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Seed Color as an Indicator of Flavanol Content in *Theobroma cacao* L.

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Abstract

Seed color has long been associated with quality traits of the cacao tree, *Theobroma cacao* L., namely disease resistance and the flavor of chocolate produced from its seeds. Flavanol compounds are central to many of these quality traits and this study investigates the relationship between seed color and flavanol content in the seed. Colorimetric data was collected for 200 *T. cacao* seeds, each individually analyzed for relative flavanol concentrations by HPLC MS. A significant positive relationship ($p<0.0000$) was found between total flavanols and pigmented anthocyanin compounds. Colorimetric seed color data showed that "lightness" of the outer seed surface, as measured by L*-values, is only modestly predictive of flavanol content. Individual seed extracts consistently revealed the presence of four pigmented anthocyanin compounds; in order of decreasing abundance, they 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. During this investigation, a method was 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 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 time and

similar spectral traces. Benefits of this system as a rapid method for procyanidin characterization are discussed.

Keyword Index: *Theobroma cacao* L., Malvaceae, flavanols, anthocyanins, procyanidins, catechins.

Introduction

Fresh cacao seeds range from deep purple, to pink, to white, which is thought to be associated with economically important, although hard-to-measure traits, such as chocolate flavor (Scarpari et al., 2005; Butler, 2001; Presilla, 2001; Ndoumou et al., 1996). These colors are closely linked to genetic identity (Bartley, 2005) and are widely used to distinguish the major cultivars. Anthocyanins, the chemical compounds responsible for cacao seed pigment (Ziegleder and Biehl, 1988), are flavanols themselves, synthesized in the same metabolic pathway (Stafford, 1990), and their regulation in the seed is potentially indicative of the entire class of these compounds.

Flavanols are central to the development of quality cacao. Prior to harvest, flavanols are synthesized in the growing seeds as defense compounds (Scarpari et al., 2005; Ndoumou et al., 1996). They protect the seed against oxidative damage and can act as a gustatory deterrent, keeping predators from eating the seeds (Stafford, 1990). During chocolate processing, flavanol levels decrease

substantially (Kealey et al., 1998), but those remaining contribute to the chocolate flavor through their known bitter and astringent properties (Stark et al., 2005). Flavanols also 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.

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 are due to a

mutation in the synthesis of anthocyanins or merely another form of the compounds.

Procyanidins, as a subclass of flavonoids, have gained much attention in recent years due to reported health benefits (Hannum and Erdman, 2000). Oligomeric procyanidins, specifically, 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 (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).

This study investigates how seed color may be indicative of native flavanol concentrations in the fresh seed. Many studies have looked at flavanol contents in cacao in recent years (Sánchez-Rabaneda et al., 2003, Whitacre et al., 2003; Gotti et al., 2006; Kelm et al., 2006; Niemenak et al., 2006). This study analyzes seeds individually in order to assess the genetic variation in the species. Relative concentrations of flavanols were measured for 200 cacao seeds to determine if 1) anthocyanins are coregulated with other flavanols and 2) if seed color may be used as a practical indicator of flavanol content. 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.

Results and Discussion

Seed cotyledons sampled from 16 varieties of cacao ranged in color from deep purple to ivory. L*a*b* colorspace mapping of individual seeds Figure 1 shows a weighted sampling of purple seeds consistent with the natural population (ICGD, 2002). Error due to oxidation during color measurement was estimated to be -2% for L-values, -3% for a-values and -3 % error for b-values.

HPLC revealed two major peaks with max UV absorbance at 520 nm (Figure 2). Major ions for the first peak (retention time = 14.82 min) were $[M]^+$ m/z = 449 and $[M\text{-hexoside}]^+$ m/z=287. The second anthocyanin (RT = 15.97 min) showed ions at $[M]^+$ m/z=419, $[M\text{-pentoside}]^+$ m/z = 287. These ion fragments correspond to those found for a cyanidin-3-O-glucoside and cyanidin-3-O-pentoside, respectively (Mazzuca et al., 2005).

