cacao tree and the chocolate produced from its seeds, there is reasonable incentive to learn more about their existence and how to manage their concentrations, especially in the tree itself. However, crop management of *T. cacao* is complicated by a lack of connection between genetic identity and expressed phenotypes. For example, it is not known exactly how flavanols are regulated by the cacao tree, nor is it known which varieties in the species are present in the highest amounts.

While it is not easy to distinguish between cacao varieties containing high levels of flavanols and those containing low levels, it is easy to distinguish cacao varieties by seed color. Fresh cacao seeds are brilliantly colored in shades of purple, pink and ivory, and are easy to distinguish prior to processing. These colors are closely linked to genetic identity and are widely used to distinguish the major cultivars. Moreover, the chemical compounds responsible for color are flavanols themselves, which provides ample reason to believe that seed color may be indicative of the total concentration of flavanols in the raw seed.

The purpose of this review is to introduce flavanols as a potential marker for economically important traits in *T. cacao*. By combining the diverse literature on flavanol functionality in various contexts, a case is made for the probable involvement of flavanols in the survival of fresh seeds, during post-harvest processing, and in the final chocolate product. Integration of this information in crop management is discussed and a research strategy is proposed for the development of flavanols as a potential field marker.

2.3 FLAVANOL FUNCTIONALITY

Flavanols are naturally occurring plants compounds (Borchers et al., 2000) characterized by a common three-ring structure, labeled A, B and C by convention (Figure 2-1). Flavanols consist of three subgroups: anthocyanins, catechins and procyanidin polymers (Figure 2-2).

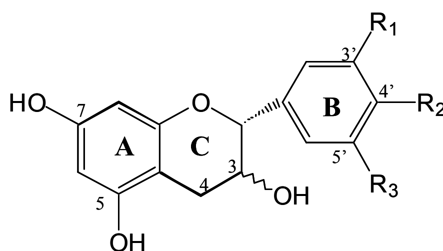

Figure 2-1. Basic structure and standard numbering system for flavanols (Stafford, 1990).

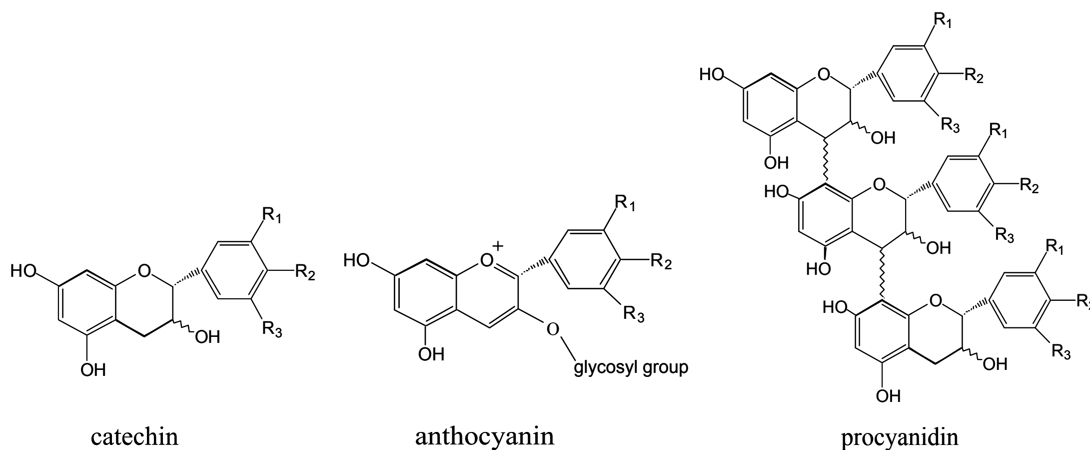

Figure 2-2. Basic structure of flavanol subclasses: catechins, anthocyanidins and procyanidins. R groups signify either a -OH or -H moiety (Stafford, 1990).

In fresh *T. cacao* seeds, flavanols represent approximately 13.5% of the seed dry weight (Ziegleder and Biehl, 1988). Those which have been specifically identified in cacao are listed in Table 1. Despite their substantial concentrations, for a long time, the function of flavanols in plant tissues remained elusive. It was hypothesized that they might act as signaling compounds, but as secondary metabolites, they were not generally considered to be an essential part of plant metabolism (Stafford, 1990).

Today, flavanols are recognized as multi-functional compounds. Their structure confers free-radical scavenging ability (Bravo, 1998), the ability to complex with protein (Stafford, 1990) and light absorbing qualities (Chen and Hrazdina, 1981). In humans, as has been well publicized, they may have the potential to aid against certain diseases. In the plant, these functions can translate into a defense response. In chocolate, the bitter and astringent properties of flavanols have been recognized and integrated into the sensory profile crucial to consumer acceptance. There already exist excellent reviews concerning the functionality of chocolate flavanols in the human body (Borchers et al., 2000), but the following two sections delineate the current progress in understanding the lesser emphasized functionality of flavanols in terms of seed resistance in *T. cacao* and chocolate flavor development.

Table 2-1. Flavanols found in dry cacao seeds (Ziegleder and Biehl, 1988).

Catechins (flavano-3-ols)	(+) - catechin (-) - epicatechin (+) - gallocatechin (+) - epigallocatechin
Anthocyanins	3-α-L- arabinosidyl cyanidin 3-β-D-galactosidyl cyanidin
Procyanidins (leucocyanidins, proanthocyanidins)	polymers of the above

Flavanols in T. cacao seeds resistance

Flavanols are generally known to act as defense chemicals in plants (Stafford, 1990). But in considering the role of flavanols in the part of the plant most relevant to chocolate, namely, the *T. cacao* seed, studies on the effect of flavanols on resistance have been few to none. The argument made here, that flavanol content in the seed may offer some resistance, is based on studies dealing with the functionality of flavanols and experiments conducted in other species.

To begin with, flavanol catechins and procyanidins have been found to possess bitter and astringent properties (Delcour et al., 1984), bestowing upon them the ability to act as gustatory deterrents, staving off potential predators from feasting on an otherwise nutritious seed (Stafford, 1990). The research of Delcour et al. (1984) showed that bitterness and astringency due to catechins and procyanidins seem to be on a continuum driven by the degree of polymerization. These studies were conducted with respect to human taste, but since the offensive "drying" sensation is caused by a flavanol polymer complexing with proteins on the tongue (Lawless and Hildegarde, 1988), it is likely that some animals find flavanols to be somewhat of a deterrent.

The ability of flavanol polymers to complex with proteins can also lead to other protective effects, depending on when and where it occurs. Free procyanidins allowed to complex with digestive enzymes, for example, may significantly inhibit a predator's digestive capabilities, depending on the organism. Flavanols in complex with plant

proteins prior to ingestion can also decrease the absorption of amino acids decreasing nutritional quality and discouraging continued consumption (Stafford, 1990).

Others have suggested that certain flavanols are able to disrupt DNA (Chisey et al., 1988). This could definitely play a defense role against attacking microbes, but is a surprising result considering the number of other studies that taut flavanols as antioxidant "protector" molecules (Borchers et al., 2000).

One final mechanism for plant defense via flavanols involves cell detoxification. It was recently pointed out that flavanol synthesis in plant cells mirrors the mechanism for cellular detoxification by exocytosis (Marrs, 1996). Marrs (1996) has proposed that since flavanol synthesis is so similar to the flushing of cell toxins, perhaps up-regulation of flavanol synthesis in response to stress is synonymous, or linked to, cell detoxification as another method of defense.

All the above theories are based on known characteristics of flavanols alone, but in an actual plant system, low procyanidin contents have been correlated with low seed vigor in a number of other legumes: fava bean (Kantar et al., 1996), chickpea (Knights and Mailer, 1989), peas (Powell, 1989), and French beans (Dickson and Petzoldt, 1988). In these studies, low phenolic contents in seeds apparently lead to cracks in the seeds which imbibe water and encourage mold growth. In fava beans, it was demonstrated that most zero-procyanidin seeds which performed well in the laboratory performed very poorly out in the field (Kantar et al., 1996). Other supporting studies show an inverse relationship between high procyanidin concentration in sorghum and mold infection (Jambunathan et al., 1986).

In summary, let it be pointed out that disease resistance in a plant is the sum total of many different defense strategies, inducible under various conditions and specific to each individual. At present, the exact mechanism by which flavanol content may contribute to disease resistance in cacao seeds is not known, although based on the evidence given above, it seems probable that the presence of flavanols in the *T. cacao* seed indicates some type of selective advantage against disease.

Flavanols in Chocolate Flavor

Chocolate flavor is the combined effect of aroma, taste and textural sensory attributes (Lawless and Hildegarde, 1988). It does not fully develop until after a long post-harvest process which includes fermentation, roasting and conching (Hoskin and Dimick, 1994). Flavanols are present during the entire process, from the fresh seed to the final chocolate product, and are direct contributors to taste as well as indirect players in aroma development.

The flavor development process has the effect of decreasing the overall concentrations of flavanols and polyphenols in general (Figure 2-3). During the first step of processing, fermentation, anthocyanins essentially disappear (Cros, 1982b) and the level of catechins and procyanidins in the seed show dramatic reductions in the first three days (Cros, 1982a; 1989). Three major modes of depletion exist: oxidation, leaching and complexation.

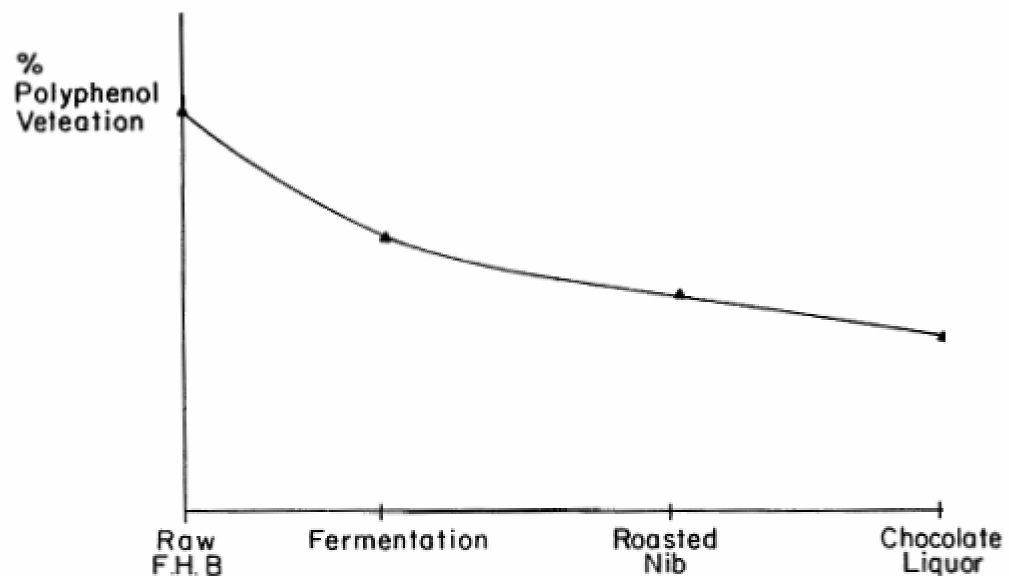

Figure 2-3. Loss of polyphenols from the *T. cacao* seed throughout different stages post-harvest processing (Kealey et al., 1998).

Oxidation begins as the pH level of the seed drops and oxygen enters the seed during fermentation. This catalyzes enzymatic and non-enzymatic oxidation in which flavanol monomers react and polymerize, creating large complexes called melanins and melanoproteins. Incidentally, it is these complexes which are responsible for the brown chocolate color (Wong et al., 1990).

Polyphenols are quickly lost through cell membranes because of membrane disintegration initiated by cell death. Over 50% of the catechins can be lost through leaching (Wollgast and Anklam, 2000a). It is interesting to note that all catechins and procyanidins, regardless of the degree of polymerization, apparently leave the seed at the same rate (Cros, 1989).

The flavanols remaining are potent because they can potentially complex with storage protein, thus interfering with aroma development during roasting. Storage

protein in seed vacuoles is the source of important aroma precursors needed for Maillard reactions in roasting. If, during fermentation, proteins are blocked from degradative enzymes, aroma precursors are diminished which could potentially affect aroma in the final chocolate. A summary of the role of flavanols in aroma development is outlined in Table 2-2.

Decreases in flavanol content continue throughout roasting, but the flavanols that remain after processing contribute to bitterness and astringent properties of the chocolate product. Originally, the bitterness and astringency of chocolate was attributed merely to the presence of caffeine and theobromine (Drewenowski and Gomez-Carneros, 2000). Subsequent sensory studies revealed that theobromine by itself had a bitter and metallic character, not at all like the bitterness perceived in chocolate (Pickenhagen et al., 1975). Pickenhagen et al (1975) more accurately described the characteristic bitterness found in chocolate as being due to theobromine in the presence of diketopiperazines. Incorporation of flavanols into the sensory profile has expanded the bitter concept even further.

Table 2-2. Summary of aroma development in chocolate.

Process	Cellular reactions	Flavanol Concentration	flavor protein precursors
pre-harvest	seeds is synthesizing molecules for growth	increasing	increasing
fermentation	acidity increases cell barriers break down	leaching out complexation polymerization	complexation degradation into small peptides and amino acids
roasting	heat initiated Maillard reaction	polymerization	amino acids and sugars lead to chocolate aroma

Preliminary data exists to support real effects of flavanols on flavor properties (Figure 2-4). Linear regressions fitted to the sensory scores of chocolate made from different bean varieties demonstrated that high catechin contents in chocolate correspond to a decreased chocolate aroma (r = - 0.77), and higher levels of bitterness (r = 0.90) and astringency (r = 0.83).

The knowledge of flavanol functionality in both disease resistance and flavor development in the seeds of *Theobroma cacao* L. points to certain relationships between expressed traits in the species. It seems that flavanols are capable of offering benefits to the seed in terms of resistance, yet they do not seem to promote an especially favorable flavor potential; flavanols in seeds are consistent with bitterness and

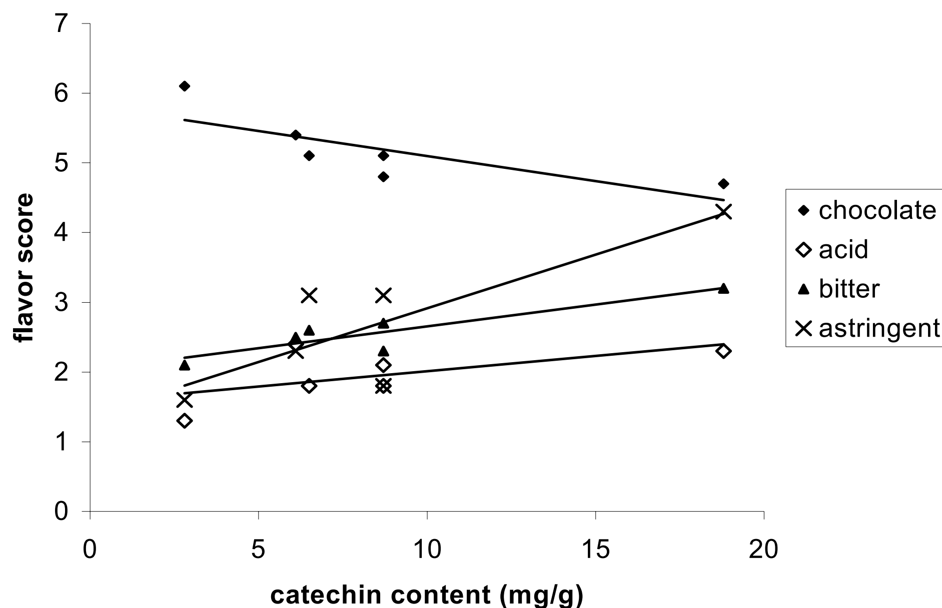

Figure 2-4. Effect of chocolate liquor catechin content on sensory attributes of the final chocolate product (Clapperton et al., 1994).

astringency and decreased production of aroma precursors. This potential contraposition of favorable traits increases the imperative for taking a closer look at flavanols with respect to crop management.

2.4 FLAVANOLS IN CROP MANAGEMENT

Character traits of the major T. cacao cultivars

A first scan of *T. cacao* as a raw material typically begins with a broad grouping into three major cultivars, or horticultural races: Forastero, Criollo, and Trinitario. These groupings are based on traditional assessments of morphological characteristics and geographic origin (Cheesman, 1944; Enriquez and Soria, 1967). Although not all traits fit perfectly into this classification scheme, fresh seed color can act as a good general guide.

