CHEMICAL CHARACTERIZATION, BIOACTIVE PROPERTIES, AND PIGMENT STABILITY OF POLYPHENOLICS IN AÇAI (*EUTERPE OLERACEA* MART.)

A Dissertation

by

LISBETH ALICIA PACHECO PALENCIA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2009

Major Subject: Food Science and Technology

CHEMICAL CHARACTERIZATION, BIOACTIVE PROPERTIES, AND PIGMENT STABILITY OF POLYPHENOLICS IN AÇAI (*EUTERPE OLERACEA* MART.)

A Dissertation

by

LISBETH ALICIA PACHECO PALENCIA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Stephen T. Talcott
Committee Members,	Susanne Talcott
	Joseph Sturino
	Luis Cisneros-Zevallos
Intercollegiate Faculty Chair,	Jimmy Keeton

May 2009

Major Subject: Food Science and Technology

ABSTRACT

Chemical Characterization, Bioactive Properties, and Pigment Stability of Polyphenolics in Açai (*Euterpe oleracea* Mart.). (May 2009)

Lisbeth Alicia Pacheco Palencia, B.S., Zamorano University;

M.S., University of Florida

Chair of Advisory Committee: Dr. Stephen T. Talcott

Phytochemical composition, antioxidant activity, pigment stability, bioactive properties, and in-vitro absorption of polyphenolics in açai fruit (*Euterpe oleracea* Mart.) were investigated. Detailed characterization of phenolic compounds present in açai fruit, açai fruit pulp, and a polyphenolic-enriched açai oil were conducted by HPLC-ESI-MS^n analyses and their stability and influence on antioxidant capacity determined. Anthocyanins were predominant in açai fruits, which also contained several flavone and flavonol glycosides, flavanol derivatives, and phenolic acids. In-vitro absorption and antiproliferative effects of phytochemical extracts from açai pulp and açai oil were determined as a function of chemical composition. Polyphenolic mixtures from both açai pulp and açai oil extracts significantly inhibited HT-29 colon cancer cell proliferation, also inducing the generation of reactive oxygen species. In-vitro intestinal absorption using Caco-2 cell models demonstrated that phenolic acids and monomeric flavanol derivatives are readily transported through cell monolayers in-vitro.

The influence of polyphenolic cofactors on the stability of anthocyanins in açai fruit under varying conditions of temperature and pH was evaluated. Significant time, temperature, and pH-dependent anthocyanin losses were observed in all models, yet the presence of phenolic acids, procyanidins, and flavone-*C*-glycosides had a positive influence on anthocyanin stability. External addition of flavone-*C*-glycosides significantly enhanced visual color, increased anthocyanin stability during exposures to high pH or storage temperatures, and had comparable effects to those of a commercial anthocyanin enhancer.

Anthocyanin polymerization reactions occurring during storage of açai fruit juice models were investigated and potential mechanisms and reaction products identified. Polymeric anthocyanin fractions contained several anthocyanin-flavanol adducts based on cyanidin or pelargonidin aglycones and their presence was related to increased anthocyanin sulfite bleaching resistance and to the appearance of large, unresolved peaks in HPLC chromatograms. A reaction mechanism involving the nucleophilic addition of anthocyanins in their hydrated form to flavanol carbocations resulting from cleavage of interflavanic bonds was proposed for the formation of flavanol-anthocyanin adducts in açai fruit juices. Antiproliferative activity and in-vitro absorption of monomeric and polymeric anthocyanin fractions were also evaluated. Both fractions inhibited HT-29 colon cancer cell growth in a similar, concentration-dependent manner, yet in-vitro absorption trials using Caco-2 intestinal cell monolayers indicated the presence of anthocyanin polymers may influence anthocyanin absorption in açai fruit products.

To my family, my greatest gift.

ACKNOWLEDGEMENTS

I will always be so grateful to Dr. Steve Talcott, my advisor, for guiding me through my Ph.D. adventures. I thank him for believing so much in me and for all the opportunities he made possible during this time. I always felt so fortunate to have him as my advisor, and I know I will always feel that way. I am also very grateful to Dr. Susanne Talcott, for introducing me to the cell culture world, and Dr. Joseph Sturino and Dr. Luis Cisneros for their guidance and support, and for all the valuable time devoted to me.

I also wish to thank my friends and lab siblings, Youngmok, Chris, Jorge, Kimmy and my big sister, Flor. Their friendship has been a joy to my life, and I thank them so much for all the happy memories we shared.

My dearest thanks go to my family, particularly to my parents, Dr. Eugenia Palencia and Dr. Pablo Pacheco, my little brothers, Luis Pablo and Kevin, and my grandparents, Albertina and Manuel, for inspiring me through their example. I'm also greatly indebted to my uncles, aunts, and cousins, particularly my Godfather, Ing. Adolfo Palencia, for their always loving, unconditional support. No words can ever thank my family for everything I have been given throughout my life.

Finally, I extend my most loving thanks to my best friend, the love of my life, and my precious husband, Jolián Rios. I am so blessed to have you by my side, and I thank you with all my heart for being always there for me, and for letting me know how much you loved me, every single step of the way. You are and always will be all I want, more than I deserve, and everything I need. I love you so much.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

Page

Page

Page

LIST OF TABLES

Page

Page

CHAPTER I

INTRODUCTION

Functional foods and beverages are achieving global success, mainly due to modern consumer trends toward health maintenance. Particular attention has been given to the protective effects of polyphenolics in fruits and vegetables and their potential roles in the prevention of degenerative diseases, including certain cancers (Riboli & Norat, 2003). Consequently, efforts to improve and retain health-supporting characteristics of fruit juices and beverages have dominated many categories and beverage manufacturers have expanded their choices to natural ingredients that add novel flavors and targeted functionality to their products.