Positive identification of procyanidin compounds was established through negative ion mass spectra (Figure 3). Detection at 280 nm revealed peaks eluting from the column (Figure 4) with masses corresponding to procyanidin monomers through pentamers (Figure 5: monomers $[M]^+$ m/z = 289; dimer $[M]^+$ m/z = 577; trimer $[M]^+$ m/z = 865, $[M\text{-monomer}]^+$ m/z = 577; tetramer $[M]^+$ m/z = 1153, $[M\text{-dimer}]^+$ m/z = 576; pentamer $[M]^+$ m/z = 1441). The pentamer showed a dominant ion fragment at m/z = 720.38 likely due to fission

by a reverse Diels-Alder reaction. Proposed cleavage sites between base units and in ring B of the oligomers are known fragmentation patterns of procyanidins, Figure 6 (Friedrich et al., 2000). Pure (-)-epicatechin run on the polyethylene glycol column showed similar retention times to that of the compound identified as the monomer. Furthermore, the UV spectral absorbance of the standard was almost a perfect match for all the procyanidins identified.

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., 2001; Wollgast et al., 2001) as well as our own studies (not included), 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, comprising short polyethylene glycol chains, separated the procyanidins according to chain length with an unusually long retention time between dimers and trimers (Figure 4). 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, 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 in a cacao seed extract (Peng et al., 2001), of which there are many (Hannum and Erdman, 2000) it is difficult to determine the exact identity of these minor 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. Separation of this impurity, confirmed by the peak in the monomer's fragmentation spectra (Figure 3), could perhaps be improved through a modification of the mobile phase or by injecting smaller sample volumes.

Two major anthocyanin species were identified in extracts for nearly every seed sampled. MS results were consistent with the presence of anthocyanidins previously identified in cacao, 3-β-D-galactosidyl cyanidin and 3-α-L-

arabinosidyl cyanidin (Niemenak et al., 2006; Forsyth and Quesnel, 1957). Relative anthocyanin concentrations measured by peak area were most closely correlated with L* values of the seed surface demonstrating an inverse linear relationship between anthocyanin content and lightness of the seed (Figure 7).

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. It is known that the color of anthocyanins can be modified based on pH (Figure 8), metal chelation, co-pigmentation with proteins and other flavanols (Gross, 1987).

Other phenolics separated on a polyethylene glycol column and visualized at 280 nm were recognized as cacao procyanidins based on similar traces from chocolate extracts using other column systems (Hammerstone et al., 1999; Wollgast et al., 2001). Negative ion mass spectrometry identified monomers through pentamers with maximum chain length likely limited by the solubility of large polymers in the extraction. A relative measure of total procyanidin

content for each seed was based on the sum of peak areas for all procyanidins polymers.

The relationship between anthocyanins and procyanidins within a single seed was positive ($p < 0.0000$) with high variance ($r^2 = 0.5346$) (Figure 9). This result is a strong indicator that the chemical classes are generally co-regulated in the seed. The high degree of variance, however, suggests additional mechanisms for fine-tuning control at the point where their metabolic pathways branch (Stafford, 1990). One Amelonado pod from Cameroon (AML) showed an inverse relationship with low procyanidin contents even for more pigmented seeds and represents an interesting lead for further study. In general, spectroscopic L-values were only modestly predictive of procyanidin content (Figure 10).

Experimental

General Experimental Procedures. Colorimetric measurements were made using either a Minolta Chroma Meter CR-200 or Spectrophotometer CM-3500d (Minolta, Ramsey, NJ, USA). Seed extract preparation included lyophilization in a VirTis Genesis 25 XL Freeze-Drier (SP Industries, Gardiner, NY, USA). Acidified extracts were injected into a Hewlett Packard series 1100 HPLC (Brinkmann Instruments, Westbury, NY, USA) coupled to an ESI-TOF

PerSeptive Biosystem Mariner Mass Detector (Perseptive Biosystem, Boston, MA, USA).

*Cacao seed samples***.** Two hundred fresh cacao seeds were obtained from clonal accessions identified by cacao research stations and included the following varieties (number of pods in parentheses): from IRAD, Cameroon: AML(2), SNK 10, SNK 16, SNK 413, UPA 134 (2); from CRU, Trinidad: DR 2 (2), ICS 16, ICS 39, ICS 40; from USDA, Puerto Rico: ICS 16, ICS 39 (3), ICS 40 (2), P 19, PA 4, SCA 6 (4), SCA 12, UF 613.