In *T. cacao*, fresh seeds are pink, purple or ivory. As a heritable trait in the species, these colors paint the story of cacao from its earliest origins, beginning in the upper Amazon rainforest of South America (Cheesman, 1944). Forastero, a purple seeded cultivar, is the term used to describe most divergent strains from this origin. Criollo, the one divergent strain of Forastero not grouped with the rest (Motamayor et al., 2000), is distinguished by white or rose colored seeds and considered a separate cultivar.

During the early 20th century, a third major cultivar was created through a breeding strategy in Trinidad.

As a group, Forastero trees may be generalized as being more vigorous, high yielding, with flat, purple seeds (Figueira et al., 1997). Because of their vigorous quality, Forastero varieties of cacao, especially the Lower Amazon type (Motamayor et al., 2000), are used in some 95% of the commercial chocolate produced today (Hancock and Fowler, 1994).

Criollos are native to Venezuela, low yielding (Cheesman, 1944) and famous for their white/light purple seeds (Figueira et al., 1997) and fine flavor quality (Presilla, 2001). The chocolate made from Criollo varieties has a more intense chocolate aroma than the chocolate made from Forastero; even within a mixed bag of seeds, the "Criollo perfume" is said to be distinguishable (Presilla, 2001).

Trinitarios are the result of crosses between Criollo and Forastero (Presilla, 2001). Today, after years of cross-breeding and selection, Trinitarios are commonly identified by geographical location (Figueira et al., 1997) and have come to represent the full range of segregation between Forastero and Criollo with seed colors ranging from purple to white (Figueira et al., 1997; Sukha, 2001).

Not surprisingly, after 2000 years of human cultivation and breeding, the distinctions between cultivars are blurry due to open-pollination and inconsistent naming, and controversy exists today over the usefulness of such terms as Criollo and Forastero (Motamayor et al., 2000). The origin of the words, in fact, were first proposed in 1882 (Motamayor et al., 2000) and only signified foreign vs. native materials with respect to Venezuelan cocoa producers. Lines of ancestry cross back-and-forth and are difficult to

trace (Presilla, 2001). The general trends emphasized here and listed explicitly in Table 3 are only the most general descriptors.

Table 2-3. Character traits of the major cultivars.

Forastero	Trinitaro	Criollo
dark purple seeds resistant to disease bulk chocolate quality	representing the full range of ← segregation →	white seeds susceptible to disease fine flavor chocolate

It is interesting to note that the general characteristics of the major *T. cacao* cultivars demonstrate an inverse relationship between disease resistance and flavor quality of the chocolate produced from them. As was previously stated, very similar trends were predicted based on flavanol functionality. Admittedly, there are exceptions. Important varieties such as Catongo, Nacional and Arriba, for example, violate the rules given in Table 3 (Jackson, 1994). These varieties demonstrate that the rules for cultivar classification are not clear cut. There are, however, plenty of varieties which confirm the trends. The Criollo Porcelana variety has ivory/rose colored seeds and is known for its low acidity and distinctive nutty aroma, like a "buttery macadamia" with "delicate notes of spice" (Presilla, 2001). Evidence of such relationships between traits, however tentative, are ripe areas for further investigation as rewards may be great in terms of efficient use of *Theobroma cacao* L. as a raw material.

History of crop management in cacao

Since the beginning of cacao crop cultivation, *Theobroma cacao* L. has undergone a selection process that has markedly shaped the species. Records show that formally directed breeding strategies began around the 1890's (Bartley, 1994), although *T. cacao* growers were likely selecting for superior genotypes well before then (Cheesman, 1944). Crop improvement strategies centered on clonal propagation through cuttings and seed reproduced cultivars (Bartley, 1994) produced primarily at research stations whose primary intent was to provide local farmers with improved raw materials. For the most part, selection criteria have been based almost exclusively on traits that could be observed in the field. High yields of seeds, resistance to disease and growth efficiency were the traits most valued in the crop (Cros, 1998).

High yield and disease resistance, however, are not the only valuable traits in the *T. cacao* tree. Characteristics apparent down the line in the chocolate product, like chocolate flavor, and, more recently, antioxidant capacity, are important as well. Quality of the chocolate product begins with the genetic source materials, but because of a long post-harvest process which separates chocolate from its original source, it is often difficult to link chocolate quality to the genetic identity of its raw material. Breeders are not likely to select for traits that cannot be easily identified in the field. Furthermore, issues of yield and disease resistance are too pressing for farmers, and too challenging for breeding programs at present for attention to turn elsewhere.

Still, the rapidly growing field of biotechnology promises new tools for crop management in agriculture, and with powerful breeding techniques on the horizon,

narrowly focused breeding programs become increasingly worrisome. Already, the Criollo cultivar of the species, prized for its superior flavor quality (Presilla, 2001), is extremely rare, existing in only a few small pockets of the world (Sukha, 2001; Motamayor et al., 2000), mostly due to a decision made in the 1890's to select for the more robust Forastero varieties (Bartley, 1994). Important varieties could be better protected and utilized if there were a viable marker in the field that would be able to guide breeders to genetic traits that cannot be easily observed in the field.

2.5 COLOR AS A MARKER FOR FLAVANOL CONTENT

The cotyledons of fresh cacao seeds exist in a continuous spectrum of purple, pink and ivory colors. In the rainforest (their natural state), they are hidden in pods filled with white pulp and encased by the seed testa, yet these colors have the potential to act as visible indicators of unexpressed phenotypes. Seed color is a heritable trait and is closely related to the genetic identity of the seed.

For a long time, it has been suspected that seed color had some significance for the chocolate product. The trends given in table 3 are repeated throughout the literature like folklore. Here we describe seed color in cacao and how it might be used as an indicator of traits in cacao through its connection with flavanol compounds.

Fresh seed color in T. cacao.

Seed colors in cacao are due primarily to the presence of the anthocyanin subclass of flavanols. The only group of flavanols with color properties, the cation on the center ring allows for wavelength absorbance in the visible spectrum. They are stored in vacuoles of pigmented cells which are estimated to make up 11-13% of the seed tissue (Jardine, 1999). In cacao, two specific anthocyanins are identified in the literature as being responsible for the purple seed color: 3-α-L-arabinosidyl cyanidin and 3-β-D-galactosidyl cyanidin (Forsyth and Quesnel, 1957). The structures of the two species are shown in Figure 2-5.

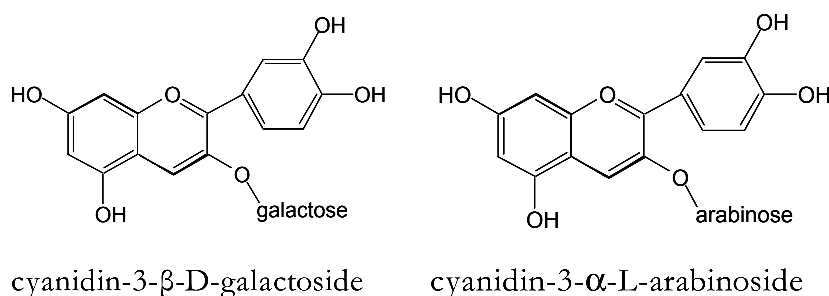

Figure 2-5. The two major anthocyanins in fresh cacao seeds (Forsyth and Quesnel, 1957).

Anthocyanins, as members of the flavanol class of compounds, carry out some of the same functions in the seed as other flavanols. This should not be surprising since their structures are so similar. Like catechins, anthocyanins also complex to create large chain procyanidins (Stafford, 1990) and may complex with protein. These procyanidins contribute to astringency and decreased aroma potential in the final product.

Anthocyanins also participate in polymerization reactions during fermentation which leads to the production of long chain brown pigments (Cros, 1982b).

Anthocyanins, as the only group of flavanols with color properties, are an excellent candidate marker for flavanols in general. Because anthcyanins are so closely related to other flavanol compounds in structure, it is likely that they are under similar metabolic control. Whether seed color can act as a marker for flavanols, (and by extension, flavor quality and disease resistance) depends on how the anthocyanins are genetically regulated with respect to other flavanols.

Regulation of flavanols

All flavanols, including anthocyanins are produced in cacao seeds through a common synthetic pathway (Stafford, 1990). The metabolic proximity of each subclass increases the likelihood that all flavanols are under similar systems of genetic control.

Although the flavanol pathway is one of the most well-characterized synthetic pathways (Winefield, 2002), regulation of the pathway, especially in the case of cacao, is not completely understood. *T. cacao* has not been used as model system for biochemical research. Instead, species, like maize, petunia, arabidopsis have been used to experimentally derive what is currently known about flavanol biosynthesis (Winkel-Shirley, 2001).

An abbreviated sketch of the pathway is shown in Figure 2-6. From this figure it is apparent that all groups of flavanols, anthocyanins, catechins and procyanidin

polymers, arise from a common precursor. The first committed step for flavanoid biosynthesis occurs at chalcone synthase (CHS) (Winefield, 2002), in which a ring structure common to all flavanols is formed. This basic structure undergoes modifications until the branch point, where dihydroflavanols are converted into a common flavan-3,4-diols intermediate before conversion into anthocyanin, catechin or procyanidin end products.

Accumulation of anthocyanins versus other flavanols depends on how resources are shunted through this branch point of the pathway. For *T. cacao*, the ratio of anthocyanins to catechins and procyanidins produced in actuality is not known.

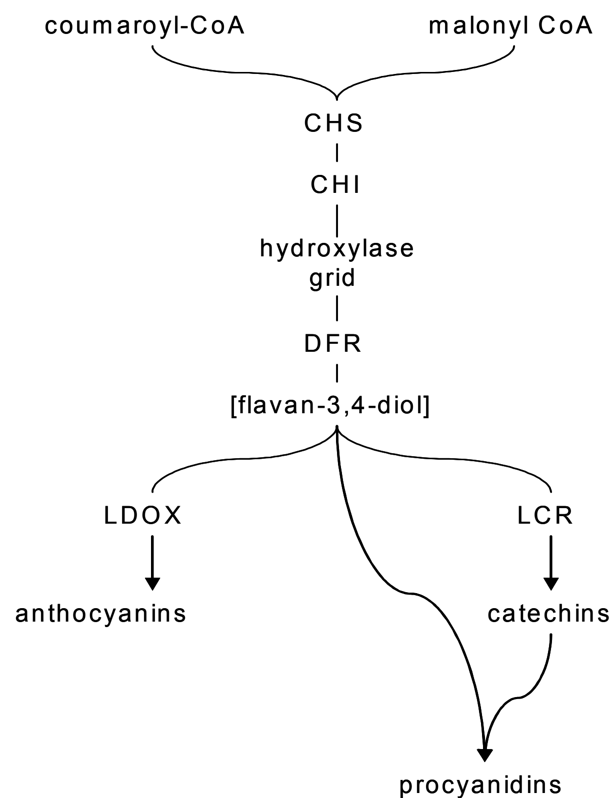

Figure 2-6. Branch point of flavanol biosynthesis. Enzymatic sequence to produce anthocyanins, catechin and procyanidin products. Enzyme abbreviations are as follows: chalcone synthase (CHS), chalcone isomerase (CHI), dihyroflavanol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), leucoanthocyanidin reductase (LCR). (modified from Winkel-Shirley, 2001).

If most of the regulation occurs upstream of the branch point, it would be expected that anthocyanins and other flavanols are regulated in tandem. The extent to which high levels of anthocyanins imply high levels of all flavanols is related to the viability of seed color is as a marker for flavanol content in the fresh seeds.

2.6 RESEARCH DIRECTIONS

Development of fresh seed color as a marker for certain character traits in cacao depends not only on co-regulation of anthocyanins and other flavanols, but also on how influential flavanols actually are in the expression of useful traits in cacao. The literature reviewed in this paper establishes groundwork for a hypothesis which states that flavanols are a contributing factor to disease resistance and flavor potential of the seeds. Recognizing that the mechanisms for disease resistance and flavor development are complex and dependant on many factors, the extent to which flavanol content in the seed contributes to expression of these traits remains to be established.

In terms of disease resistance, it is not known whether seed concentrations of flavanols actually confer a significant resistance either to the seed, or to the plant as a whole. The type of resistance flavanols could effectively guard against is not known either. *Theobroma cacao* L. is a crop susceptible to a number of different types of infections and disease. *Phytophthora* canker, for example, is a fungus that can enter through the pod and infect the tree (Dand, 1993). If flavanols really do have the

antifungal ability mentioned above, it would stand to reason that the presence of flavanols could deter the ability of the fungus to infect the seeds in the pod. Ceratocytosis wilt, on the other hand, is a fungus that infects through wounds made in pruning (Dand, 1993). It is unlikely that a marker for flavanol contents in the seeds would have anything to indicate about susceptibility to Ceratocytosis. Still, the need exists to characterize seed flavanols as defense compounds in *T. cacao*.

Investigating the real effects of seed flavanols on flavor quality is a second challenge that presents itself in this line of research. Defining the effects of fresh seed flavanol contents on the flavor of the final chocolate product is complicated by the variable factors introduced during processing. A comparative study between seeds of varying flavanol contents would require an identical chocolate processing procedure. Model systems are another possible way to gain information of flavanol effects on flavor.

The information presented in this review demonstrates the importance and relevance of flavanols, not only in chocolate, but also for the raw material. Continued investigation of flavanols in fresh seeds in terms of what they may indicate for characteristics of the species promises great returns. The potential to develop flavanols as an easy visible marker for seed characteristics in the field would be an asset to breeders and future management of the cacao crop. At the very least, recognition of the role of flavanols throughout processing creates a basis for more informed and effective crop management *T. cacao* and has implications for bean blending for chocolate flavor or antioxidant properties as well.

ACKNOWLEDGMENT

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CHAPTER 3

IDENTIFICATION OF NOVEL ANTHOCYANINS INVOLVED IN *THEOBROMA CACAO* SEED COLOR

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3.1 ABSTRACT

Fresh seed colors for *Theobroma cacao* L. range from deep purple, to pink, to white, and are thought to be associated with economically important, but hard-to-measure traits, such as chocolate flavor. In order to provide a framework for the investigation of seed color as a potentially useful field marker, a more thorough understanding of seed color, in terms of both the observable phenotype and its chemical constituents, is needed. In this study, colorimetric and chemical analyses were performed on two hundred *Theobroma cacao* L. seeds. Seeds were plotted based on light reflectance data as well as on measures of perceptible color. Individual seed extracts consistently revealed the presence of four pigmented anthocyanin compounds. Listed in order of decreasing abundance, the anthocyanins were a cyanidin arabinoside, cyanidin galactoside, cyanidin rutinoside, and cyanidin pentoside. The last two, whose identifications were confirmed by tandem MS, were previously unidentified in cacao. In correlating visual data with chemical data, purple seeds were consistently higher in total anthocyanins, while white seeds contained lower levels of total anthocyanins. Anthocyanin concentrations from seed extracts were not well correlated with variations in hue, suggesting that there must be other factors involved in the expression of fresh seed color. *In vivo* effects that modify absorbance like vacuolar pH and copigmentation, are suggested as possible explanations and are demonstrated with cacao seed extracts.

KEYWORDS: *Theobroma cacao*; Sterculiaceae; cacao; fresh seed color; anthocyanins; 3-α-L-arabinosidyl cyanidin; 3-β-D-galactosidyl cyanidin; cyanidin rutinoside; cyanidin pentoside.

3.2 INTRODUCTION

Under the rainforest canopy, the cacao tree, *Theobroma cacao* L., produces brilliantly colored seeds, covered in a white mucilagenous pulp and encased in pods. These seeds, before they are harvested for chocolate production, can be purple, pink, white, or a mottled mixture of the three. Probably because cacao seeds are better known in their processed, brown form, the brilliant colors of the fresh seed have thus far escaped extensive study. Nevertheless, a number of accounts in the literature point to their importance as a potential indicators of disease resistance and chocolate flavor (Chapter 2). In order to fully appreciate the significance of color in fresh cacao seeds, a more thorough chemical understanding of the phenotype should first be established.

Pigments in unfermented seeds were first characterized during polyphenol analyses conducted in Trinidad during the period 1949-1968 (Ziegleder and Biehl, 1988). Paper chromatographic techniques were used to separate extracted purple pigments, later attributed to the anthocyanins, 3-α-L- arabinosidyl cyanidin and 3-β-D-galactosidyl cyanidin (Forsyth and Quesnel 1957). Each was estimated to comprise 0.3% and 0.1% of seed dry weight, respectively (Ziegleder and Biehl, 1988).