Açai (*Euterpe oleracea* Mart.), a palm fruit native to the Amazon region, has recently attracted international attention as a novel source of polyphenolics, particularly anthocyanins, and may offer a promising alternative to synthetic dyes for food and beverage applications. However, few attempts (Gallori, Bilia, Bergonzi, Barbosa, & Vincieri, 2004; Lichtenthaler, Belandrino, Maia, Papagiannopoulos, Fabricius, & Marx, 2005; Schauss et al., 2006; Pacheco-Palencia, Hawken, & Talcott, 2007a; Pacheco-Palencia, Hawken, & Talcott, 2007b) have been made to characterize its phytochemical composition in detail and assess its bioactive properties.

Moreover, the poor stability of anthocyanins remains a major problem facing the beverage industry, as oxidation and polymerization reactions during processing, storage,

---

This dissertation follows the style of *Food Chemistry.*

and distribution lead to the formation of brown pigments (Monagas Gomez-Cordoves, & Begoña, 2006; Pacheco-Palencia et al., 2007a), which negatively impact color and overall quality perception. Furthermore, anthocyanin polymerization reactions may not only affect quality attributes, but potentially influence the intestinal absorption and bioactive properties of anthocyanins present in these products.

These studies evaluated the polyphenolic composition, antioxidant properties, chemical stability, antiproliferative activity and in-vitro absorption properties of açai fruit and its main commercial products; emphasizing key relations between polyphenolic structure and in-vitro absorption and antiproliferative activity. In addition, factors influencing anthocyanin stability were examined and potential mechanisms for anthocyanin polymerization reactions and their influence on antiproliferative activity and in-vitro absorption determined.

Specific objectives were:

1- To characterize polyphenolic compounds present in the two main commercial açai species (*Euterpe oleracea* Mart. and *Euterpe precatoria* Mart.) and assess their thermal stability under common thermal processing conditions.

2- To assess the polyphenolic composition and stability of polyphenolic compounds from açai oil and evaluate their impact on antioxidant capacity.

3- To evaluate the antiproliferative activities of açai polyphenolic extracts against colon cancer cells and determine their intestinal absorption using in-vitro cell models.

4- To investigate the influence of non-anthocyanin polyphenolic components on anthocyanin stability and overall pigment intensity.

5- To determine potential mechanisms, reactants, and products involved in anthocyanin polymerization reactions in açai juices.

6- To investigate potential relations between anthocyanin polymerization reactions and antiproliferative properties and in-vitro intestinal absorption of anthocyanins in açai.

CHAPTER II

LITERATURE REVIEW

Açai Fruit Generalities and Composition

Açai (*Euterpe oleracea* Mart.) is a slender, multistemmed, monoecious palm, widely distributed in the Amazon estuary floodplains, and particularly abundant in the Northeastern regions of Brazil (Muñiz-Miret, Vamos, Hiraoka, Montagnini, & Mendelsohn, 1996). Açai palms can reach a height of over 25 meters and produce from 3 to 6 kg of fruit per year (Clay & Clement, 1993). Fruits appear in clusters, and are rounded, measure between 1.0 and 1.4 cm in diameter, have a large seed (up to 80% of total mass) and generally mature to a dark purple color (Rogez, 2000). Fruiting occurs throughout the year, with heavy seasonal production between July and December (Muñiz-Miret et al., 1996). Harvesting açai fruit is an arduous and frequently dangerous task, done by individuals accustomed to climb the açai palms using a fiber ring to support their feet, and manually collect all ripe fruit bunches in baskets (Rogez, 2000). Fruits are generally transported to the markets and commercialized in less than 24 hours to prevent significant nutritional and quality losses (Rogez, 2000).

Once harvested, the edible pulp of açai fruits is typically macerated with water to produce a thick, purple beverage of creamy texture, oily appearance, and characteristic flavor, which is also commonly referred to as “açai”. The most important trade qualities are based on total solids and are known as “açai grosso” (>14% total solids), “açai medio” (>11% total solids) and “açai fino” (>8% total solids) (Muñiz-Miret et al., 1996).

Nutrient composition may vary among fruit sources and processing methods, but according to Clay & Clement (1993), açai fruits generally contain ~12.5 protein, ~10.0% lipid, ~1% sugar, 0.05% calcium, 0.03% phosphorous, and traces of iron, vitamins A and B1. In addition, açai fruit pulps are rich sources of anthocyanins, particularly cyanidin-3-rutinoside and cyanidin-3-glucoside, and contain lower concentrations of phenolic acids and flavanol derivatives (Schauss et al., 2006; Pacheco-Palencia et al., 2007b).

Due to its highly perishable nature, consumption and commercialization of açai fruit and açai fruit pulps had long been restricted to a regional level; however, increased international interest and expanded distribution has made açai fruit pulp and various retail products made from açai fruit pulp, widely available to the general public. Considerable attention has been generated by health related benefits associated to its high anthocyanin content and antioxidant capacity (Gallori et al., 2004, Coisson, Travaglia, Piana, Capasso, & Arlorio, 2005), and numerous açai-containing functional ingredients and beverages are already gaining popularity in European, Asian, and North American markets.