Colorimetric analysis. Colorimetric data for fresh seed cotyledons was taken 1-13 days after harvest. Reflectance measurements were made within 2 minutes of peeling seed testa, twice on seed outer surface using an 11mm diameter aperture and excluding specular reflection. Extensive preliminary experiments were carried out to determine the effect of time after pod opening and seed peeling on the colorimetric data, and estimates of the error associated with browning reactions is given in the results.

Seed storage. Immediately following colorimetric analysis, seeds were quick frozen in liquid nitrogen and stored at -70°C. 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 masses were recorded in a humidity-controlled environment.

Polyphenol extraction. A polyphenol extraction procedure was adapted from (Adamson et al., 1999, Wollgast and Anklam, 2000). Individual seeds were ground 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). Seeds were individually ground at a controlled speed for 10 seconds. Lipids were removed in three hexane washes, using a Beckman GPR Centrifuge (Global Medical Instrumentation, Inc., Albertville, MN) for 10 min, 4000-5000 RPM at <0°C to settle the particulate between each wash. After the final hexane supernatant was discarded, polyphenols were extracted in 10 mL of HPLC grade MeOH:H_2O (75:25, v/v) for 24 hours at 5°C. Recovery in the second extract were determined to be less than 5-10% of the first extract, and not included in the analysis.

HPLC MS analysis. 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, and stored at -70°C. Injection samples consisting of extracts dissolved in 10% HCO2H solution (Fisher Scientific, Pittsburgh, PA, USA) (1:4, v/v) were placed in 2mL screw cap vials (Agilent Technologies, Palo Alto, CA, USA).

Ten μL of filtered extract were injected by autosampler into a Hewlett Packard series 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). Anthocyanins 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% HCO2H solution, solvent B: H_2O/HCO2H/acetonitrile (4/1/5), and solvent C: MeOH. Samples were run at a flow rate of 0.750 mL/min and visualized at 520 nm with a diode array detector.

Procyanidin monomers through heptamers were separated on a Supelco® polyethylene glycol column ((1.0 x 50mm) 120 Å) using a reverse phase gradient and visualized at 280 nm. 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. Relative amounts of anthocyanins, catechins and procyanidins were quantified in terms of catechin equivalents by comparison of peak areas with an authentic (-)-epicatechin standard.

Statistical analysis. All regression and principal component analysis performed on data was accomplished using Minitab software, version 13.32 (Minitab, Inc., State College, PA, USA). Statistics on non-linear regressions for the correlation of anthocyanins with seed color were performed using SAS software, version 8.2 (SAS Institute, Inc., Cary, NC, USA).

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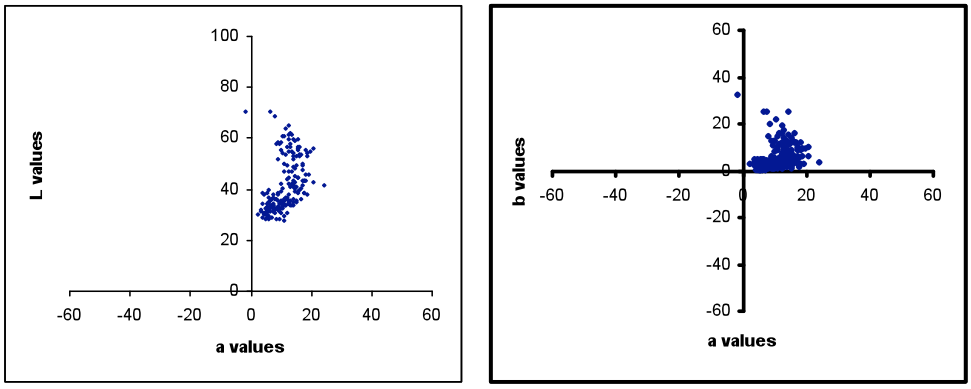
Figure 1.