Despite the fact that the chemical agents responsible for purple color have been identified, an explanation for the full range of *T. cacao* seed color is still lacking. Anthocyanins identified in purple seeds do not explain the existence of white, pink or mottled seeds. One might assume that lighter seed colors are due to decreased levels of anthocyanins, yet under certain conditions, anthocyanins may appear colorless (Stafford,

1990). Therefore, it is not known whether white seeds due to a mutation in the synthesis of anthocyanins or merely another form of the compounds.

This experiment aims to find a chemical explanation for the full range of fresh seed color observed for *Theobroma cacao* L. Seeds across the species are investigated and categorized in terms of their light absorbing ability as well as how they are seen by a standard observer. Pigmented molecules are then extracted from each seed to see how these chemical constituents relate to the colorimetric data. In the process, a full picture of cacao seed color is defined, including two previously unidentified compounds.

3.3 RESULTS

Colorimetry

Reflectance spectra recorded for the outer and cut surfaces of cacao seeds confirmed that primary differences in seed colors (ranging from deep purple to white) were due to a shift in "lightness", or total reflectance. Seeds also showed characteristic reflectance curves depending on chromaticity (Figure 3-1). In pink seeds, a dip in the curve was observed around 540 nm, on average. More purple seeds generated a curve that gravitated towards a base level of reflectance over the range 400 nm - 600 nm.

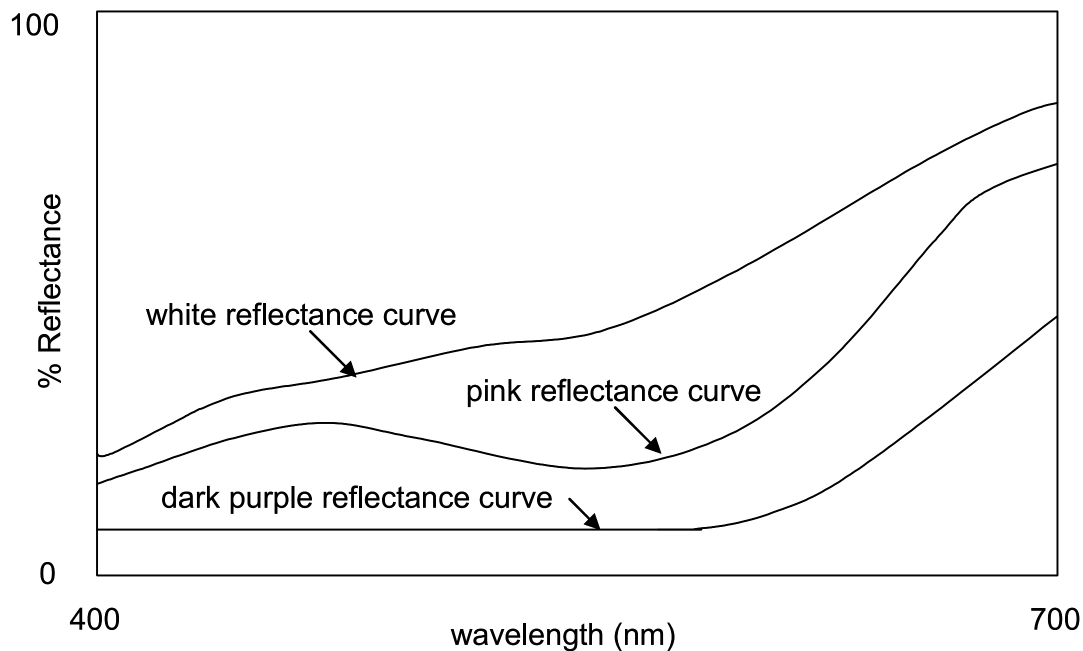

Figure 3-1. Schematic for the interpretation of reflectance spectra obtained for outer and cut surfaces of fresh *T. cacao* seeds.

Reflectance spectra were converted into tri-stimulus values according to the CIE (Commission Internationale de l'Eclairage) L*a*b* system that specifies a color in a 3-D colorspace (Figure 3-2). This colorspace was created with respect equal spacing of perceived color differences (Minolta, 1998). A distance greater than 2 between any two points indicates a perceptible difference to a standard observer (Billmeyer and Saltzman, 1981). Mapping of seeds onto these coordinates showed that the primary distinguishing factor between seeds was the "lightness", or L-value.

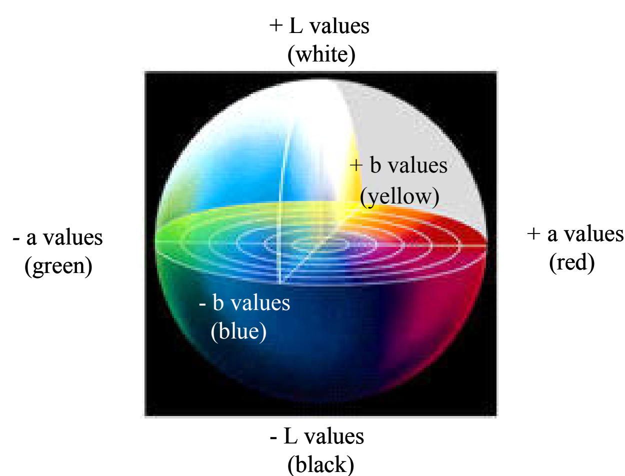

Figure 3-2. The CIEL*a*b* color space. Spectral reflectance is converted into L-values, a-values, and b-values, which correspond to individual scales of lightness-darkness, red-green and yellow-blue, respectively. Source: Minolta (1998).

Identification of cacao seed pigments

HPLC-MS analysis on *T. cacao* seed extracts led to the identification of 4 anthocyanin species, varying in concentration, but present in almost every seed. Anthocyanins were recognized by their characteristic UV-Vis spectra recorded during HPLC with a diode array detector. The spectra were nearly indistinguishable, all showing a maximum absorbance at 520 nm in the acidified extract.

The HPLC-MS revealed ion fragments at *m/z* 287 for compounds **1**, **3**, and **4**, corresponding to the cyanidin aglycone base. Compound **2** may or may not have shown this -- it's concentrations were too small. Additional molecular ions distinguished the compounds according to their glycosylated adjunct. In this way, compound **1** (*m/z* 449) was determined to be a cyanidin hexoside; compound **2** (*m/z* 595), a cyanidin

diglucoside; and compounds **3** and **4** (*m/z* 419), cyanidin pentosides. Compounds **1** and **3,** highest in abundance, were thought to correspond to previously identified 3-β-D-galactosidyl cyanidin and 3-α-L- arabinosidyl cyanidin, respectively.

Table 3-1. Identification of unknown compounds from acidified methanolic cacao seed extract.

	On-line HPLC			*ES-MS*	
Compound	Vis-max (nm)	Local UV-max (nm)	t_R *(min)*	M^+ *m/z*	A^+ *m/z*
1	520	?	14.82	449	287
2	520	256	15.54	595	too small
3	520	?	15.97	419	287
4	520	?	17.59	449	287

3.4 DISCUSSION

Correlations between anthocyanin concentrations and colorimetric values showed that lightness of the outer surface of the seed was most indicative of the actual anthocyanin content. The darker the seed was on its outer surface, the more anthocyanins

were present in the seed extract. The relationship was logarithmic with a certain level of saturation whereby increased levels of anthocyanins did not result in an appreciably darker seed (Figure 3-3).

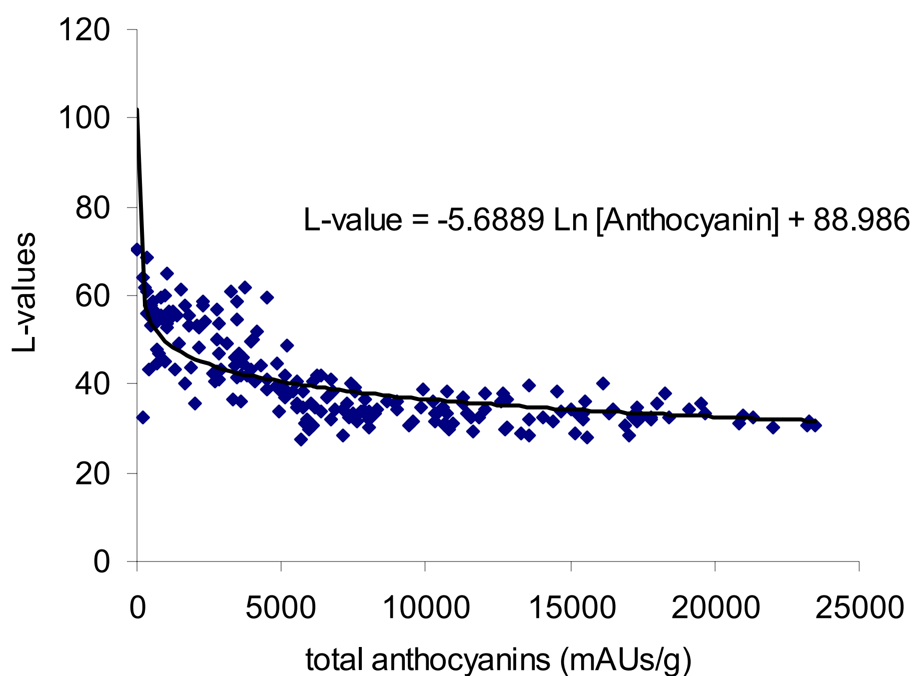

Figure 3-3. Relationship between "lightness" of the outer seed surface, as measured by L-values, and total anthocyanin content, given in relative units of milliAbsorbance Units · seconds per gram seed dry weight. $R^2 = 0.58$

No relationships specific to anthocyanin type were observed. Instead, anthocyanins with the highest concentrations, i.e. - the cyanidin arabinoside, showed the highest correlation with the final color of the seeds. Redness, or a-values, however, were not highly correlated with anthocyanin concentrations, especially for the lighter, inner seed surface. A-values for the cut seed surface were not correlated significantly with anthocyanin concentrations.

From these results, it seems that the intensity of seed color, concentrated on the outer surfaces of the seed, is significantly related to anthocyanin concentrations of the total seed. Anthocyanin contents are not perfectly correlated with colorimetric data, however. This could be due to the difference in analytical techiniques; colorimetric measurements were taken on specific surfaces of the seed, while anthocyanin concentrations were a result of a total seed extraction. One important factor to consider are other factors involved in the expression of seed color.

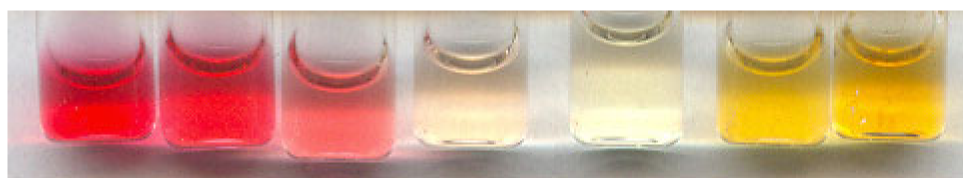

Figure 3-4. Demonstration of the effect of pH on anthocyanin color. Seed extracts were placed in different buffered solutions, pH 1-7, from left to right.

It is known that the color of anthocyanins can be modified based on pH, metal chelation, co-pigmentation with proteins and other flavanols (Gross, 1987). In fact, two of these effects were demonstrated with our cacao seed extracts, *in vitro*. Figure 3-4 shows the change in colors with a change in pH. Figure 3-5 shows the change when catechins are added to the extract.

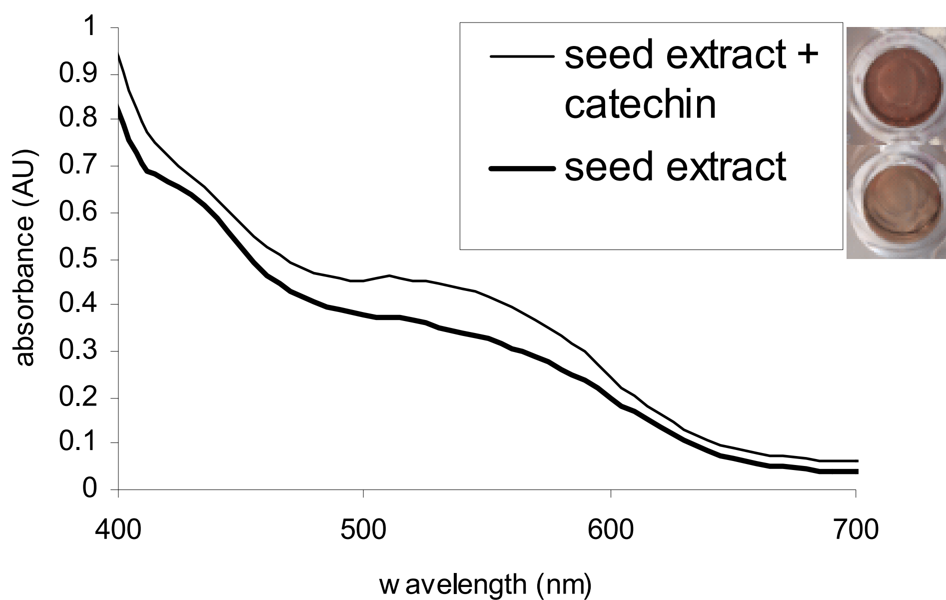

Figure 3-5. Effect of adding catechin to cacao seed extract. Absorbance of extract in a 96 well plate was determined using a SpectraMax 190 and recorded in absorbance units.

The data on anthocyanin content and colorimetric observations give good indication that color intensity is largely related to the concentration of anthocyanins. Chromatic nuances in the seed color likely depend on conditions of the cellular environment, demonstrated *in vitro*. The modified concept of cacao seed color accounts for purple, pink and white seeds using 4 anthocyanins, 2 previously unidentified, each differing in their glycosylation.

3.5 EXPERIMENTAL

General Experimental Procedures

Colorimetric measurements were made on fresh cacao seeds using either a Minolta Chroma Meter CR-200 (Minolta, Ramsey, NJ, USA) or a Minolta Spectrophotometer CM-3500d (Minolta, Ramsey, NJ, USA). Spectrophotometers were standardized against a white tile (Y = 93.2, X = 0.3142, y = 0.3219) and set to exclude specular reflection from shiny seed surfaces. Sample preparation included quick freezing of seeds in liquid nitrogen followed by lyophilization in a VirTis Genesis 25 XL Freeze-Drier (SP Industries, Gardiner, NY, USA). Freeze dried seeds were weighed and fat removed through 3 hexane washes. Polyphenols were extracted in methanol/water (75:25, v/v) and filtered through a 0.45 micron PTFE membrane (VWR International, Inc., Bridgeport, NJ, USA). Acidified extracts were injected into a Hewlett Packard series 1100 HPLC (Brinkmann Instruments, Westbury, NY, USA) coupled to PerSeptive Biosystem Mariner Mass Detector (Perseptive Biosystem, Boston, MA, USA). Absorbance spectra on extracts were read using SpectraMax 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

Samples

All seed cotyledons were taken from live pods 1-13 days after harvest (depending on shipping time). Cultivars were clonal accessions identified by research station and included the following varieties (number of pods in parentheses): from IRAD, AML(2), SNK 10, SNK 16, SNK 413, UPA 134 (2); from CRU, DR 2 (2), ICS 16, ICS 39, ICS 40; from USDA, ICS 16, ICS 39 (3), ICS 40 (2), P 19, PA 4, SCA 6 (4), SCA 12, UF 613. Access information may be found in Appendix B.

High performance liquid chromatography

Extracts were separated on a Keystone BetaBasic C18 column (1 x 150mm) using a mobile phase based on a gradient program which included solvent A: 10% formic acid solution, solvent B: H_2O/formic acid/acetonitrile (4/1/5), and solvent C: methanol. Samples were run at a flow rate of 0.750 mL/min and visualized with a diode array detector.

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CHAPTER 4

METHOD FOR THE ANALYSIS OF CACAO PROCYANIDIN OLIGOMERS USING A NOVEL POLYETHYLENE GLYCOL COLUMN

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4.1 ABSTRACT

A new method has been developed for the efficient quantification of procyanidins. These compounds, of interest in many foods, were analyzed from fresh cacao seed extracts. Comparative procyanidin profiles for each seed were obtained using a Supelco® polyethylene glycol column to separate oligomers. Data on retention times and mass chromatographs led to the identification of procyanidin monomers through pentamers with putative identifications of hexamers through decamers based on retention times and similar spectral traces. Benefits of this system as a rapid method for procyanidin characterization are discussed.