Polyphenolics: Structure, Classification, and Bioactive Properties

Polyphenolics are a wide array of secondary metabolites synthesized by vascular plants, and encompass more than 8,000 compounds connected to a variety of physiological functions such as nutrient uptake, photosynthesis, enzyme activity, protein synthesis, microbial resistance, pigmentation, and sensory properties (Robbins, 2003).

Structurally, phenolic compounds are derivatives of benzene with one or more hydroxyl substituents, often accompanied by functional substitutions such as esters, methyl esters, glycosides and others (Visioli, Borsani, & Galli, 2000). Phenolic compounds are synthesized via the plant aromatic pathway, beginning with the production of aromatic amino acids through the shikimate pathway. Some aromatic amino acids continue to the phenylpropanoid pathway, yielding cinnamic acid derivatives, which may enter the flavonoid route to give rise to a wide diversity of flavonoid compounds (De Bruyne, Pieters, Deelstra, & Vlietinck, 1999).

Based on their structural properties, phenolic compounds may be divided into various groups, including phenolic acids, flavonoids, tannins, and other phenylpropanoid derivatives (Skerget, Kotnik, Hadolin, Hras, Simonic, & Knez, 2005). Phenolic acids are characterized by one carboxylic acid functional group and two constitutive carbon frameworks, the hydroxycinnamic and hydroxybenzoic structures, commonly substituted with one or more hydroxyl groups (Herrmann, 1995). Flavonoids are the most common and widely distributed group of plant phenolics (Le Marchand, 2002). To date, over 5,000 different flavonoids have been identified and are classified into at least 10 chemical groups (Whiting, 2001). Among them, flavonols, flavones, flavanones, catechins, anthocyanidins, and isoflavones are particularly common (Cook & Samman, 1996). The basic flavonoid structure is the flavan nucleus, which consists of 15 carbon atoms arranged in three rings (C6-C3-C6), commonly referred to as A, B and C (Fig. 1). The level of oxidation and pattern of substitution of the C ring distinguish the various flavonoid classes, while individual compounds within a class are differentiated by

substitutions in the A and B rings, which may include hydrogenation, hydroxylation, methylation, malonylation, sulphation, and glycosylation (Cook & Samman, 1996). In addition, the pyran C ring can also be opened in chalcone forms and recyclized into a furan ring (Skerget et al., 2005).

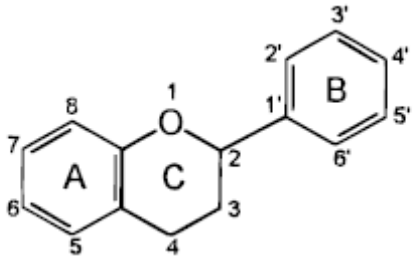


Fig. 1. Basic flavonoid structure.

An additionally important group of polyphenolic compounds are tannins, defined as a group of water-soluble phenolic compounds of relatively high molecular weight (500 to 3000 daltons), that have the ability to precipitate alkaloids, gelatin and other proteins (Cos et al., 2003). Tannins are commonly classified based on their structural characteristics into hydrolysable tannins, consisting of polyesters of gallic or ellagic acid, and condensed or non hydrolysable tannins, commonly referred to as proanthocyanidins, and composed of flavan-3-ol nuclei polymers (De Bruyne et al., 1999).

Besides their physiological roles in plants, polyphenolics are important components in the human diet. Depending on the diet, polyphenolic intake may range from 50 to more than 800 mg/day, exceeding that of vitamin C (~70 mg/day) or vitamin E (~10 mg/day) (Pietta, 2000). Moreover, polyphenolic-rich diets have been associated

with a reduced incidence of oxidative damage diseases, including cancer and coronary heart disease (Visioli et al., 2000). Much of these effects have been attributed to the ability of polyphenolics to inhibit oxidative reactions in-vivo (Chun, Dim, Smith, Schroeder, Han, & Lee, 2005). Proposed mechanisms of antioxidant action by phenolic compounds include the suppression of enzymes and trace elements involved in free radical production, scavenging of reactive oxygen or nitrogen species, and protection of natural antioxidant defenses (Pietta, 2000). Polyphenolics have shown to inhibit the enzymes responsible for radical production, including xanthine oxidase and protein kinase C, cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, and NADH oxidase, and to efficiently chelate trace metals that play important roles in the generation of reactive oxygen species (Cos et al., 2003). Yet the predominant mode of antioxidant activity is believed to be scavenging of reactive species, via hydrogen atom or electron donation (Shahidi & Wanasundara, 1992). According to Rice-Evans, Miller, & Paganga (1996), structural properties of polyphenolics, in terms of the availability of hydrogen-donating groups, determine their antioxidant activity, while substituents in the aromatic ring affect the stabilization of the resulting phenolic radical and thus, the radical-quenching ability of phenolic compounds.

Anthocyanin Properties and Pigment Stability

Anthocyanins are considered one of the largest and most important plant pigments visible to the human eye (Kong, Chia, Goh, Chia, & Brouillard, 2003). They

belong to the flavonoid group of phenolic compounds and are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenyl-benzopyrylium or flavylium salts (Brouillard, 1983). The flavylium cation constitutes the main part of the anthocyanin molecule and contains conjugated double bonds responsible for light absorption around 500 nm, causing pigments to appear red to the human eye (Markakis, 1974). The aglycones, called anthocyanidins, are usually penta- (3,5,7,3´,4´) or hexa-substituted (3,5,7,3´,4´,5´) (Francis, 1989). More than twenty naturally occurring anthocyanidins have been identified; however, six of them (Fig. 2) are by far, the most common in foods: pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp) (Jurd, 1972).