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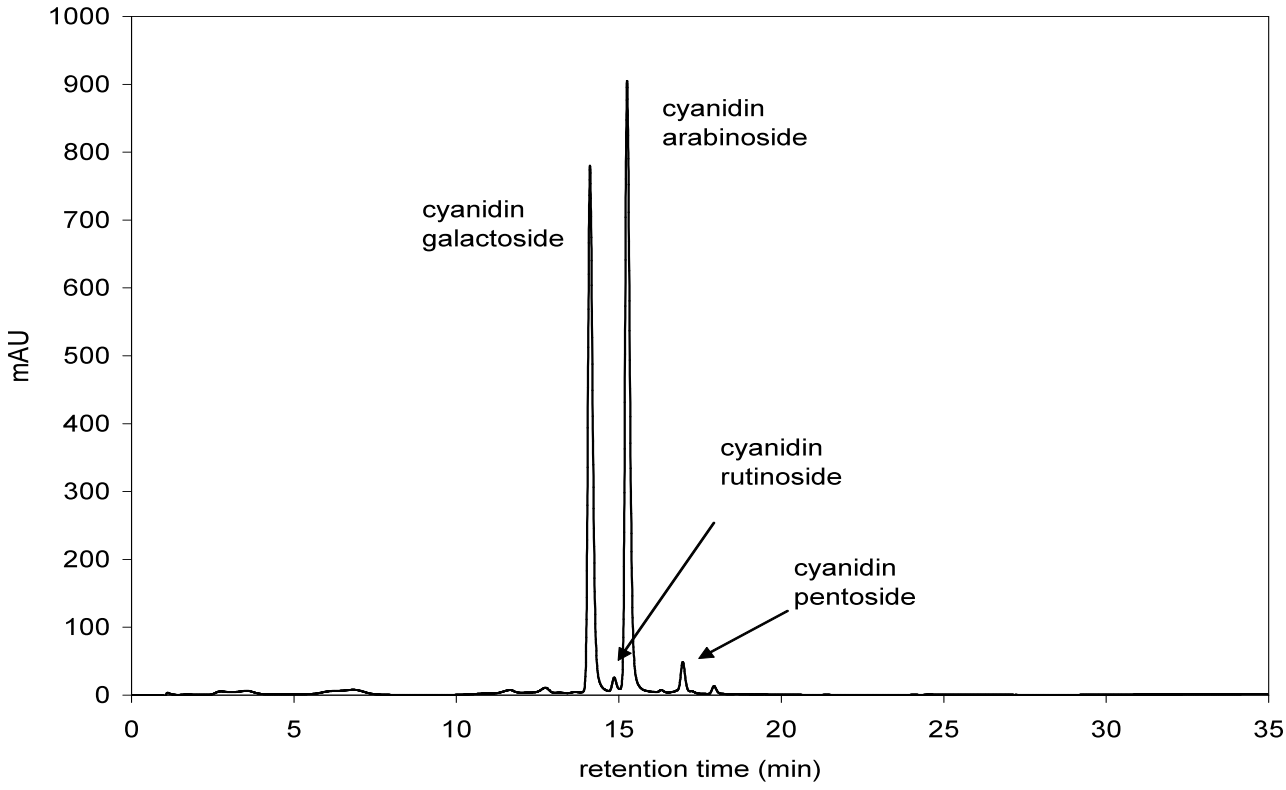