KEYWORDS: cacao, procyanidins, catechins, flavanols, HPLC, polypropylene glycol

4.1 INTRODUCTION

Procyanidins, as a subclass of flavonoids, have gained much attention in recent years due to reported health benefits (Hannum and Erdman, 2000). Oligomeric procyanidins have been shown to decrease blood pressure and confer protective effects in the veins (Zhu et al., 2002). Their natural occurrence in plant-based foods like red wine, sorghum, apples, cranberry, blueberry and tea have led to procyanidin characterization in a number of food products (Degenhardt et al., 2001; Gu et al., 2002; Guyot et al., 2001; Peng et al., 2001). Chocolate has recently been recognized as a food high in procyanidins and has been a leading model system used to develop techniques for their analysis (Hammerstone et al., 1999; Adamson et al., 1999; Wollgast et al., 2001).

Analysis of procyanidins presents a number of challenges. First, extraction of these compounds from natural sources is limited by their solubility. As chain length increases, procyanidins are increasingly insoluble in extraction solvents. Second, procyanidins are unstable, easily polymerizing, especially in response to oxidative conditions (Rohr et al., 1999). Finally, the number of isomers generated during polymerization creates a significant problem for chromatographic separation. Resolution is typically poor for procyanidins (Rohr et al., 1999) with higher mass procyanidins spread out over the whole chromatogram (Peng et al., 2001; Rohr et al., 1999).

Column choice plays a significant role in separation of analytes. Previous attempts have been made to separate procyanidins on both polar (Hammerstone et al., 1999); Adamson et al., 1999) and non-polar (Degenhardt et al., 2001; Wollgast et al., 2001) stationary phases. These studies have shown poor selectivity for procyanidins on

non-polar columns (Degenhardt et al., 2001), but a satisfactory separation using polar columns (Hammerstone et al., 1999; Adamson et al., 1999). Unfortunately, polar columns require a long analysis time, show evidence of column damage over time, and require detection by fluorescence (Adamson et al., 1999).

The analytical technique presented here was developed for a comparative study of the procyanidin contents in 200 fresh cacao seeds (raw materials for chocolate). Since so many samples needed to be run, the technique had to be consistent and fast. For these purposes, a medium polarity column containing a polyethylene glycol stationary phase was used to separate procyanidin oligomers from monomers to decamers. Use of this column is presented here as an alternative method for procyanidin analysis.

4.2 EXPERIMENTAL

Chemicals and materials

Two-hundred fresh cacao seeds from pods harvested at the ripe or newly ripe stage were obtained from cacao germplasm stations in three geographical locations: USDA Tropical Research Station, Mayaguez, Puerto Rico; CRU, University of the West Indies, Trinidad; and IRAD, Yaounde, Cameroon. Hexane used for fat extraction was Optima grade (Fisher Scientific, Pittsburgh, PA, USA). All other solvents (acetonitrile, formic acid and methanol) were of HPLC grade (Fisher Scientific, Pittsburgh, PA, USA).

Water used in the preparation of solvents was purified to 18.2 MΩ-cm using a NANOpure Ultrapure Water System (Barnstead International, Dubuque, IA, USA).

Sample preparation

Seeds were removed from pods 1-13 days after pod harvest, depending on shipping time. In order to control the sample set for dead or germinating seeds, any browning seeds or any seeds with the radical sticking out more than 2 mm were not used in the study. Seeds were separated from the pulp and testa were removed. Within 2 minutes of exposure, seeds were quick frozen in liquid nitrogen, and then freeze-dried in a VirTis Genesis 25 XL Freeze-Drier (SP Industries, Gardiner, NY, USA). Dry weight was recorded and each seed was individually ground under hexane with a Brinkmann Polytron Kinematica GmbH PCU1 (Brinkmann Instruments, Westbury, NY, USA). Lipids were removed in three hexane washes using a Beckman GPR centrifuge (Global Medical Instrumentation, Albertville, MN, USA) for 10 min at 4000-5000 RPM to settle the particulate between each wash. The precipitate remaining for each seed was extracted with 10 mL methanol:H_2O (75:25, v/v) for 24 hours at 5°C. Polyphenols concentrations in second extracts of the precipitate were determined to be negligible (data not shown). Polyphenol extracts were passed through a non-sterile 15 mm syringe filter with a 0.45 micron PFTE membrane (VWR International, Bridgeport, NJ, USA) and combined with 10% formic acid solution (1:4, v/v) for injection samples. Twenty µL of each injection

sample (~20 mg seed dry weight/mL) was injected by autosampler into the HPLC. (-)-epicatechin standard (100 μg/mL) was injected at a volume of 15 μL.

HPLC-MS equipment

Individual seed extracts were separated using a Hewlett Packard series 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). Peaks were visualized using both a diode array detector at 280 nm and a direct on-line coupled Mariner API-TOF MS workstation (Applied Biosystems, Foster City, CA, USA). The limit of detection for the diode array detector operating in the linear range of 0.24-700 ng/mL was 1.2 pg at a signal-to-noise ratio of 2.

4.3 RESULTS AND DISCUSSION

Fresh cacao seed extracts run through the polyethylene glycol column resolved a number of peaks absorbing in the phenolic range. These peaks, visualized at 280 nm, are shown in Figure 4-1. Mass chromatographic data revealed peaks eluting from the column (Figure 4-2) with masses corresponding to procyanidin monomers through pentamers (Table 4-1). Positive identification of these compounds was established through an analysis of negative ion mass spectra (Figure 4-3).

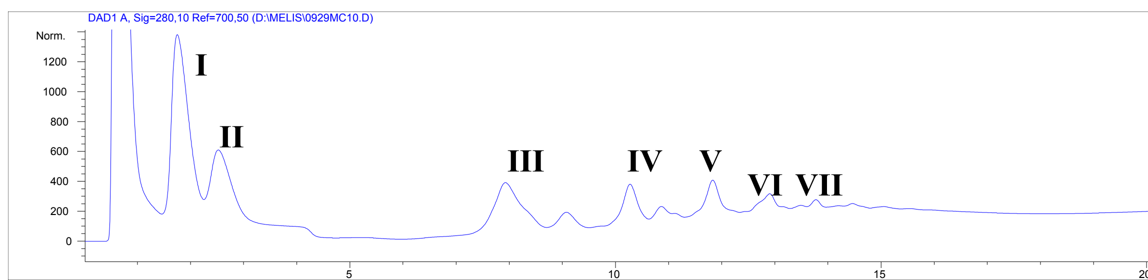

Figure 4-1. Absorbance at 280nm of peaks eluting from the Supelco® polyethylene glycol column (1.0 x 50 mm) 120 Å. Compound I: catechin monomers, compounds II- V: procyanidin oligomers (dimers through heptamers), compounds VI-VII: procyanidin hexamers, heptamers (putative identifications).

Table 4-1. Identification of procyanidins by main masses observed in the MS chromatograph.

procyanidin	molecular mass (a.m.u.)	pseudomolecular ion m/z
monomer	290	289
dimer	578	577
trimer	866	865
tetramer	1154	1153
pentamer	1442	1141

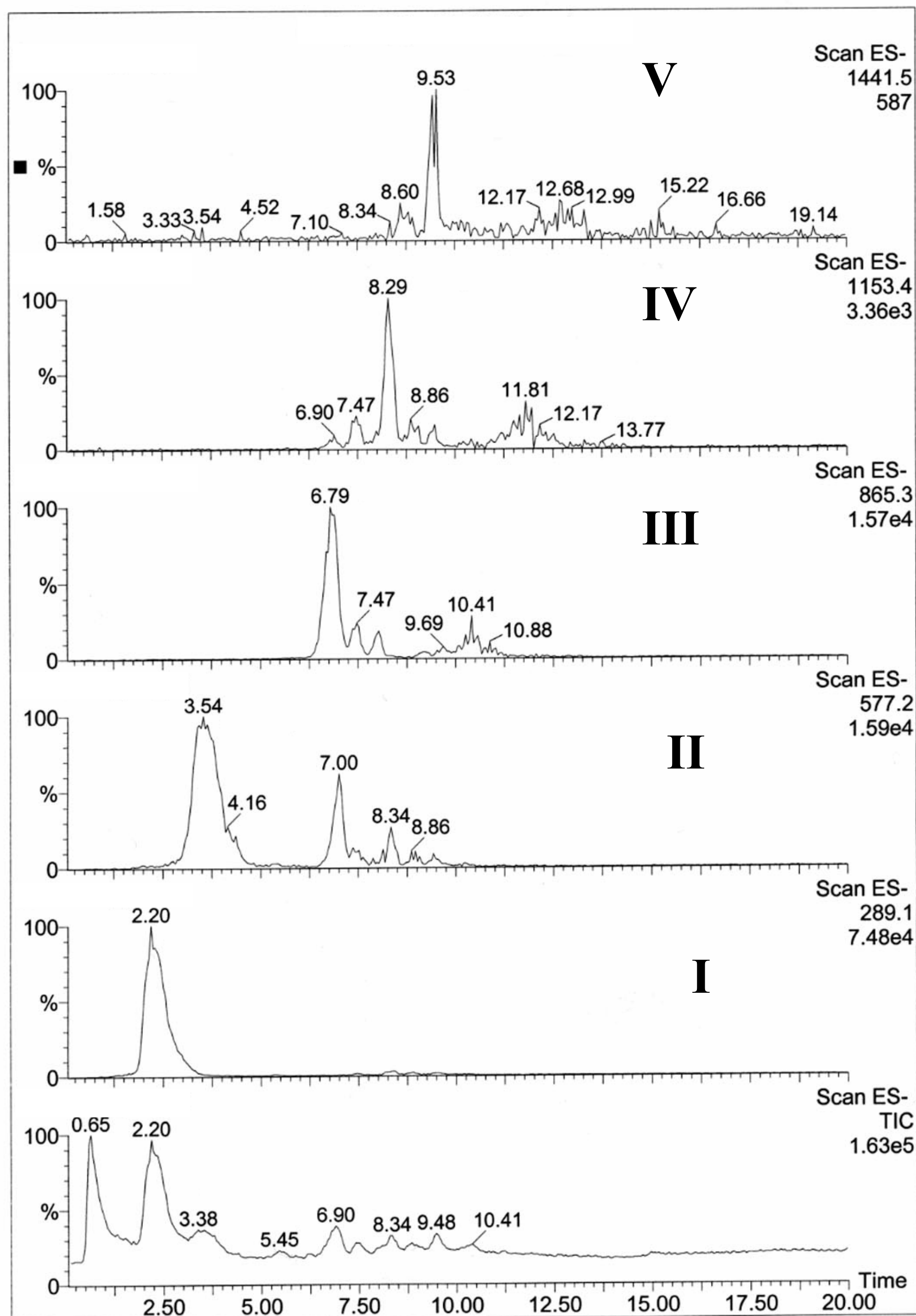

Figure 4-2. Mass chromatograms of catechin monomers (m/z 289) and procyanidin oligomers (dimer through hexamers m/z 577 to 1729).

Fragmentation spectra for compounds I, II and III (Figure 4-4) showed an abundance of peaks corresponding to the $[M-H]^-$ ion for monomers, dimers and trimers, respectively (see Table 4-1). Compounds IV and V also showed peaks for the $[M-H]^-$ ion, although competing fragmentation peaks were also observed. The dominant peak at m/z 576.29 in the spectrum for compound V is likely due to cleavage between the interflavonic bond for the tetramer leading to two dimeric ions. Compound V shows a predominance of a peak at m/z 720.38. This indicates fission of the pentamer by a reverse Diels-Alder reaction. Proposed cleavage sites between base units and in ring B of the oligomers (see Figure 4-3) are known fragmentation patterns of procyanidins (Friedrich et al., 2000) and can be used to explain most other peaks observed in Figure 4-4. The compounds identified here are further supported by those previously found in chocolate extracts using other column systems (Hammerstone et al., 1999; Wollgast et al., 2001).

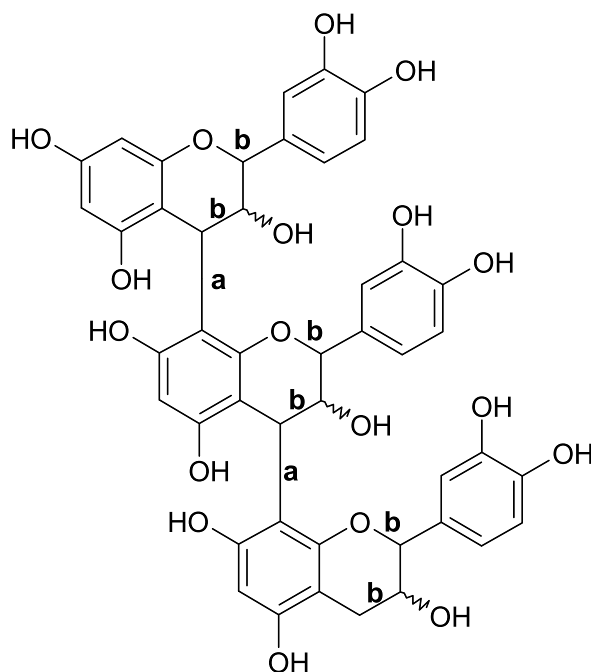

Figure 4-3. Likely fragmentation patterns for procyanidin oligomers (Friedrich et al., 2000): cleavage of the interflavonic bond (a), Retro-Diels-Alder fission of the C ring (b).

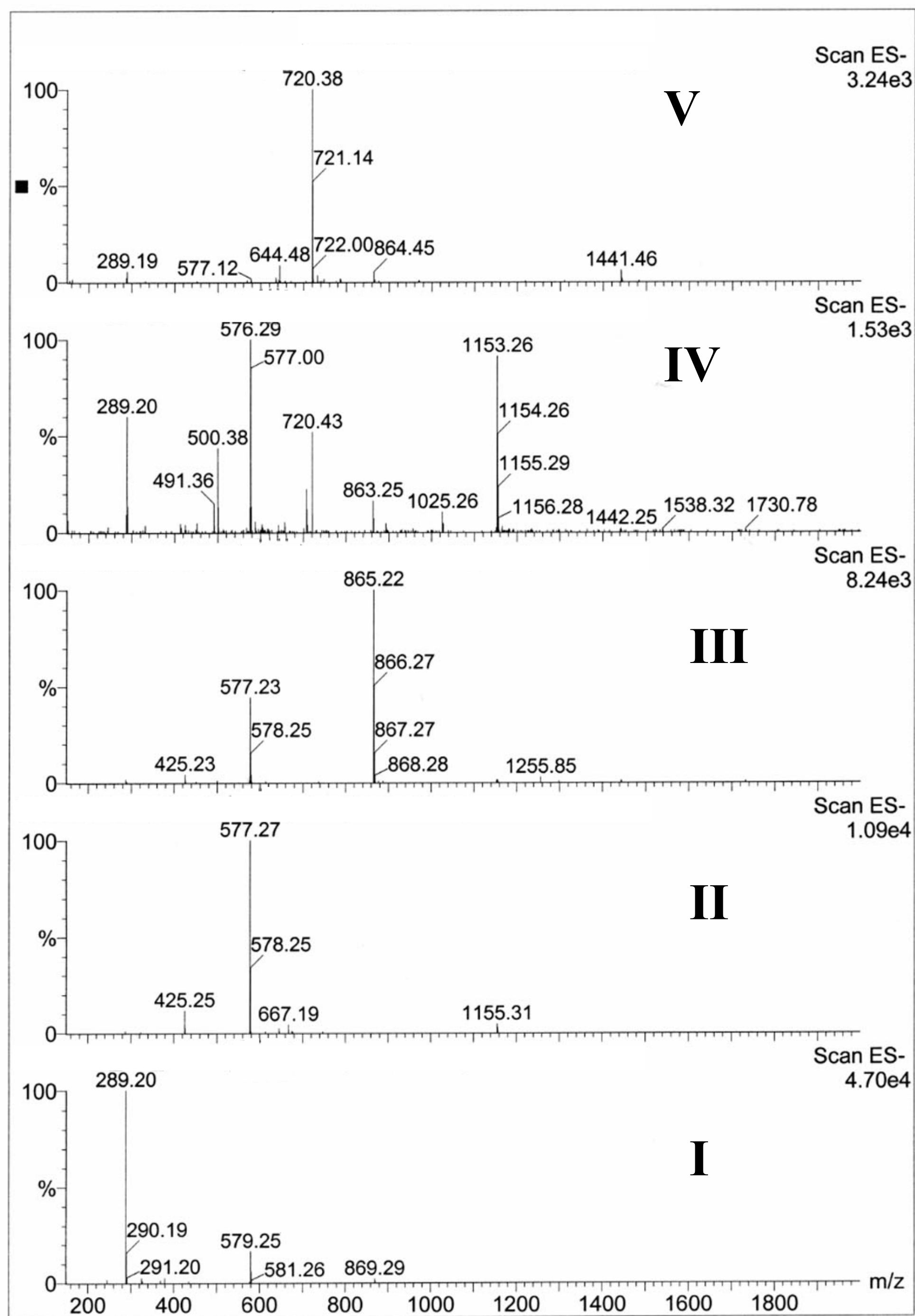

Figure 4-4. Negative ion mass spectrum of fresh cacao seed extract. Compound I: catechin monomers, compounds II-VI: procyanidin oligomers (dimer through hexamer)

On the basis of a single peak on the mass chromatograph for compound I (Figure 4-2), the column did not seem to be able to separate stereoisomers such as (-)-epicatechin and (+)-catechin. Therefore, one isomeric species was deemed sufficient as a standard. Pure (-)-epicatechin run on the polyethylene glycol column (Figure 4-5) showed similar retention times to that of the compound identified as the monomer (Figure 4-1). Furthermore, the UV spectral absorbance of the standard was almost a perfect match for all the procyanidins identified (data not shown).