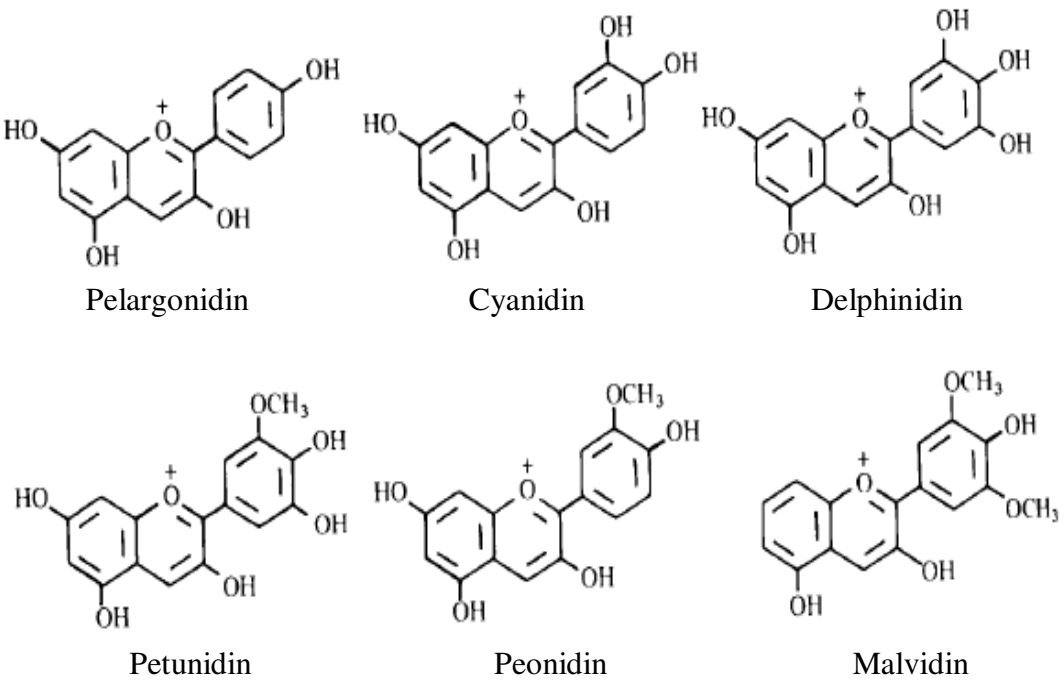


Fig. 2. Chemical structures of the most abundant anthocyanidins.

Anthocyanidins are rarely found in nature as such, and occur most commonly in their glycosylated forms, as anthocyanins, which are more soluble and stable in aqueous solutions (Harborne, 1964). Common glycoside moieties include glucose, rhamnose, galactose, arabinose and xylose; however complex glycosides such as rutinose, sophorose, and sambubiose may also occur naturally (De Ancos, Gonzales, & Cano, 1999). Anthocyanins may be also differentiated by the nature and number of organic acids attached to the anthocyanin glycosyl units, typically aromatic phenolic acids or aliphatic dicarboxyl acids or a combination of both. The most common acylating agents include derivatives of hydroxycinnamic acids, hydroxybenzoic acids, and a range of aliphatic acids (Clifford, 2000).

In addition to their role as secondary metabolites in the pigmentation of plants, anthocyanins have gained increased interest as functional ingredients for food applications and as prospective, potent protective agents against oxidative stress (Stintzing, Stintzing, Carle, Frei, & Wrolstad, 2002). However, being electron deficient, the flavylium nucleus of anthocyanins is highly reactive and undergoes undesirable structural changes during processing and storage (Jurd, 1972). Color stability of anthocyanins is thus, strongly affected by a variety of factors, including anthocyanin structure, concentration, pH, temperature, light, and the presence of enzymes, oxygen, copigments, metallic ions, ascorbic acid, sugar and their degradation products (Rodriguez-Saona, Giusti, & Wrolstad, 1999). Anthocyanin structure, particularly related to the number and position of glycosyl units and acyl groups attached to the aglycone, significantly influences the reactivity of the anthocyanin molecule and

generally results in improved color stability (Baublis, Spomer, & Berber-Jimenez, 1994; Turker, Aksay, & Ekiz, 2004). Increased anthocyanin concentration has also shown to promote higher color stability via self-association reactions among anthocyanins (Dao, Takeoka, Edwards, & Berrios, 1998). Due to their ionic nature, anthocyanins are strongly influenced by changes in pH, which enable structural changes and result in different colors and hues at different pH values (Jurd, 1972; Clifford, 2000). As a result, the intensely red colored flavylium cation, being the predominant anthocyanin form in very acidic media, hydrates as the pH is increased, yielding the colorless carbinol form and decreasing color intensity. Further pH increases lead to ring opening of the carbinol form, yielding the colorless chalcone (Markakis, 1974; Brouillard, 1983).