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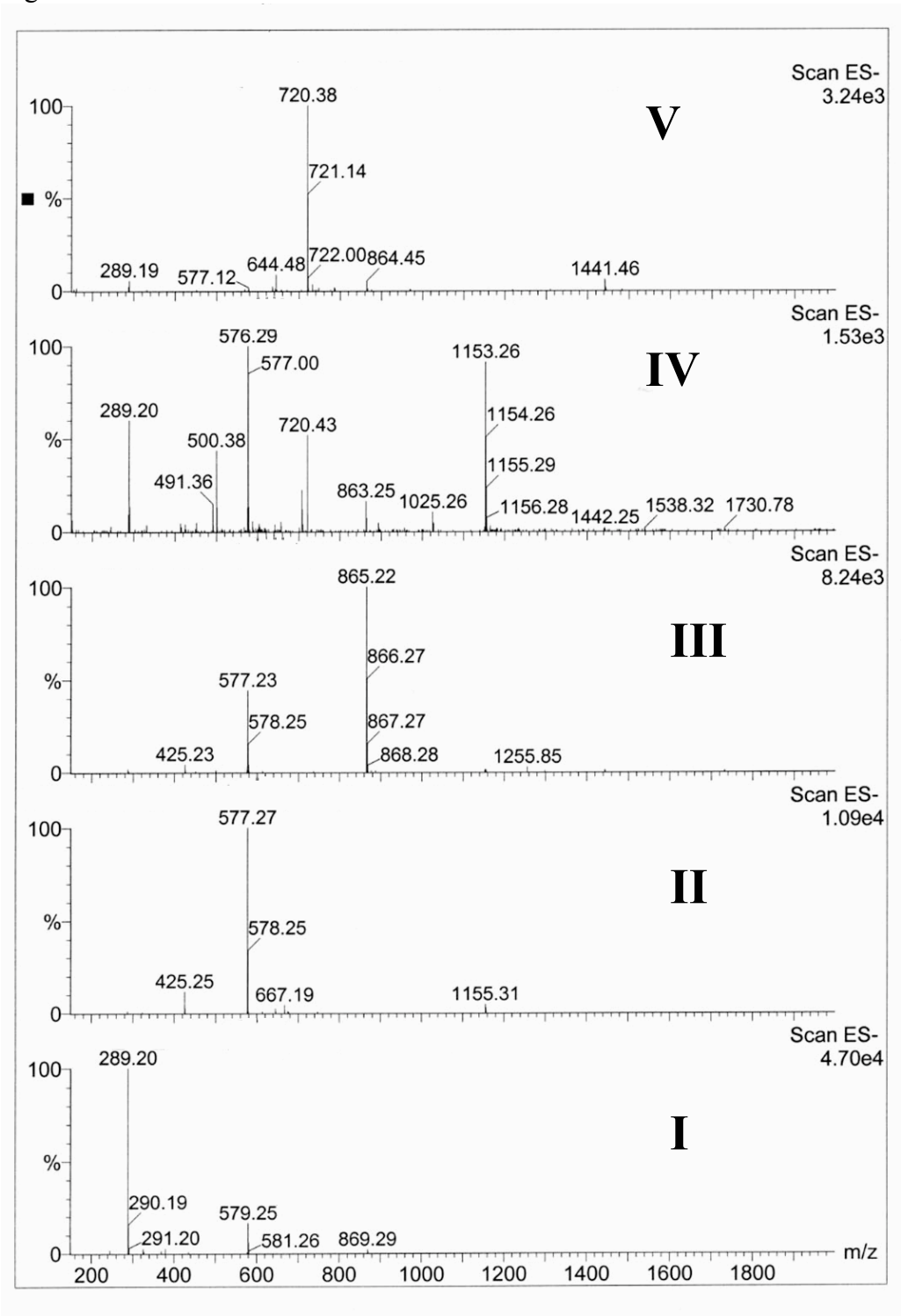
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Figure 4.