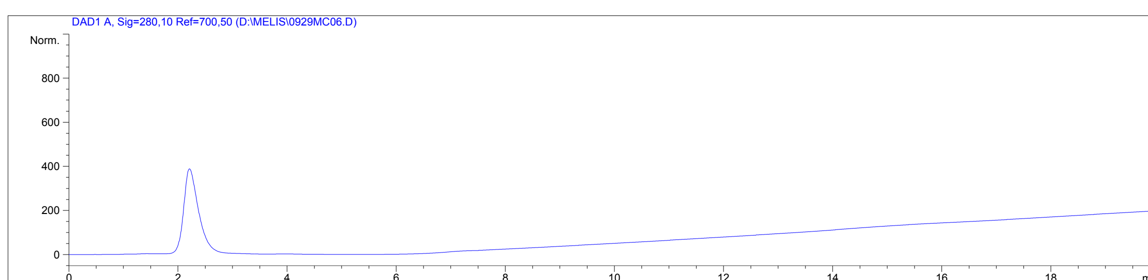

Figure 4-5. (-)-epicatechin standard run on Supelco® polyethylene glycol column. Retention time: 2.30 min.

Although a reverse-phase gradient was used, retention patterns of the identified procyanidins seem to indicate that the polyethylene glycol column works like a normal-phase separation. Previous reverse-phase studies using non-polar C18 columns (Degenhardt et al., 1999; Wollgast et al., 2001) as well as our own studies (not shown), have shown that procyanidins are not separated well based on hydrophobicity. Since solubility of procyanidins in the mobile phase seems to be very similar, separation of procyanidins on the polyethylene glycol column is likely mediated by interactions with the stationary phase.

The column stationary phase, comprised of short polyethylene glycol chains, separated the procyanidins according to chain length with an unusually long retention time between dimers and trimers (Figure 4-1). It seems that procyanidins with a degree

of polymerization equal to or greater than three interact differently with the column in comparison with monomers and dimers. While lower molecular weight procyanidins pass easily through the column with the mobile phase, trimers demonstrate an added degree of interactions with the column stationary phase.

There is also reason to believe that the polyethylene column is able to separate isomers of the procyanidins. Unidentified, paired peaks were observed for compounds III and IV in Figure 4-1, and sometimes observed for higher oligomers as well. These peaks could well contain structural isomers of procyanidins, perhaps glycosylated forms. Because the absorbance at 280 reveals any type of phenolic compound present a cacao seed extract (Peng et al., 2001), of which there are many (Hannum et al., 2000), it is difficult to determine the exact identity of these compounds.

One advantage of the polyethylene glycol column is that procyanidins can be reasonably separated using solvents which are friendly to the mass spectrometer. For extracts in which there were large quantities of procyanidins, there was some difficulty in separating monomers from dimers. Incomplete resolution of the monomer, confirmed by the 577 peak in the monomer's fragmentation spectra (Figure 4-4), could perhaps be improved through a modification of the mobile phase or by injecting smaller sample volumes.

Analysis of 200 fresh cacao seed extracts showed the presence of procyanidin monomers through pentamers with putative identification of hexamers up to, in some cases, decamers, based on retention times and similar spectral traces. The current study demonstrated resolution of procyanidin polymers to the decamer level, although

maximum chain length is likely limited by the solubility of large polymers in the extraction.

Use of the polyethylene column is recommended for the characterization of procyanidins for a large number of samples. The 15 minutes it takes to resolve procyanidin oligomers is a significant improvement over the 50 minutes it takes to analyze the same compounds using a polar silica column (Hammerstone et al., 1999). The sufficient separation and short analysis time makes the polyethylene glycol column an adventageous method for the characterization of procyanidins.

ACKNOWLEDGEMENT

Contribution and development of the polyethylene glycol column was done by Supelco®. Special thanks is also due to Dr. Greg Ziegler and Dr. Mark Guiltinan for their input into the research design, and the Hershey Foods Corporation for funding of the research.

CHAPTER 5

COLOR AS AN INDICATOR OF FLAVANOL CONTENT IN THE FRESH SEEDS OF *THEOBROMA CACAO* L.

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5.1 ABSTRACT

Flavanols are critical compounds in *T. cacao* seeds, raw materials for chocolate. They have bioactive properties which confer disease resistance to the seed prior to harvest and play a role in flavor development during chocolate processing. Because some flavanols have color properties and contribute to the pigmentation of fresh seeds, seed color has potential to be utilized as a potential indicator of total flavanol content which could aid breeders in selection of crops with favorable traits. To test the viability of fresh seed color as a potential indicator of flavanol content, 200 *T. cacao* seeds from 14 different varieties were surveyed for observable color and relative flavanol concentrations. Comparison of seeds in the sample set revealed a significant positive relationship ($p<0.0000$) between pigmented anthocyanin flavanols and other flavanols (catechins, procyanidins). Thus, high concentrations of pigmented compounds in the fresh seed was consistent with high levels of total flavanols. Anthocyanins and flavanols seem to be generally co-regulated during synthesis in the fresh seeds, although the high variance ($r^2 = 0.5346$) points to additional mechanisms for fine tuning control. Quantitative seed color data showed that "lightness" of the outer seed surface was the most indicative of anthocyanin content. To a lesser degree, a descriptive relationship was also observed with fresh seed color and total flavanol content. In conclusion, lightness of the outer seed surface, as measured by L-values, can be used to predict flavanol content with a standard error of 4.0µg catechin equivalents per gram seed dry weight.

KEYWORDS: *Theobroma cacao*, chocolate, fresh seed color, flavanols, anthocyanins, procyanidins, catechins.

5.2 INTRODUCTION

Chocolate has recently gained attention for its potential health benefits attributed to naturally high concentrations of flavanol antioxidants. Numerous studies have investigated the effectiveness of chocolate-derived flavanols in curbing certain degenerative diseases specifically through inhibition of oxidative damage (Verstraeten et al., 2003), thrombosis (Pearson et al., 2002), cardiovascular disease (Kris-Etherton and Keen, 2002), and cancer proliferation (Carnesecchi et al., 2002). Simultaneous efforts have been directed towards the preservation and isolation of these compounds from the raw material; the ultimate goal being to develop chocolate as a functional food (Romanczyk et al., 2002; Kealey et al., 2001).

Flavanols are not only important in terms of human health. Their presence provides functionality to the raw material and likewise affects chocolate flavor development. Before harvest for chocolate production, flavanols are synthesized as defense compounds (Stafford, 1990) in the seeds of *Theobroma cacao*. They protect the seed against oxidative damage and can act as a gustatory deterrent, keeping predators from eating the seeds (Stafford, 1990). Flavanols are known to protect other seeds during growth from mold infection (Kantar et al., 1996), and likely confer a selective advantage to *T. cacao* seeds as well. During chocolate processing, flavanol levels decrease substantially (Kealey et al., 2001), but may still contribute to the flavor of chocolate through bitterness and astringency. Finally, flavanols may alter the course of flavor development by binding with protein and enzymes and decreasing the number of aroma precursors generated (Zak and Keeney, 1976). The significance of flavanols in flavor

development, disease resistance and bioactive properties make them a potentially important indicator of favorable traits for selection and breeding of cacao.

Flavanols in *T. cacao* seeds are composed of three classes (Figure 2-1), catechins, anthocyanins and polymers of the same. They are synthesized in the same pathway (Figure 2-6) with a branch point leading to the slight modifications which distinguish catechins, anthocyanins and procyanidins. Anthocyanins are the only pigmented flavanol molecules, and have been identified as the source of the purple seed color (Ziegleder and Biehl, 1988). Because all flavanols either have color properties themselves or are metabolically related to those that do, it may be possible to use color as a marker for total flavanol content in cacao. For a long time is has been thought that the white, pink and purple colors of fresh *T. cacao* seeds were indicative of phenotypic traits in cacao, although this has never been scientifically proven (Butler, 2001).

This paper takes up the issue of fresh seed color as a potential marker for traits in cacao. Motivated by the centrality of flavanols in many important traits in *T. cacao*, the research investigates how seed color may be indicative of native flavanol concentrations in the fresh seed. Here a survey of 200 cacao seeds is conducted to find out if 1) anthocyanins are coregulated with other flavanols and 2) seed color may be used as a practical indicator of flavanol content.

5.3 MATERIALS AND METHODS

Cacao seed samples

A total of 200 fresh cacao seeds representing 14 different varieties were obtained from cacao research stations IRAD (Cameroon), CRU (Trinidad) and USDA (Puerto Rico). (A complete list of pod varieties sampled is available in the supplemental information section.) In the absence of complete genetic information, the sample set was chosen based on color, judged by comparison with seed colors listed in the International Cocoa Germplasm Database (ICGD, 2002). Cacao pods were harvested at the ripe, or newly ripe stages and sent to the Pennsylvania State University where they were kept in a temperature-controlled environment at 18°C. Seeds were removed from pods 1-13 days after pod harvest (depending on shipping time) for colorimetric analysis.

Colorimetric analysis

Two instruments were used interchangeably for colorimetric measurements on fresh seed cotyledons: a Minolta Chroma Meter CR-200 (Minolta, Ramsey, NJ, USA) or a Minolta Spectrophotometer CM-3500d (Minolta, Ramsey, NJ, USA). Spectrophotometers were standardized against a white tile (Y = 93.2, X = 0.3142, y = 0.3219) and set to exclude specular reflection from shiny seed surfaces. In order to capture color variation throughout the seed, reflectance measurements were made by

placing sections of the outer and cut surfaces of the seed on an aperture 11 mm in diameter. Two measurements on the outer surface of each seed were recorded within 2 minutes of exposure, and averaged. Measurements on the cut seed surfaces were made within 15 seconds after cutting. The error in colorimetric values due to seed oxidation was estimated to be -2 to -3% for the outer surface. Enzymatic oxidation on the cut surfaces resulted in a -7% error in L-values, +3% error in a-values and +74% error in b-values (see Appendix).

Seed storage

Immediately after colorimetric analysis, seeds were quick frozen in liquid nitrogen, placed in Whirl-Pak® bags (VWR, Bridgeport, NJ, USA) and stored at -70°C. Trays of aluminum pans were placed on a pre-cooled shelf (<0°C) in a VirTis Genesis 25 XL Freeze-Drier (SP Industries, Gardiner, NY, USA). Liquid nitrogen was allowed to evaporate before the chamber was shut. Lyophilization was accomplished under vacuum (300 mT) through a cycle of -40°C, 120 min; -12°C, 2230 min; 0°C, 130 min; 10°C, 75 min. Freeze-dried seed mass was measured in a humidity controlled atmosphere.

Polyphenol extraction

A polyphenol extraction procedure was adapted from Adamson et al. (1999) and Wollgast and Anklam (2000a). Seeds were ground individually using a Brinkmann Polytron Kinematica GmbH PCU 1 with a probe 3/4" in diameter (Brinkmann Instruments, Westbury, NY) under 10 mL hexane Optima grade (Fisher Scientific, Pittsburgh, PA, USA) in a 50 mL polypropylene tube (Dimensions: D = 30 mm, length = 115 mm) (VWR International, Inc., Bridgeport, NJ, USA). Equal grinding for each seed was approximated by grinding seeds for 10 seconds each. Lipids were removed in three hexane washes, using a Beckman GPR Centrifuge (Global Medical Instrumentation, Inc., Albertville, MN) for 10 min, 4000-5000 RPM and <0°C to settle the particulate between each wash. After the final hexane supernatant was decanted, polyphenols were extracted in 10 mL of HPLC grade methanol:H_2O (75:25, v/v). Samples were extracted for 24 hours at 5°C. Polyphenols in the second extract were determined to be less than 5-10% of the first extract, and therefore not used in the analysis.

Sample Preparation

Polyphenol extracts were passed through a non-sterile 15 mm syringe filter with a 0.45 micron PTFE membrane (VWR International, Inc., Bridgeport, NJ, USA) into 2 mL microfuge tubes, stored at -70°C. Injection samples placed in 2mL screw cap vials

(Agilent Technologies, Palo Alto, CA, USA) consisted of extracts dissolved in 10% formic acid solution (Fisher Scientific, Pittsburgh, PA, USA) (1:4, v/v).

HPLC analysis

Ten μL filtered extracts was injected by autosampler into an HPLC (Hewlett Packard series 1100). Each extract was run on two columns, one for the separation of anthocyanins, and the other for the separation of procyanidins (Chapters 3 & 4). Relative amounts of anthocyanins, catechins and procyanidins were quantified by estimating peak area on chromatographs.

Statistical analysis

All regression and principal component analyses performed on data were accomplished using Minitab software, version 13.32 (Minitab, Inc., State College, PA, USA). Statistics on non-linear regressions to correlate anthocyanins with seed color were performed using SAS software, version 8.2 (SAS Institute, Inc., Cary, NC, USA).

5.4 RESULTS AND DISCUSSION

Quantification of observable seed color

Table 5-1. Qualitative color assessment of cacao seed sample set. Total number of seeds sampled is 200.

ICGD color category	Number of fresh *T. cacao* seeds sampled
white	23
grey-white	3
pink	15
violet	21
light purple	28
purple	35
mottled	21
dark purple	54

Appropriate sampling of the fresh seed color for the *T. cacao* species was judged through comparison with seed colors listed in the ICGD (2002). The sample set contained representatives of all color classes currently listed (Table 5-1), although the grouping of seeds into such categories was a bit arbitrary since cacao seed colors exist on

a continuum from deep purple to white.

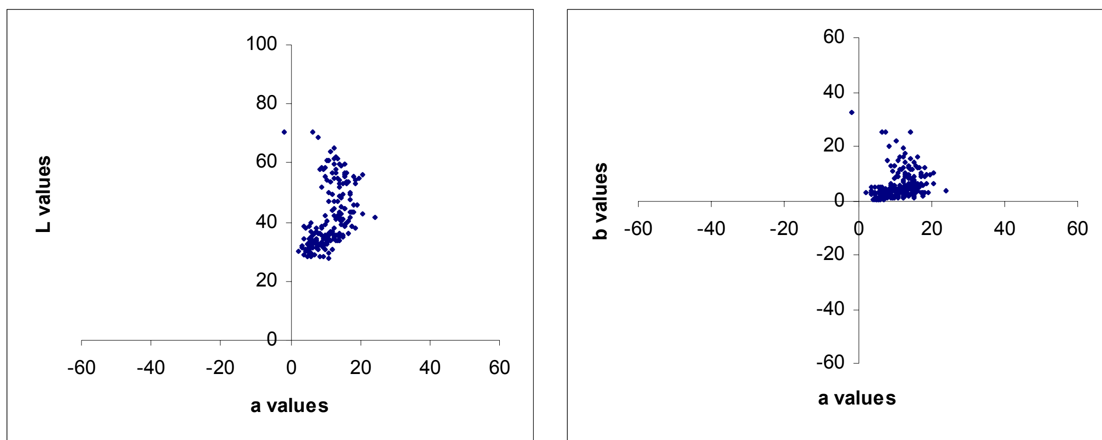

Figure 5-1. *T. cacao* seed color measurements (average of two data points on outer surface) mapped onto a 3-dimensional colorspace where "lightness" of the seed is indicated by L-values (L=0 is black, and L=100 is pure white), and chromaticity coordinates are given by a-values and b-values (+a is red, -a is green, +b is yellow, -b is blue). Any distance of 2 equals a perceptible difference based on a standard observer.