Temperature and the presence of oxygen are important factors influencing anthocyanin degradation rates during processing and storage. Increased processing and storage temperatures are thought to induce hydrolysis of glycosidic bonds in anthocyanin molecules, leading to the formation of unstable aglycones which degrade rapidly in aqueous systems (Maccarone, Maccarone, & Rapisarda, 1985). Deleterious effects and accelerated anthocyanin losses in the presence of oxygen have been attributed to direct or indirect oxidation reactions (Jackman, Yada, Tung, & Speers, 1987). Moreover, the presence of certain enzymes common in food systems, including glycosidases, peroxidases, and phenolases, have also been shown to accelerate anthocyanin degradation. Glycosidases have the ability to break covalent bonds between glycosyl residues and aglycones, leading to the rapid degradation of the unstable aglycone, while peroxidases and phenolases can oxidize phenolic compounds in the media to their

corresponding quinones, which then react with anthocyanins, and lead to brown condensation products (Kader, Irmouli, Nicolas, & Metche, 2001). Finally, the presence of sugars, ascorbic acid, and their degradation products are known to decrease anthocyanin stability via oxidative reactions (Krifi, Chouteau, Boudrant, & Metche, 2000) and to enhance the formation of large polymer pigments (Poei-Langston & Wrolstad, 1981).

In addition to anthocyanin losses due to oxidation and degradation reactions, the formation of anthocyanin polymers and resulting changes in color, from bright red to darker hues, has been a long-observed phenomenon (Somers, 1971). However, the nature and mechanism of formation of these polymeric compounds are yet poorly understood and current information on anthocyanin polymerization is largely limited to reactions occurring in red wine systems, which may differ from those occurring in juice systems. Anthocyanin polymers have been shown to form during thermal processing and storage in a time and temperature-dependent manner, accelerated by the presence of oxygen, light, ascorbic acid, and flavanol derivatives (Skrede, Wrolstad, Lea, & Enersen, 1992; Garzon & Wrolstad, 2002; Tsai, Delva, Yu, Huand, & Dufosse, 2005; Pacheco-Palencia et al., 2007a). Interactions between anthocyanins and sugar or ascorbic acid degradation products, particularly furfurals and other aldehydes, are thought to influence the formation of brown pigments during storage of fruit-based, anthocyanin-containing beverages (Es-Safi, Cheynier, & Moutounet, 2000). Aldehydes have also been shown to promote anthocyanin polymerization with flavonols, flavan-3-ols, and their derivatives resulting in the formation of both colorless and yellow-colored compounds that

contribute to anthocyanin browning reactions and decreased color stability in wines (Timberlake & Bridle, 1976; Es-Safi, Cheynier, & Moutounet, 2002). Moreover, anthocyanin reactions with tannins are thought to be responsible for polymeric pigments formed in the course of wine aging (Cheynier et al., 2006) and various products resulting from direct condensation reactions of anthocyanins with tannins have been detected in wines (Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000; Salas, Dueñas, Schwarz, Winterhalter, Cheynier, & Fulcrand, 2005). However, several of these anthocyanin-tannin adducts are colorless (Remy et al., 2000). Moreover, anthocyanin reactions that do not involve procyanidins or tannins have also been demonstrated. The resulting products include uncharacterized, large anthocyanin polymers (Salas et al., 2005) and smaller molecules such as pyranoanthocyanins (Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998), and anthocyanin-caftaric acid adducts formed through enzymatic oxidation reactions (Sarni-Manchado, Cheynier, & Moutounet, 1997).

HPLC-ESI-MS^n as a Tool for Polyphenolic Characterization

High Performance Liquid Chromatography (HPLC) coupled with electrospray ionization (ESI) and tandem mass spectrometry (MS^n) has gained popularity in recent years as a fast and effective method for the identification and structural characterization of polyphenolics in a variety of foods and beverages. Until a few years ago, polyphenolic characterization was mainly performed using diode-array detection coupled with HPLC (Pati, Losito, Gambacorta, La Notte, Palmisano, & Zambonin,

2006). Nevertheless, structural similarities among polyphenolics based on the same constitutive units led to similar UV-visible spectra and possible co-elution, which along with a lack of commercially available standards, made their identification very difficult (Gu et al., 2003). Moreover, due to their ubiquitous presence as complex mixtures in plants, extensive fractionation procedures and hyphenated techniques were often needed (Careri, Mangia, & Musci, 1998).

Mass spectrometry has proved to be an effective tool in the structural characterization of polyphenolics, providing experimental evidence for structures that were only hypothesized until a few years ago (Flamini, 2003). Liquid chromatography-mass spectrometry coupling has led to the development of new interfaces, expanding the possibilities for the analysis of non-volatile, thermally labile phenolic compounds (Careri, Bianchi, & Corradini, 2002). Major advances in ionization techniques having the ability to analyze high molecular mass compounds with high sensitivity have been key to the development of such new interfaces (Careri et al., 1998). Among them, electrospray ionization has been introduced as a soft ionization technique for the analysis of phenolic compounds, as it has proved suitable for non-volatile, thermally labile compounds, in a relatively wide mass range (Niessen, 1999).