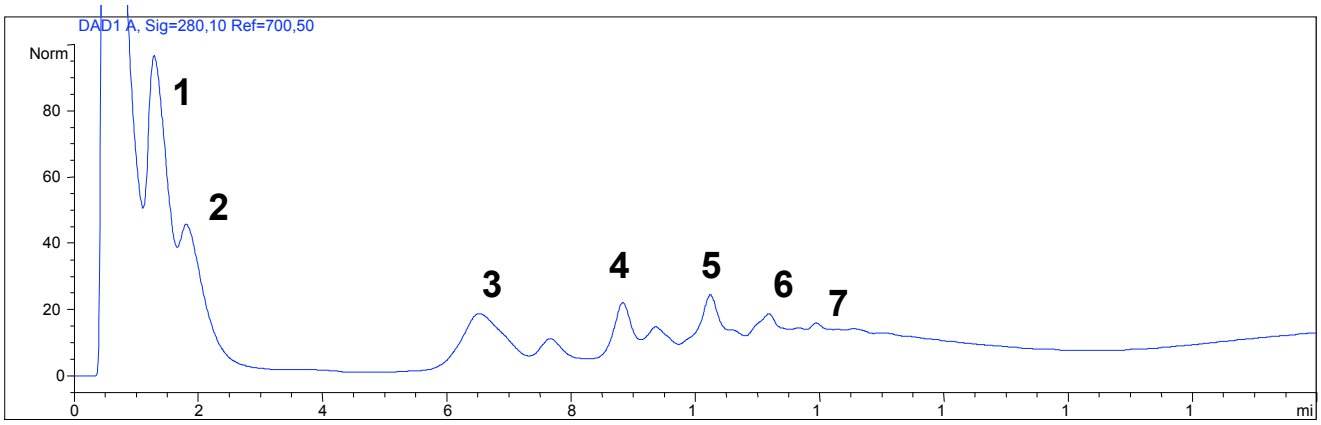
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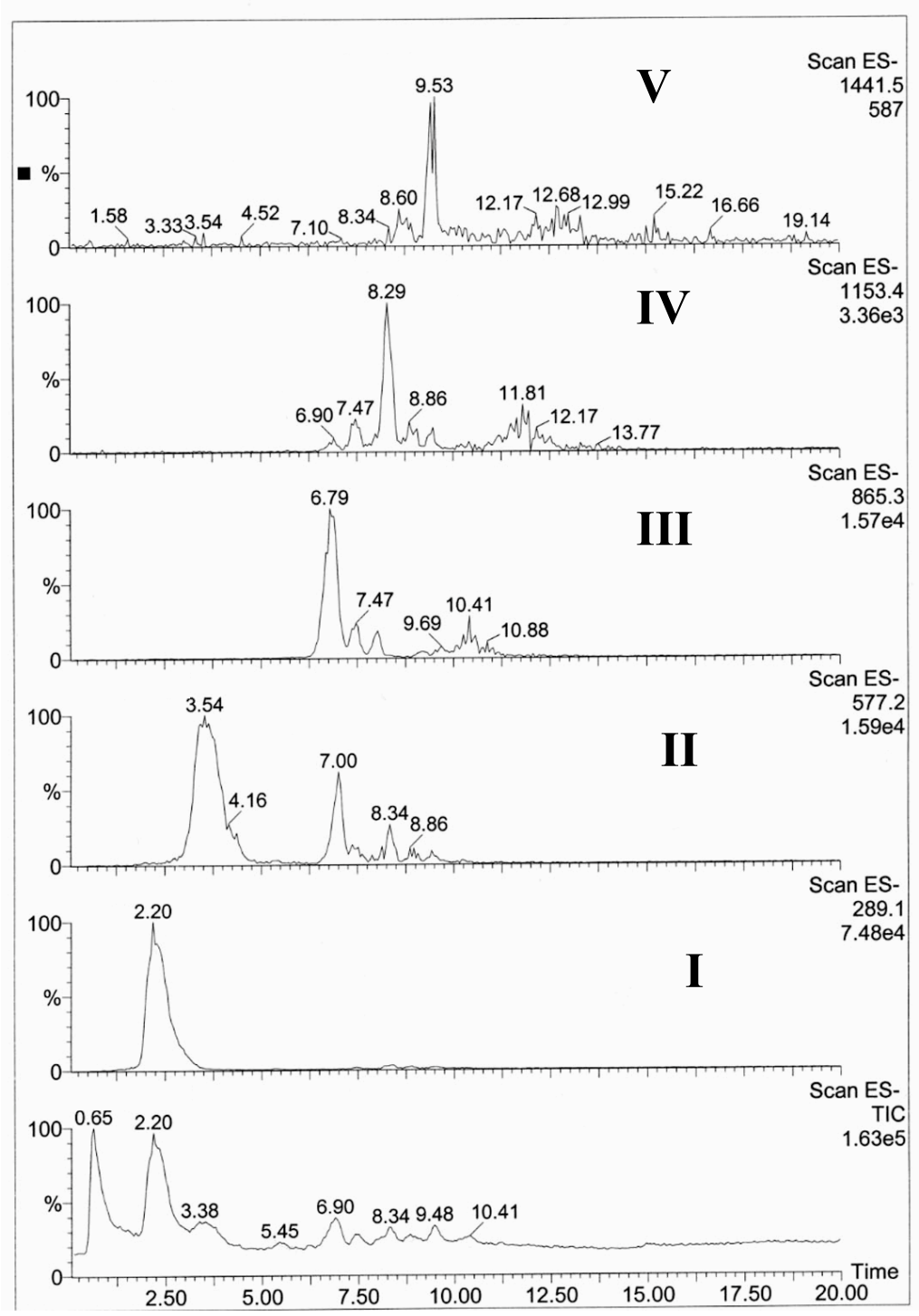
Figure 5.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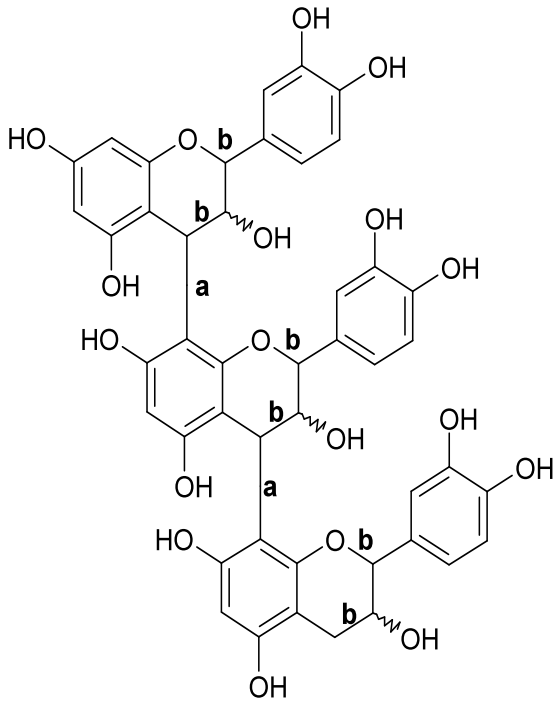