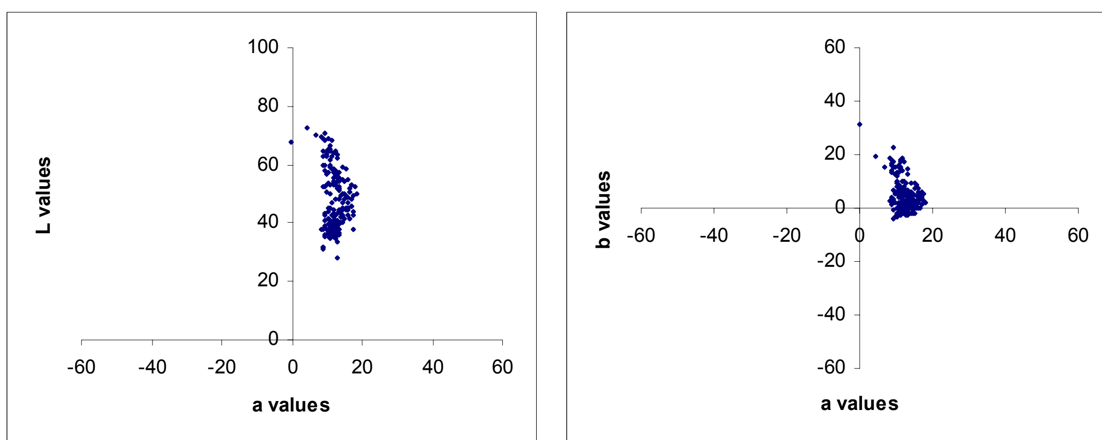

Figure 5-2. *T. cacao* seed color measurements (average of two data points on cut surface) mapped onto a 3-dimensional colorspace (as described in legend for Figure 5-1).

Observable seed colors were better analyzed using quantitative data based on reflectance spectra. Each seed was mapped onto a three-dimensional colorspace, where equal distance corresponds to equal perceptible distance. Figures 5-1 and 5-2 confirm that the seeds sampled comprise a continuum of colors ranging from deep purple to

white. The primary distinguishing factor for seeds colors on both the outer and inner surfaces seem to be their "lightness", as described by L-values. The greater proportion of darker, purple seeds in the sample set is a reflection of the dominance of purple seeds in the natural population.

Characterization of anthocyanins

Pigmented anthocyanin compounds were analyzed to give more information on seed color. Details on characterization of these compounds can be found in Chapter 3, but in summary, HPLC-MS analysis on *T. cacao* seed extracts led to the identification of 4 anthocyanin species, two of them identified for the first time. The extracted anthocyanins varied only in glycosylation of the aglycone base (Figure 5-3) and the relative amounts of each were fairly consistent from seed to seed. Concentration of total anthocyanins, however, varied appreciably.

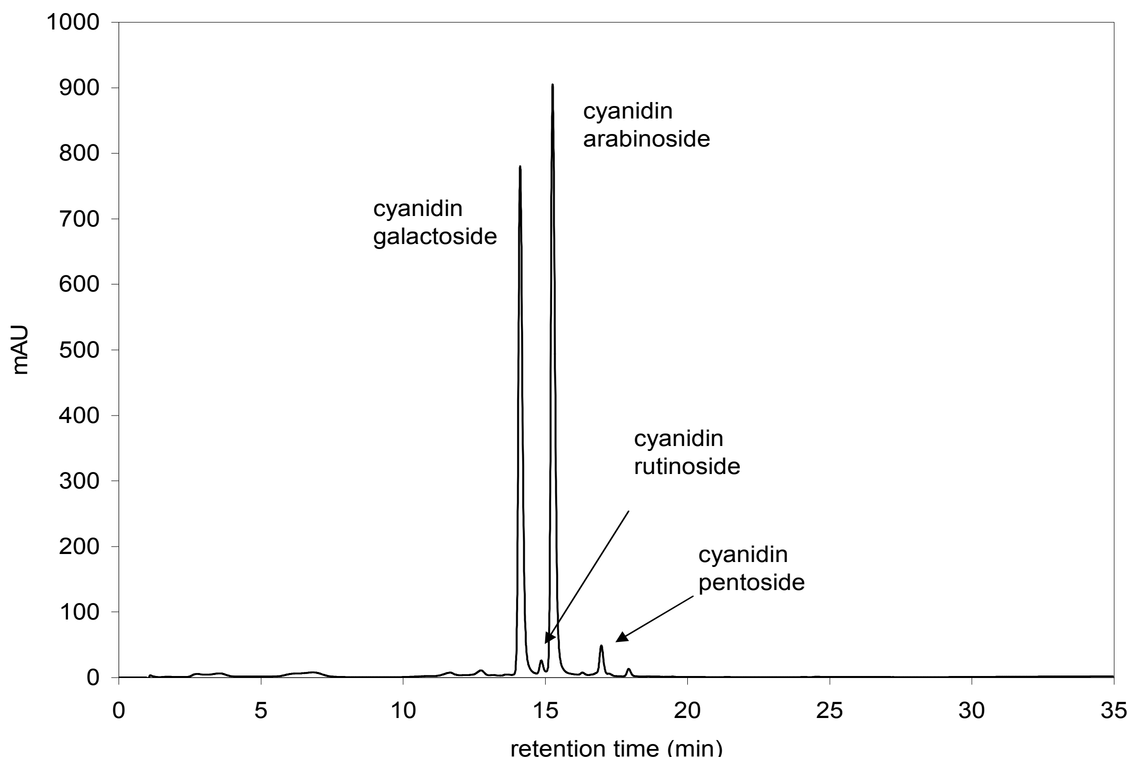

Figure 5-5. Chromatograph of *T. cacao* seed extract resolved on a Betabasic C18 HPLC column. Absorbances are observed at 520 nm and anthocyanins identified by tandem MS.

Correlations of anthocyanin concentrations with observable color revealed that anthocyanin concentrations from fresh seed extracts could not entirely explain the expression of color in the *T. cacao* seed. This is because it is not only concentration, but position of the molecule within the cell which contributes to the final seed color (Chapter 3). Anthocyanin concentrations from extracts were, however, well correlated with L-values of the outer seed surface. Seeds which appeared darker on their outer surface were likely to have higher concentrations of anthocyanins in the total seed extract. The relationship found was logarithmic with a certain level of saturation whereby increased concentrations of anthocyanins did not result in an appreciably darker seed (Figure 3-3).

Characterization of procyanidins

Other flavanol groups were characterized through HPLC-MS analysis (Chapter 4). Chromatographic separation of *T. cacao* seed extracts led to the identification of catechin monomers through heptamers (Figure 5-4). Total procyanidin concentrations varied from seed to seed, but the relative proportions of procyanidins within a single seed was fairly constant. This suggests that procyanidins are synthesized in the seed in such a way that oligomer ratios remain constant, or perhaps that certain oligomers were preferentially extracted according to their solubility. Solubility of procyanidins in aqueous solvents decreases as chain length increases (Pasch, 1998). Either way, since the relative proportions of procyanidins was relatively constant for each seed, total procyanidin content as a sufficient relative measure of catechin/procyanidin synthesis.

Procyanidins are known to modify color properties *in vivo* through copigmentation effects, resulting in either an increase or shift in total absorbance (Chen and Hrazdina, 1981). Because procyanidins were measured as extracts, it was not possible to isolate a color characteristic with a unique correlation with procyanidins. Instead, procyanidins plotted against colorimetric values gave similar correlations to those found between color variables and anthocyanins, although with a greater degree of variance. Therefore, the data collected demonstrates a relationship between anthocyanins and procyanidins total contents more than it does between procyanidins and color.

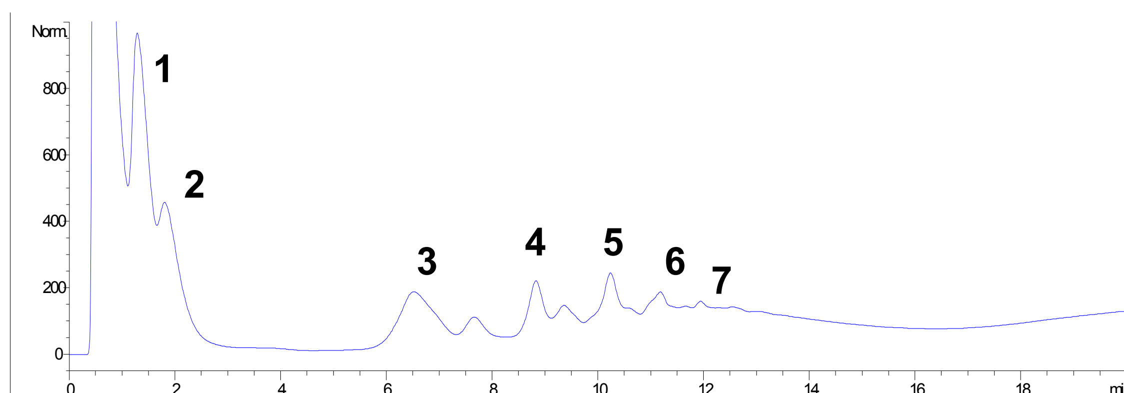

Figure 5-4. Chromatograph of procyanidins. *T. cacao* seed extracts were resolved on Supelco® polyetheylene glycol column and observed at 280 nm. Peaks were identified by mass spectroscopy and are labeled according to degree of polymerization.

Comparison of flavanol metabolites

Relative concentrations of anthocyanins and procyanidins for each seed shows a positive relationship ($p < 0.0000$) with high variance ($r^2 = 0.5346$). The positive relationship observed in Figure 5-5 suggests that anthocyanins and procyanidins are generally co-regulated throughout the species; when anthocyanins are upregulated in the living seed, higher amounts of procyanidins will likely be seen as well. There is, however, a fair degree of variance, suggesting additional mechanisms for fine-tuning control at the branch point.

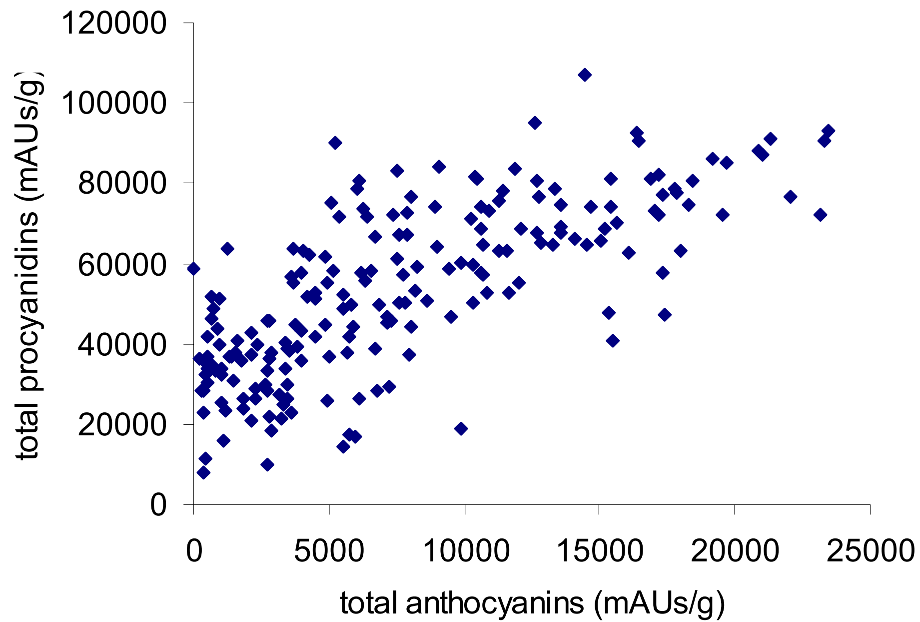

Figure 5-5. Comparison of relative amounts of anthocyanins and procyanidins for 200 *T. cacao* seed extracts.

Breaking down the data by pod (Figures 5-6, 5-7) yields more clues to how flavanols are synthesized during development. Moving forward on the assumption that seeds from the same pod will be more closely genetically related than seeds from different pods, linear regression lines were fitted to seeds from each pod (in which 10 or more seeds were sampled). All except one pod (Figure 5-7) confirmed a positive relationship between anthocyanins and procyanidins. Variations in fit, showed that pods are able to alter the proportion of anthocyanins and procyanidins from seed to seed, perhaps in response to environmental effects. The AML pod is evidence of exceptions to strict co-regulation.

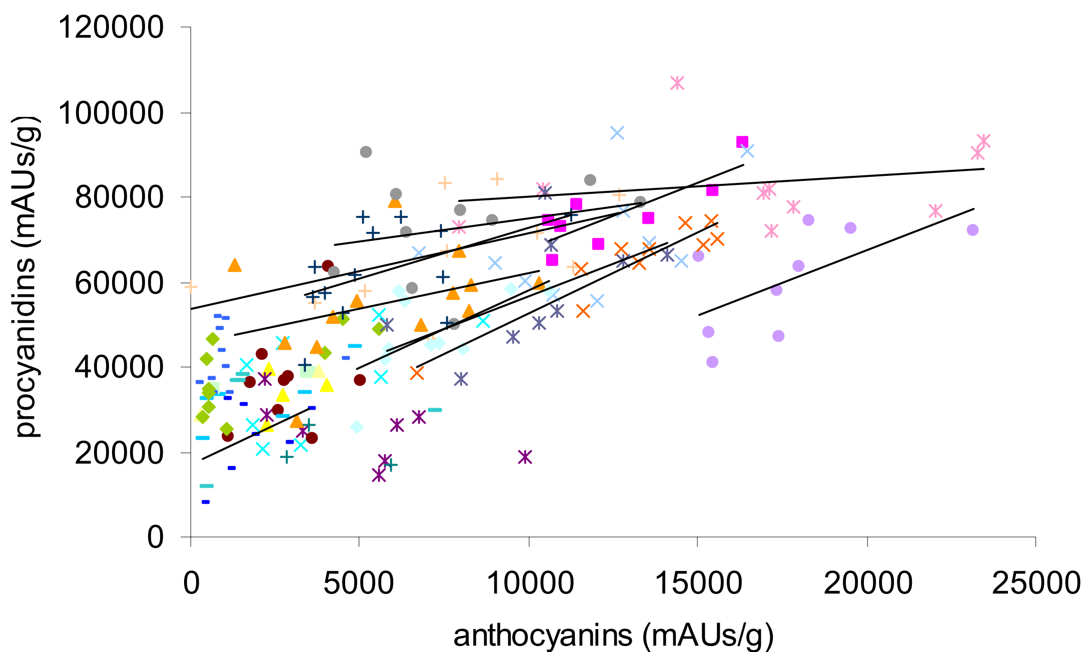

Figure 5-6. Relative contents of procyanidins versus anthocyanins for *T. cacao* seeds, grouped by pod. Linear trendlines are shown for those pods in which 10 or more seeds were sampled.

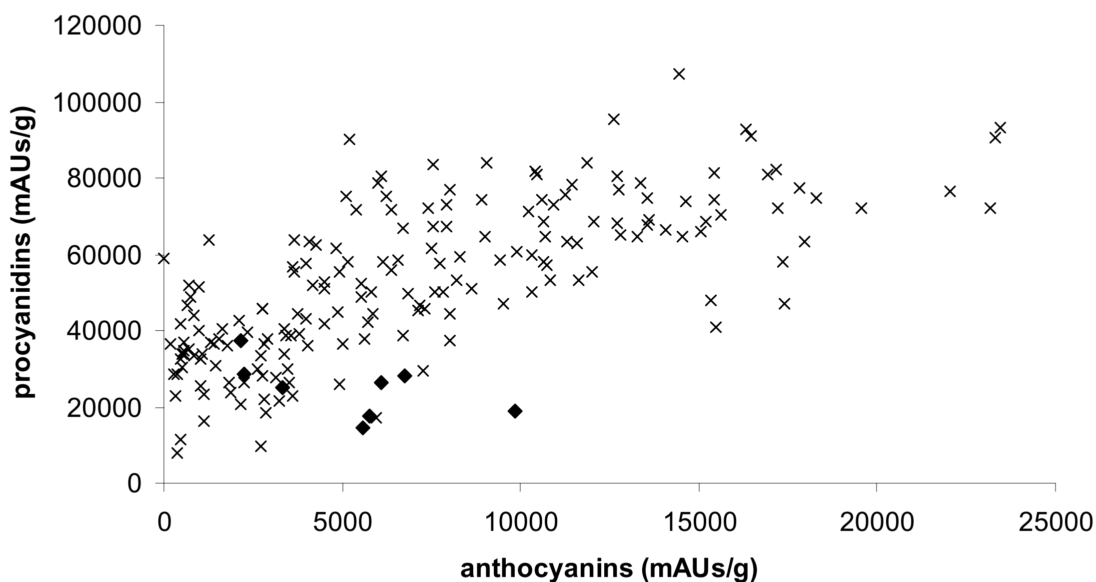

Figure 5-10**.** Negative relationship between procyanidins and anthocyanins observed for 7 seeds sampled from an Amelonado pod from Cameroon. AML seeds are indicated by (♦) with respect to seeds from the entire sample set (x).