Early ESI applications were predominantly involved with the detection of molecular ions (Guyot, Doco, Souquet, Moutounet, & Drilleau, 1997; Hammerstone, Lazarus, Mitchell, Rucker, & Schmitz, 1999), as ESI offered the possibility of generating molecular ions without any fragmentation (Friedrich, Eberhardt, & Galensa, 2000). Ions were measured both in the positive ionization mode ($[M+H]^+$) and in the

negative ionization mode ($[M-H]^-$) (Hammerstone et al., 1999; Wu, Wang, & Simon, 2003). However, due to the weakly acidic character of most polyphenolics, dissociation is generally favored over protonation, and the negative ionization mode is often associated with better sensitivity and selectivity in polyphenolic analysis (Friedrich et al., 2000). The use of formic or acetic acids in the HPLC eluents has shown improved chromatographic resolution and ion formation efficiency in the negative ionization mode; however, it often leads to formate or acetate adducts which may complicate data interpretation (Wu et al., 2003). In addition, multi-charged species ($[M-2H]^{-2}$ or $[M-3H]^{-3}$), dimeric ions, and cluster ions have also been reported when using ESI-MS^n operating in the negative mode (Poon, 1998; Le Roux, Doco, Sarni-Manchado, Lozano, & Cheynier, 1998). Despite these difficulties, instruments having ESI coupled with tandem MS capabilities have been extensively employed in polyphenolic identification and structural elucidation. ESI-MS^n analyses are known to yield characteristic and consistent fragmentation patterns for each particular compound in both positive and negative ionization modes, making it sensitive and reliable method for structural polyphenolic analyses (Gu et al., 2003).

In-Vitro Models for Polyphenolic Absorption

Polyphenolic bioavailability is a key factor when trying to relate in-vitro chemoprotective and antioxidant properties to in-vivo outcomes in cancer and inflammatory disease prevention (Balimane, Chong, & Morrison, 2000; Yi, Akoh, Fischer, & Krewe, 2006). Currently, the most extensively characterized and widely used

in-vitro model for intestinal absorption and permeability is the Caco-2 cell line (Balimane et al., 2000). Caco-2 cells, originally obtained from a human colon adenocarcinoma, undergo a process of spontaneous differentiation in long-term culture, leading to the formation of cell monolayers that express several morphological and functional characteristics of mature intestinal enterocytes (Pinto et al., 1983). Thus, Caco-2 cells grow in monolayers, show a cylindrical morphology, with microvilli on the apical side, tight junctions between cells, and express intestinal hydrolase enzyme activities on the apical membrane (Sambuy, De Angelis, Ranaldi, Scarino, Stammati, & Zucco, 2005). Caco-2 cells are commonly cultured on permeable supports that allow free access of nutrients to both sides of the cell monolayer, which resemble the conditions in the intestine in-vivo and lead to improved morphological and functional differentiation (Artursson, 1990). In addition, transepithelial electrical resistance and permeability of marker molecules have been routinely used to monitor the integrity of the cell monolayer (Sambuy et al., 2005).

Caco-2 cell monolayer models have been employed to assess intestinal transport of several groups of polyphenolics, including several phenolic acids (Konishi, Kobayashi, & Shimizu, 2003; Konishi & Shimizu, 2003), flavonoids (Walgren, Walle, & Walle, 1998; Vaidyanathan & Walle, 2001; Yi et al., 2006), and procyanidins (Deprez, Mila, Humeau, Tome, & Scalbert, 2001). Overall, all polyphenolic groups tested have been shown to be transported from the apical to the basolateral side of cell monolayers, and transport efficiency has been strongly influenced by chemical structure, concentration, and the presence of additional matrix components (Konishi et al., 2003;

Walgren et al, 1998; Yi et al., 2006; Deprez et al., 2001). Transport efficiency is usually higher for low molecular weight compounds and decreases as the molecular mass increases (Konishi et al., 2003). Thus, while low amounts of procyanidin dimers and trimers have been reported to pass through Caco-2 cell monolayers, polymers with a higher degree of polymerization have not (Scalbert, Deprez, Mila, Albrecht, Huneau, & Robot, 2000). According to Shoji et al. (2006), the absorption of polyphenolics of higher molecular weight and the limits of absorption in-vivo, in relation to the degree of polymerization, is still unclear.

CHAPTER III

PHYTOCHEMICAL, ANTIOXIDANT, AND THERMAL STABILITY OF TWO AÇAI SPECIES, *EUTERPE OLERACEA* AND *EUTERPE PRECATORIA*

Introduction

Açai fruit, *Euterpe oleracea* Mart. and *Euterpe precatoria* Mart., are an important economic palm fruit export from the Brazilian Amazon (Galotta & Boaventura, 2005) and constitute the majority of açai pulp exported for food and ingredient applications. The predominant species of açai exported from Brazil is *E. oleracea*, or locally referred to as "açai-do-Pará", frequently found in mono-specific populations common in the Amazon River estuary floodplains (Muñiz-Miret et al., 1996). The lesser available *E. precatoria* or "açaí-do-terra-firma" is widely distributed in the central and western regions of the Brazilian Amazon and is commonly found in non-flooded, upland fields (Clay & Clement, 1993). The palms of *E. oleracea* are multi-stemmed, monoecious, and may reach heights higher than 25 m to produce small, round (1.0 to 1.4 cm in diameter), dark purple fruits that are mainly harvested between July and December (Muñiz-Miret et al., 1996). By contrast, the palms of *E. precatoria* are single-stemmed and may grow to a maximum height of 22 m to produce spherical, dark purple fruits (1.0 to 1.8 cm in diameter) harvested from December to August (Clay & Clement, 1993). Fruits from both species are characterized by a single seed that comprises approximately 80% of the total volume, covered by fibrous layers and a slight oily coating under a thin (0.5 to 1.5 mm. thick), edible mesocarp (Muñiz-Miret et al., 1996;