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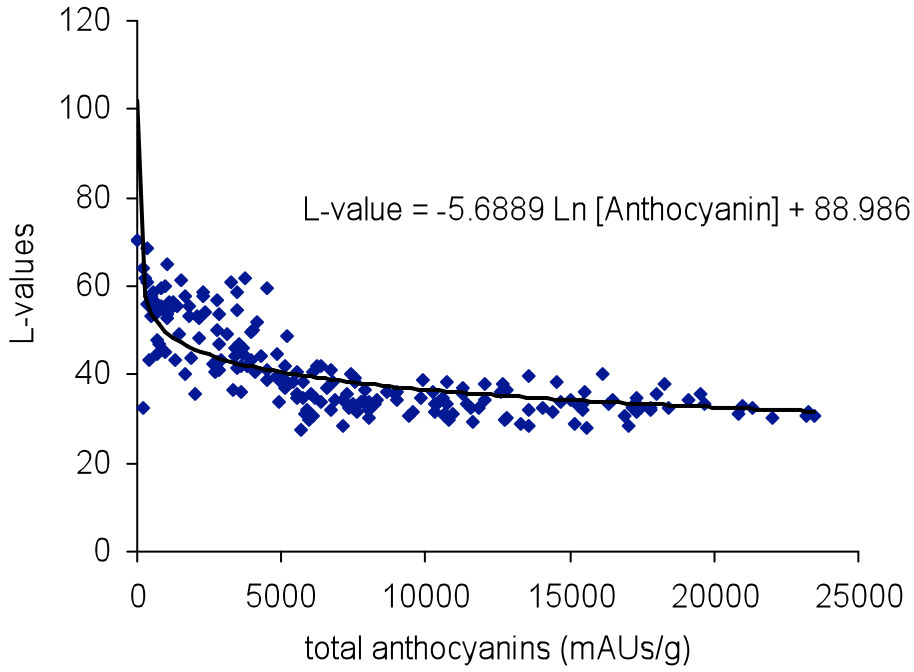

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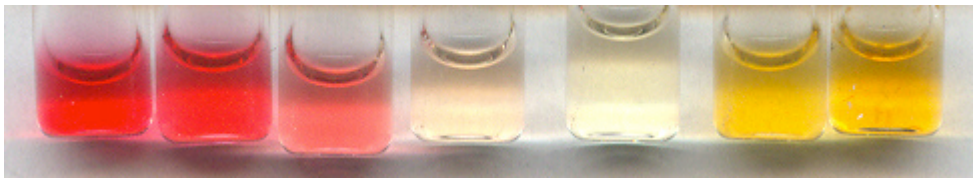