Color as a practical indicator of flavanol content

Finally, in order to use fresh seed color as a marker for flavanol content, a practical relationship must exist between procyanidin content and observable color. L-values of the outer seed surface provided the most descriptive relationship. Figure 5-8 shows that L-values are less predictive at lower concentrations of procyanidin content, and more closely related at higher levels of procyanidin content. Maximum error was ± 10 µg catechin equivalents per gram seed dry weight, but on average, procyanidin contents may be predicted with a variance of ± 3.57 µg catechin equivalents per gram dry seed weight. Essentially, color may be used as only a rough indicator of flavanol content.

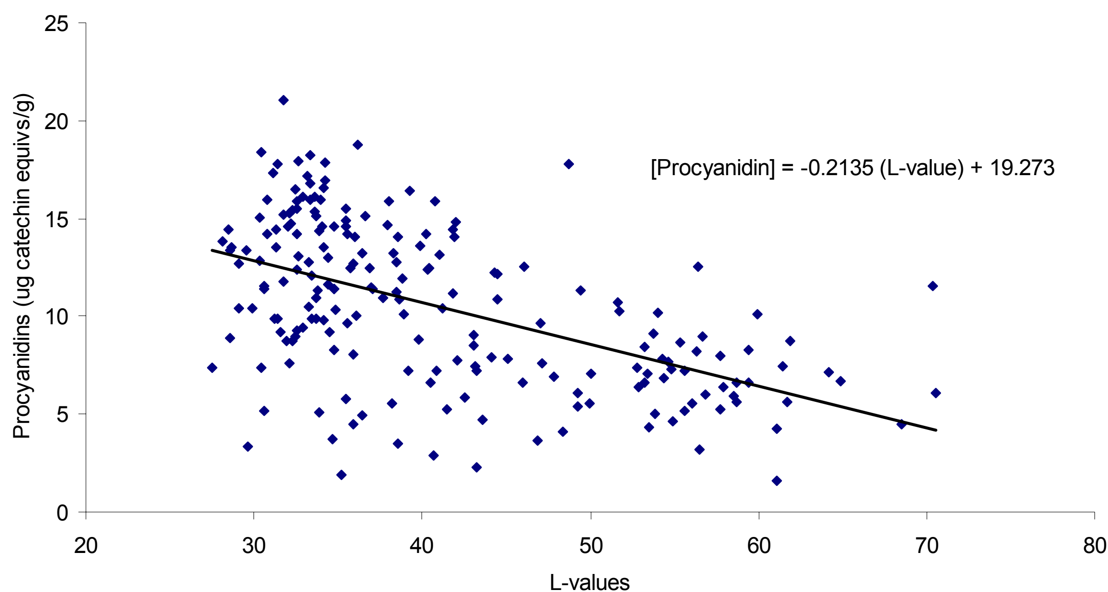

Figure 5-8. Prediction of procyanidin content using L-values for the outer seed surface. $R^2 = 0.28$

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5.5 SUPPORTING INFORMATION

Table 5-2. Identity and origin of fresh cacao seeds sampled.

pod variety	source location code[1]	pod number	# of seeds sampled
AML	cm	2	10
AML	cm	3	8
DR 2	tr	2	4
DR 2	tr	3	8
ICS 16	pr	3	5
ICS 16	tr	1	3
ICS 39	pr	1	9
ICS 39	tr	3	3
ICS 39B	pr	2	7
ICS 39B	pr	3	6
ICS 40	pr	2	3
ICS 40	pr	3	2
ICS 40	tr	1	10
P 19	pr	1	10
PA 4	pr	1	10
SCA 6A	pr	7	10
SCA 6B	pr	5	5
SCA 6B	pr	6	10
SCA 6B	pr	8	2
SCA 12	pr	2	10
SNK 10	cm	1	13
SNK 16	cm	1	10
SNK 413	cm	1	9
UF 613	pr	3	10
UPA 134	cm	2	13
UPA 134	cm	3	10

[1]Pod sources and locations are indicated by the following codes: cm - IRAD, Cameroon; tr - CRU, Trinidad; pr - USDA, Puerto Rico.

CHAPTER 6

FINAL REMARKS

Flavanols are bioactive molecules, able to sequester free radicals, complex with proteins, and interact with taste receptors to give a bitter sensation in food products. This thesis has described how, in the context of chocolate production, flavanols contribute to the color of the fresh cacao seed, confer disease resistance to the raw material, play a role in flavor development, and eventually contribute to antioxidant capacity in the final chocolate product. Because of their wide range of functions, flavanols are introduced in this thesis as "linking compounds" highlighted as a springboard for recognizing new relationships within the *T. cacao* species. Specifically, this work focused on the relationship between fresh seed color and flavanol content and established a basis for developing fresh seed color as a marker for other economically important traits in *T. cacao*.

The experimental work was divided into subsections detailing the characterization of *T. cacao* fresh seed color, analysis of flavanol compounds, and correlations between the two. Results on colorimetric analysis showed that seed color is correlated with anthocyanin concentrations in the fresh seed, but is also dependent on the sub-cellular

location of the pigmented molecule. Two new anthocyanins were found in seeds extracts: a cyanidin pentoside and a cyanidin rutinoside. These anthocyanins were previously unidentified for *T. cacao* and were present in relatively small amounts, yet their presence underlines the fact that pigmented molecules in cacao differ only in glycosylation. It is possible that this glycosylation is another clue pointing to the importance of sub-cellular localization of anthocyanins. Glycosylation often acts as an aid for transport within a cell (Winefield, 2000). Perhaps these glycosylations help to direct anthocyanins to specific locations within the cell vacuole, leading to different variations of color due to copigmentation effects.

The task of quantifying flavanols was most complicated in the case of the catechin monomers and procyanidins. An alternative method was outlined for their separation. Using a polyethylene glycol as the solid phase, we were able to separate procyanidins quickly. These peaks were quantified for comparison from seed to seed.

In the 200 seeds tested, flavanol synthesis seemed to be coordinated with respect to anthocyanins and procyanidins. Plots for native contents of anthocyanins versus procyanidins for each seed showed a positive relationship. Even in looking at seeds of just one pod, a positive relationship was confirmed -- with the exception of one pod, where a negative relationship was seen.

The relationship between flavanols content and expression of the seed color phenotype was more loose. Seeds with darker colors did show higher levels of flavanols, but the standard error in the relationship was too high to develop a predictive model with precision. The relationship found in this thesis between flavanol content and fresh seed color is more useful in its conceptual value.

A correlation in the between anthocyanin and flavanol content in fresh cacao seeds suggests that the metabolites are generally co-regulated throughout the species. The phenylpropanoic pathway which gives rise to flavanols probably has a regulation point early in the pathway as well as a fine-tuning mechanism at the point at which flavanols deviate from anthocyanins. All these suggestions are new for cacao. The phenylpropanoid pathway is known well, but the regulation is not well known, especially in the case of cacao. This study provides the framework for additional biochemical studies to better understand regulation of the flavanol pathway. The neg. relation pod is even a good specimen to work with for comparison with a control.

The second major outcome of the research has to do with using results for breeding. Because of this paper, breeders can certainly know that purple seeds have higher levels of flavanols. But how is this useful? What can flavanol contents do for cacao.

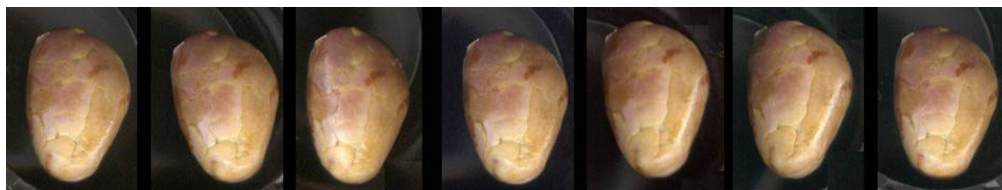
Currently, high flavanol contents are already being conserved for the making of highly fortified chocolate. These are mostly processing procedures. Selection of varieties with naturally high levels, plus the processing procedures could be useful. As outlined earlier, flavanols are also involved in disease resistance, flavor development. These links have to be more practically established. We know that flavanols are involved, but we don't know how crucial they are. This could be a method for controlling disease resistance and flavor development simultaneously. In all, we have a good stepping stone into the future for a holistic management of the cacao crop.

APPENDIX A

SEED OXIDATION STUDIES

The error in colorimetric measurements caused by seed oxidation was examined in a series of preliminary experiments. These studies had two simultaneous objectives: first, to develop methods to minimize the effect of oxidation on seeds; and second, to establish margins of error and their significance.

The first aim was to document cacao seed oxidation for both the outer and cross-sectional surfaces of the seed. Sample data from this experiment is shown in Figure A-1 through Figure A-6. Figure A-1 and Figure A-2 show oxidation of the outer seed surface while Figure A-3 and Figure A-4 explain oxidation of the cut surface. For digital photographs, a purple/white mottled seed was chosen to demonstrate oxidation of both purple and white sections simultaneously. The corresponding colorimetric readings are shown below the images. Illustrative of the general scheme, readings were taken at 15s, 30s, 1min, 2min, 5min, 10min, 15 min, 20min (and 30min for the outer surface only). In total, twelve replicate experiments were conducted from which the following assessments on seed oxidation are made.



Time (min:sec)	0:15	1:00	1:50	2:36	3:20	4:00	4:38
L	57.27	54.96	57.70	57.39	58.19	57.31	57.01
a	5.76	5.98	6.28	5.83	5.72	6.23	6.32
b	16.53	17.31	16.10	16.37	17.30	16.54	17.53

Time (min:sec)	5:20	6:03	6:48	7:25	8:04	9:25	10:02
L	57.87	57.27	56.97	57.26	55.90	56.53	54.94
a	5.96	6.51	6.51	6.28	6.64	6.65	6.31
b	17.00	15.81	16.51	16.76	18.09	16.80	19.91

Figure A-1. Example of outer surface seed oxidation.

Figure A-1 shows that oxidation for the outer surface is barely perceptible. The colorimeter does, however, pick up changes, L-values being most descriptive of the reaction (Figure A-2). Linear approximations were fitted to the L-value vs. time plots and a statistical analysis revealed that of twelve seeds, ten had slowly, but significantly decreasing L-values ($p<0.05$), the other two being relatively constant ($p<0.25$).

Chromaticity coordinates a-values and b-values were not as descriptive of oxidative color changes, although a-values also showed suggestions of decreasing values

along time. For the most part, error among replicate readings seemed to mask any real oxidation trends given by a-values and b-values.

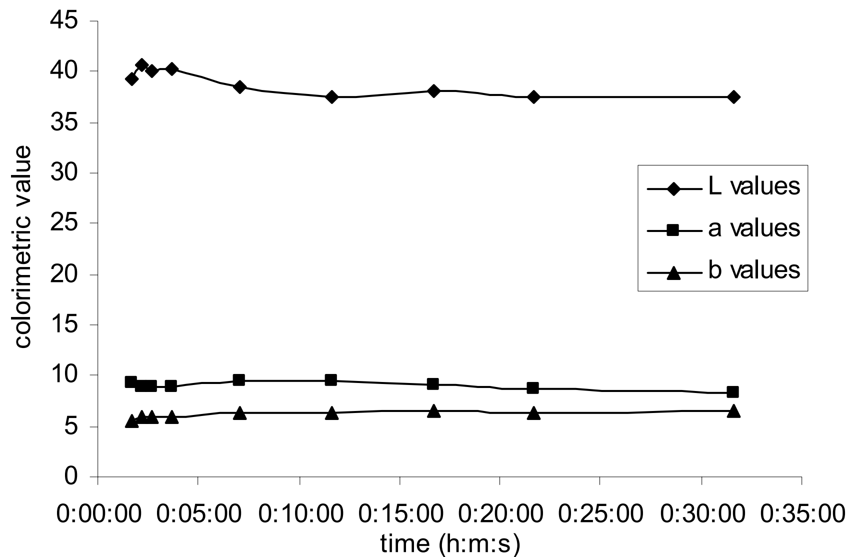
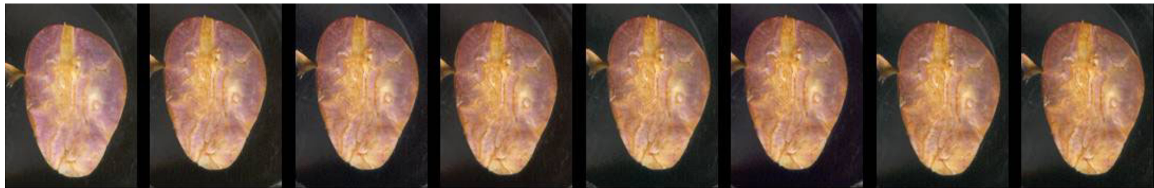


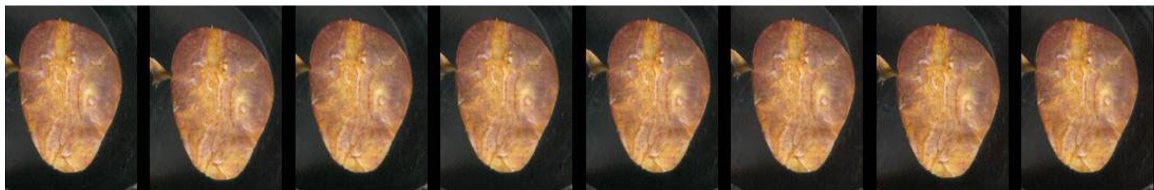
Figure A-2. Outer seed surface oxidation modeled by colorimetric CIEL*a*b* values.

Relatively modest color changes on the outer surface of the seed are attributed to the endosperm layer (shiny layer seen in Figure A-1). When the testa is peeled, and the seed exposed to oxygen, the endosperm, only a few cell layers thick, apparently protects underlying pigmented cells of the cotyledon from coming in contact with oxygen. In fact, the most noticeable color change in Figure A-1 occurs at a supposed site of endosperm damage in the upper right corner of the seed. In peeling the testa from the seed, the thin endosperm is sometimes disrupted. Besides this site of damage, on this seed and others used in the experiment, oxidation of the outer surface is considered to be minimal.

Measurements on the cross-sectional surface of the seed showed a much more dramatic color change. Figure A-3 shows that the brilliant color seen in the first image quickly dulls to a purplish brown after 1 minute. In this case, cutting of the seed initiates an enzymatic oxidation.



Time (s) 0:15	0:56	1:32	2:09	2:45	3:19	3:59	4:38
L 61.77	59.65	58.88	58.43	58.10	57.85	57.62	57.44
a 6.03	6.93	7.26	7.41	7.56	7.73	7.82	7.91
b 19.19	22.81	24.04	24.76	25.26	25.64	25.99	26.24



Time (s) 5:14	6:00	6:35	7:17	8:00	8:36	9:15	9:58
L 57.28	57.16	57.06	56.94	56.85	56.76	56.70	56.63
a 8.06	8.02	8.02	8.12	8.10	8.15	8.24	8.26
b 26.45	26.67	26.89	27.00	27.13	27.23	27.34	27.44

Figure A-3. Example of cut surface seed oxidation.

Cell damage allows for polyphenol oxidase to gain new access to substrate (Misnawi and others 2002), which is likely demonstrated here. It should be noted that an

additional blue streak was sometimes observed on the cut surface, most likely due to interactions between the metal razor blade and the cut cells because metal chelation of procyanidins is known to produce a blue color in flowers (Kondo and others 1996).

Graphs of the tri-stimulus values vs. time demonstrate these trends more clearly. In Figure A-4, color changes in a way that is consistent with an enzymatic reaction. As polyphenol oxidase gains new access to substrate, the rate of oxidative change is high, later stabilizing as available enzymes are saturated. Based on the known reaction mechanism, colorimetric values are assumed to reflect a logarithmic relationship with respect to time. Log functions fitted to the oxidation curves for L-values showed a correlation coefficient greater than 95% for all the seeds tested. Colorimetric b-values also showed good fits to a log plot. Again, a-values were not as descriptive of the reaction and remained fairly stable.