Clay & Clement, 1993). Fruits of both species ripen from green to deep purple color and are commercially pulped with added water to prepare a thick, dark purple pulp with an oily surface appearance and distinctive flavor. Locally known in Brazil as "açai" and popular in the eastern regions of South America (Brondizio et al., 2002), açai pulp has gained in commercial export markets for use in a variety of food and beverage applications, greatly favored by international consumer trends towards health, wellness, novelty, and exotic flavors. Particular attention has been given to the potential health benefits of açai, associated with its in vitro antioxidant properties (Gallori et al., 2004) attributed to its polyphenolic composition (Lichtenthaler et al., 2005). Polyphenolics from *E. oleracea* have only been recently characterized (Gallori et al., 2004; Lichtenthaler et al., 2005; Pacheco-Palencia et al., 2007a; Pacheco-Palencia et al., 2007b; Schauss et al., 2006), and no previous reports on the phytochemical composition comparing *E. oleracea* or *E. precatoria* fruits are available. Moreover, factors affecting polyphenolic stability have not been determined. Therefore, this study was conducted to assess the phytochemical composition, antioxidant properties, and thermal stability of de-seeded *E. oleracea* and *E. precatoria* fruits. Results from these studies are aimed to assist the food industry in determining potential uses of these fruits for a variety of food and supplement applications.

Materials and Methods

Frozen, manually de-seeded, fully ripe açai fruits from *Euterpe oleracea* and *Euterpe precatoria* were kindly donated by Everything Nature, Inc. (Orlando, FL) and

Turiya Corp. (Vero Beach, FL) and shipped overnight to the Department of Nutrition and Food Science at Texas A&M University. Only the edible portion of the fruit pulp was retained, and the inedible seed discarded. A composite from approximately 250 fully ripe fruits was used in these trials. Replications were made from these de-seeded fruit pulps by macerating with a known volume of water (1:5 w/v fruit/water ratio) adjusted to pH 3.5 with citric acid. Treatments were prepared by loading 10 mL of the prepared fruit puree into screw-cap tubes and sparging for 5 minutes with either nitrogen or air (as an oxygen source) until complete saturation. Dissolved oxygen content was monitored using a YSI-57 dissolved oxygen meter (Yellow Springs, OH). Purees were then heated in a water bath to an internal temperature of 80°C for 1, 30 or 60 minutes and compared to a non-heated control. Treatments were held frozen at -20 °C and analyzed within 1 week after processing. Prior to analysis, purees were centrifuged at 4°C for 15 min to separate insoluble solids and lipids from the aqueous juice fraction, which was collected and filtered through Whatman #4 filter paper to clarify. For antioxidant analyses, these aqueous extracts were partitioned into two polyphenolic fractions using ethyl acetate, which separated most phenolic acids and flavonoids (non-anthocyanin fraction) from the remaining aqueous juice (anthocyanin fraction). Ethyl acetate was removed under reduced pressure at <40°C, and the isolate re-dissolved in 0.05 M citric acid buffer (pH 3.5) and along with the anthocyanin fractions, subjected to acid hydrolysis for 90 minutes at 95°C in 2N HCl and immediately assessed for antioxidant capacity and phytochemical composition.

Polyphenolic compounds present in açai were analyzed by reverse phase HPLC with a Waters 2695 Alliance system (Waters Corp., Milford, MA), equipped with a Waters 996 photodiode array detector. Separations were performed on a 250 x 4.6 mm Acclaim 120-C18 column (Dionex, Sunnyvale, CA) with a C18 guard column. Mobile phases consisted of water (phase A) and a 60:40 methanol and water (phase B), both adjusted to pH 2.4 with o-phosphoric acid. The gradient solvent program ran phase B from 0 to 60% in 20 min; 60 to 100% in 20 min; 100% for 7 min; 100 to 0% in 3 min and final conditions were held for 2 min at a flow rate of 0.8 mL/min. Polyphenolics were identified by UV/VIS spectral interpretation, retention time, and comparison to authentic standards (Sigma Chemical Co., St. Louis, MO). Unidentified flavonoid compounds were quantified in rutin equivalents, while procyanidin concentrations were expressed in (+)-catechin equivalents.

Mass spectrometric analyses were performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher, San Jose, CA). Separations were conducted using the Phenomenex (Torrace, CA) Synergi 4µ Hydro-RP 80A (2 x 150 mm) with a C18 guard column. Mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in 50:50 methanol:acetonitrile (phase B) run at 0.25 mL/min. Polyphenolics were separated with a gradient elution program where phase B changed from 5 to 30% in 15 min, from 30 to 65% in 25 min, and from 65 to 95% in 10 min and was held isocratic for 20 min. Electrospray ionization was conducted in the negative ion mode under the following conditions: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage,

3.3 kV; capillary temperature, 250°C; capillary voltage, 1.5 V; tube lens offset, 0 V. Total anthocyanin contents were determined spectrophotometrically (Helios, Thermo Electron, San Jose, CA) at 520 nm and quantified using mg/kg equivalents of cyanidin-3-glucoside with a molar extinction coefficient of 29,600 (Pacheco-Palencia et al., 2007b). Antioxidant capacity was determined using the oxygen radical absorbance capacity assay, adapted to be performed in a BGM Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 520 nm emission), as previously described (Talcott & Lee, 2002). Results were quantified in μmol Trolox equivalents (TE) per gram of de-seeded açai fruit.

Data for each chemical analysis was the mean of three replicates, prepared independently. Analysis of variance, parametric correlations, and means separations (Tukey-Kramer HSD post-hoc test, $p < 0.05$) were conducted using SPSS version 15.0 (SPSS Inc., Chicago, IL).