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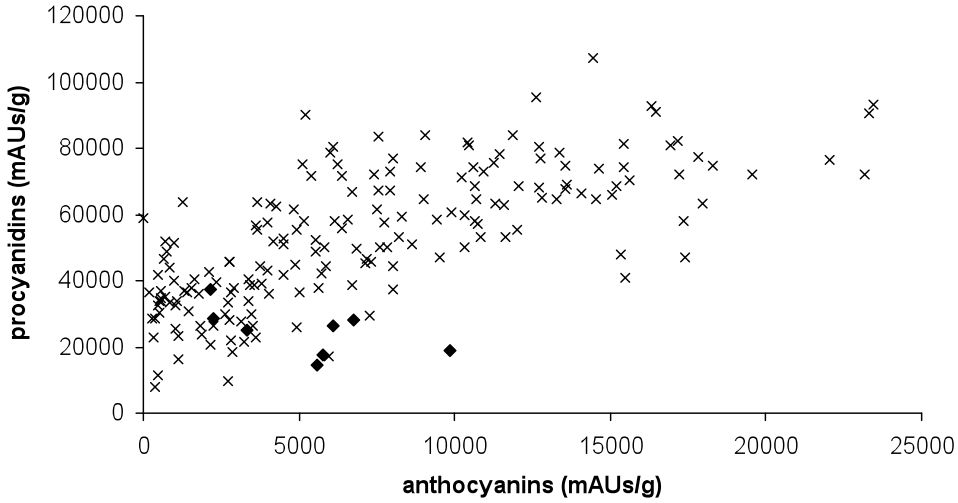

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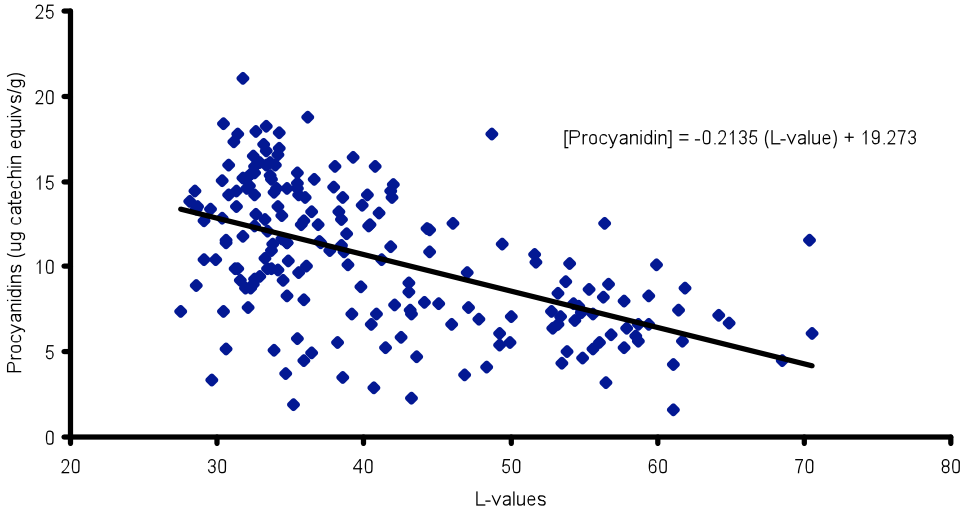

Figure Legends

Figure 1. *T. cacao* seed color measurements (average of two data points on outer surface) mapped onto an L*a*b* 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 2. Chromatograph of *T. cacao* seed extract resolved on a Betabasic C18 column. Absorbances are observed at 520 nm and anthocyanins identified by tandem MS.

Figure 3. Negative ion mass spectrum of fresh cacao seed extract. Compound I: catechin monomers, compounds II-V: procyanidin oligomers (dimer through pentamer)

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

Figure 5. Mass chromatograms of catechin monomers (m/z 289) and procyanidin oligomers (dimer through hexamers m/z 577 to 1441).

Figure 6. 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 7. Relationship between "lightness" of the outer seed surface, as measured by L-values, and total anthocyanin content, given in relative units of milli Absorbance Units sec per gram seed dry weight. R^2=0.58.

Figure 8. The effect of pH on anthocyanin color. Seed extracts were placed in different buffered solutions, pH 1-7, from left to right.

Figure 9. Comparison of relative amounts of anthocyanins and procyanidins for 200 *T. cacao* seed extracts. Figure 5-10**.** Seeds from Amelonado pod (♦) are highlighted with respect to seeds from the entire sample set (x).

Figure 10. Prediction of procyanidins using L-values for the outer seed surface.

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