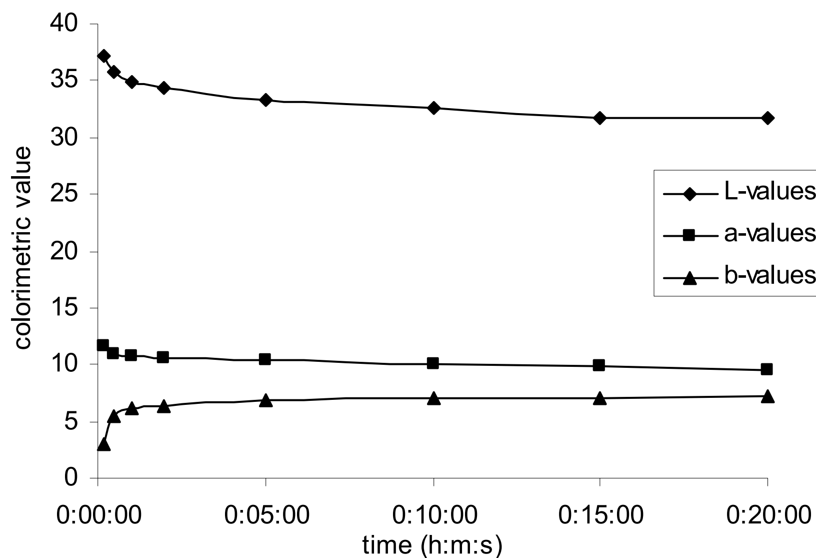


Figure A-4. Cut seed surface oxidation modeled by colorimetric CIEL*a*b* values.

Despite efforts to find general patterns of oxidation for all cacao seeds, significant variations in the course of oxidation from seed to seed were observed. In both experimental observations and in calculating the rates of oxidation, it seems that each seed has its own unique concentrations of enzyme and substrate. No strong correlations between seed color and rate of oxidation were found -- nor between variety and rate of oxidation even though oxidation rates were sometimes strikingly similar for two seeds of the same variety. There may be further ways to characterize cacao seed oxidation, but they were not apparent in the 12 seeds examined here.

Oxidative changes were also apparent on reflectance plots over the visible spectrum (Figure A-5). Changes in reflectance curves are shown with respect to all visible wavelengths for the first 15 minutes of seed oxidation.

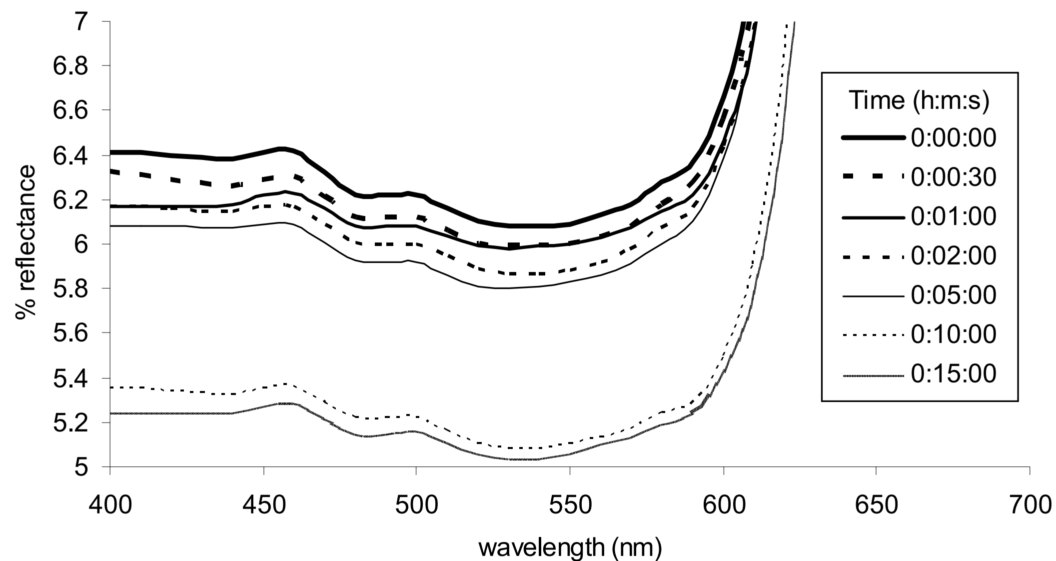


Figure A-5. Changes in reflectance curves over time during oxidation. This particular example shows oxidation of the outer surface of a purple seed (UPA134 cm1).

For the outer surface, reflectance decreases over time with little change in curve shape. The reflectance curve shifts down in increments which are relatively small; reflectance decreases by about 3% from the original curve for the first 2 minutes of oxidation. From 5 minutes to 10 minutes, there is a disproportionately large decrease in total reflectance which is corroborated by a disproportionate decrease in L-values for this particular seed. This disproportionate decrease was not observed in general for all the seeds, but does point out that each seed demonstrated its own unique oxidation pattern. Curiously, after 15 minutes, reflectance began to increase again. Although interesting, this effect is not investigated further here because oxidative changes after 15 minutes are not relevant to the discussion on error in colorimetric measurements.

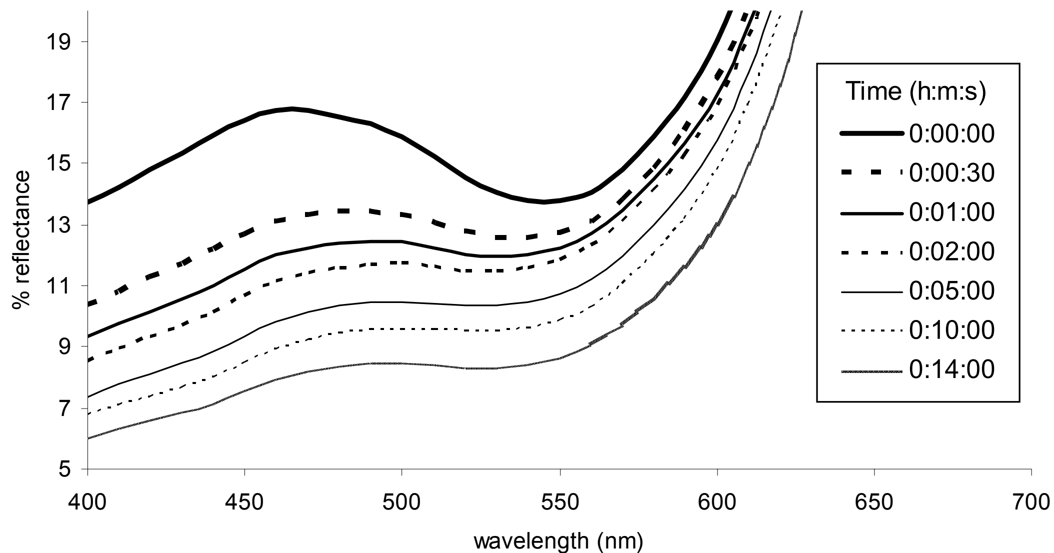


Figure A-6. Changes in reflectance curves over time during oxidation. Oxidation of the light purple cut surface of a seed (UPA134 cm1) is shown.

For the cut surface, reflectance decreases with a simultaneous "flattening" of the curve. This "flattening" is typical of a light purple seed. In general, changes are more

drastic for shorter wavelengths and higher wavelengths remain constant. On average, the reflectance curve decreases by about 20% for the cut surface in the first 2 minutes. This is considerably more significant than oxidative changes for the outer surface.

In an effort to quantitatively summarize changes in spectral reflectance for oxidation of both outer and cut surfaces, principal components analysis (PCA) was used. PCA is a statistical method that can describe the variation in large data sets in a few variables. For data shown in Figure A-7, only one principal component was needed to describe over 95% of the variation in the data.

Interpretation of principal components found is done in Figure A-7 and Figure A-8. In each figure, the most important principal components are shown and the percentage of the variation in the data they describe is given in the legend. The values on the y-axis, "absolute value of the coefficients" may be interpreted as a measure of contribution to the principal component.

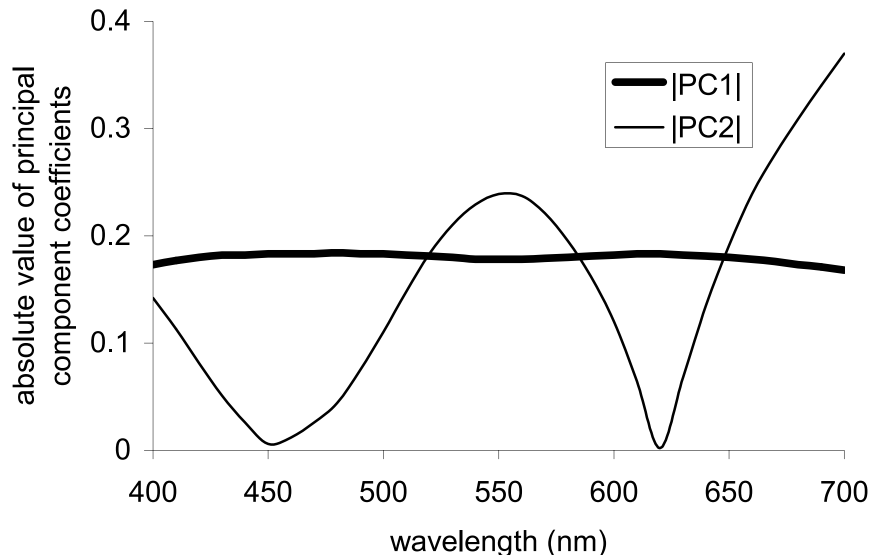


Figure A-7. Interpretation of principal components found for spectral changes during oxidation of the outer seed surface.

For example, in Figure A-7, the first principal component describes 95.9% of the variation in the data. At the same time, the almost constant plot shows that it seems to be a good general descriptor of variation for all wavelengths. This component probably corresponds to the general shift down in absorbance during oxidation as observed in Figure A-5. The second principal component, describing only 4% of the variation, is more descriptive of variation in longer wavelengths (> 650nm).

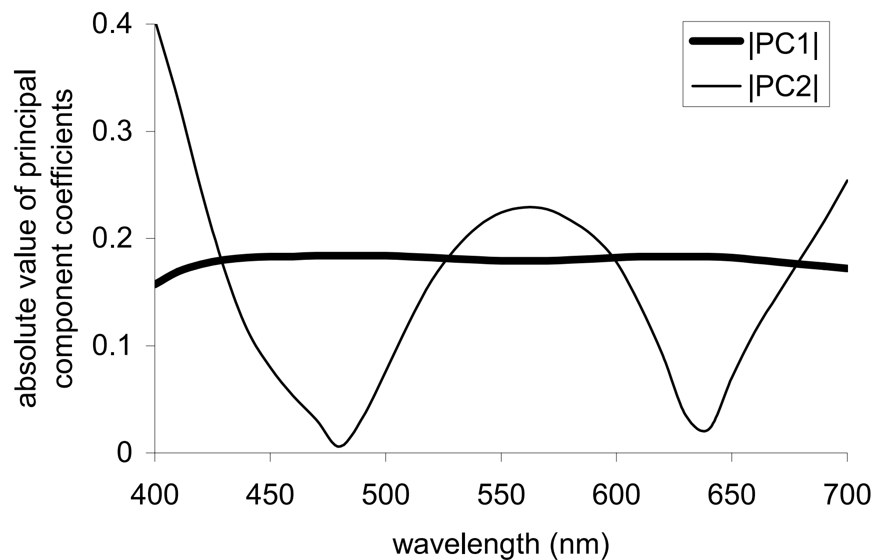


Figure A-8. Interpretation of principal components found for spectral changes during oxidation of the cut seed surface.

Principal components found for oxidation of the cut surface (Figure A-8) were quite similar to those found for the outer surface. The first principal component captured 97.6% of the variability and was also a good general descriptor of all the wavelengths.

The first principal component for both the outer and cut seed surface confirms that oxidation is best described by a general downward shift in reflectance spectra.

One remedy for error induced by oxidation is the addition of an anti-oxidant to the seed surface. Oxidation inhibitors including a mushroom preservative solution (Beelman and Duncan 1999) and lemon juice, were tested. Three additional trials used distilled water to test the effect of washing off the oxidation products.

Figure A-9 shows the effect of adding each of these to the cut surface of the seed 1 minute after cut. As can be seen, the control has a consistent course of change over the time period. Addition of anti-oxidant (1:00 min) changes the course of oxidation, returning it to a value that is arbitrarily related to the original point.

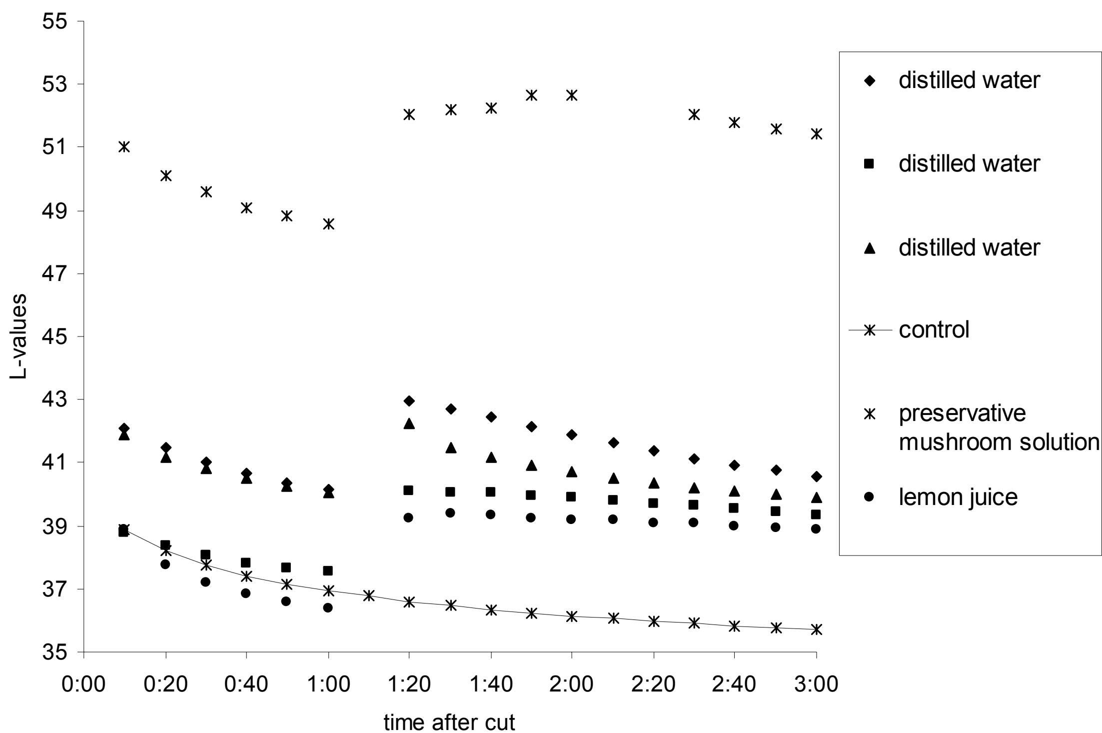


Figure A-9. Effect of anti-oxidants on seed cut surface oxidation. Anti-oxidants (water, mushroom preservative solution and lemon juice) were added to seed cut surface at one minute after cut.

Anti-oxidants did seem to reverse the oxidation process, but their effect added more uncertainty to the error. Distilled water was able to wash off oxidative products, returning the seed L-value close to its original value, but did not prevent further oxidation. Instead of using an anti-oxidant or water wash to make up for oxidative color changes, a controlled error strategy was chosen to account for oxidation error. Assuming that colorimetric measurements were taken in a uniform way for each seed assuming a maximum times of exposure no greater than 2 minutes for the outer surface and 15 seconds for the cut surface, estimates of error were calculated.

For the outer surface, color changes caused by oxidation were so slight that replicate errors dominated any errors induced by oxidation. Still, a linear approximation was used to extrapolate the colorimetric reading at time zero. Using the extrapolated value at time zero as the theoretical 'true' reading, error at 2 minutes after oxidation was found to be -2% for L-values, -3% for a-values and -3 % error for b-values for the outer surface colorimetric measurements.

Color changes on the cut surface of the seed during measurement were much more variable because of the enzymatic oxidation. The logarithmic curve is prone to very large changes in the early time points. A linear plot of colorimetric value versus the natural log of time was extrapolated to find the theoretical reading at a time less than 1 second. Error assessments for readings taken at 15 seconds of exposure showed a -7% error in L values, +3% error in a values and +74% error in b-values. Error in b-values seemed to be very high due to a portion of the seeds with very rapid browning in the early stages of oxidation (see Figure A-4).

APPENDIX B

CONTACT INFORMATION FOR CACAO SOURCES

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