Results and Discussion

From historical observations and repeated evaluations of both açai species for characteristics such as total polyphenolics, total anthocyanins, and radical scavenging activity, it was observed that *E. precatoria* has consistent and appreciably high concentrations for these attributes than *E. oleracea*. Such observations led to a more in-depth study comparing these two commercial species of açai. Commercial açai pulps can vary considerably based on processing methods, the ratio of fruit pulp to water, fruit quality, and level of fruit ripeness yet the fruit selected for these evaluations were chosen

by industrial processors to be most representative of fruit commercially available. Both açai species shared similar polyphenolic profiles yet significant variations among individual polyphenolic concentrations were detected. Anthocyanins were the predominant polyphenolic in both species, and accounted for more than 90% of their total polyphenolic contents.

Spectroscopic and mass spectrometric data under both positive and negative ionization modes, along with individual anthocyanin concentrations for each species are presented in Table 1. Cyanidin glycosides were characterized by major ion signals at *m/z*=287.1, $[M-H]^+$, and *m/z*=285.2, $[M-H]^-$, corresponding to cyanidin aglycone. Similarly, peonidin-3-glucoside resulted in peonidin aglycone ion signals at *m/z*=301.1, $[M-H]^+$, and *m/z*=299.0, $[M-H]^-$, while pelargonidin-3-glucoside produced analogous aglycone ion signals at *m/z*=271.1, $[M-H]^+$, and *m/z*=269.0, $[M-H]^-$. Total anthocyanin concentrations were over 50% higher in *E. precatoria* than in *E. oleracea* fruits, mainly due to the abundance of cyanidin-3-rutinoside (3,135 ± 47 mg/kg), although pelargonidin-3-glucoside (319 ± 1.2) and cyanidin-3-sambubioside (4.6 ± 0.8 mg/kg) were also present. The lower anthocyanin concentrations detected in *E. oleracea* fruits included cyanidin-3-rutinoside (1,256 ± 38 mg/kg), cyanidin-3-glucoside (947 ± 29 mg/kg), and peonidin-3-rutinoside (44.0 ± 3.1 mg/kg). These findings confirm previous investigations that report anthocyanins in *E. oleracea* pulp and freeze-dried concentrates (Gallori et al., 2004; Lichtenthaler et al., 2005; Pacheco-Palencia et al., 2007a; Pacheco-Palencia et al., 2007b; Schauss et al., 2006) and are the first report of the anthocyanin profile of *E. precatoria*.

Table 1. HPLC-ESI-MS^n analyses of anthocyanins present in *E. oleracea* and *E. precatoria* fruits.

RT (min)	Compound	λ_{max} (nm)	$[M-H]^+$ (*m/z*)	MS/MS^+ (*m/z*)	$[M-H]^-$ (*m/z*)	MS/MS^- (*m/z*)	Concentration (mg/kg)[1]
			Euterpe oleracea				
25.4	Cyanidin-3-glucoside	520	449.1	287.1	447.1	285.2	947 ± 29
26.5	Cyanidin-3-rutinoside	520	595.1	449.1, 287.1	593.1	447.1, 285.2	1,256 ± 38
29.1	Peonidin-3-rutinoside	520	609.0	463.0, 301.1	607.0	461.1, 299.0	44.0 ± 3.1
			Euterpe precatoria				
25.7	Cyanidin-3-sambubioside	520	581.2	371.1, 287.1	579.2	369.2, 285.2	4.60 ± 0.8
26.5	Cyanidin-3-rutinoside	520	595.1	449.1, 287.1	593.1	447.1, 285.2	3,135 ± 47
27.9	Pelargonidin-3-glucoside	515	433.2	271.1	431.2	269.1	319 ± 1.2

[1] Values represent the mean and standard error from three replicates.

Spectrophotometric determinations of total anthocyanin content of *E. oleracea* (2,056 ± 83 mg/kg) and *E. precatoria* (4,227 ± 104 mg/kg) fruit purees also revealed major differences in pigment color intensities. Additional differences between spectrophotometric and chromatographic measurements might have originated from copigmentation reactions among anthocyanins and other non-anthocyanin polyphenolics naturally present in both açai species, which are known to enhance visual color and result in higher estimates of total anthocyanin contents in spectrophotometric assays (Boulton, 2001; Wilska-Jeszka & Korzuchowska, 1996).

Non-anthocyanin polyphenolics in *E. oleracea* (Fig. 3, Table 2) and *E. precatoria* (Fig. 4, Table 3) fruits included a diversity of phenolic acids and flavonoids. Phenolic acids detected in both species included protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, and ferulic acids, with vanillic and syringic acids being equally predominant. Both individual and total phenolic acid concentrations were higher in *E. precatoria* than in *E. oleracea* fruit. Flavonoids were among the most abundant non-anthocyanin polyphenolics in both species, and included various flavone-*C*-glycosides of apigenin and luteolin.

Flavone *C*-glycosides were identified based on their spectral and mass spectrometric characteristics, particularly by their distinctive fragmentation patterns yielding product ion signals at $[M-H-60]^-$, $[M-H-90]^-$, and $[M-H-120]^-$ in negative ion mode MS^n analyses (Gattuso et al., 2007; Caristi et al., 2006; Pereira et al., 2005; Ferreres et al., 2003; Voirin et al., 2000).

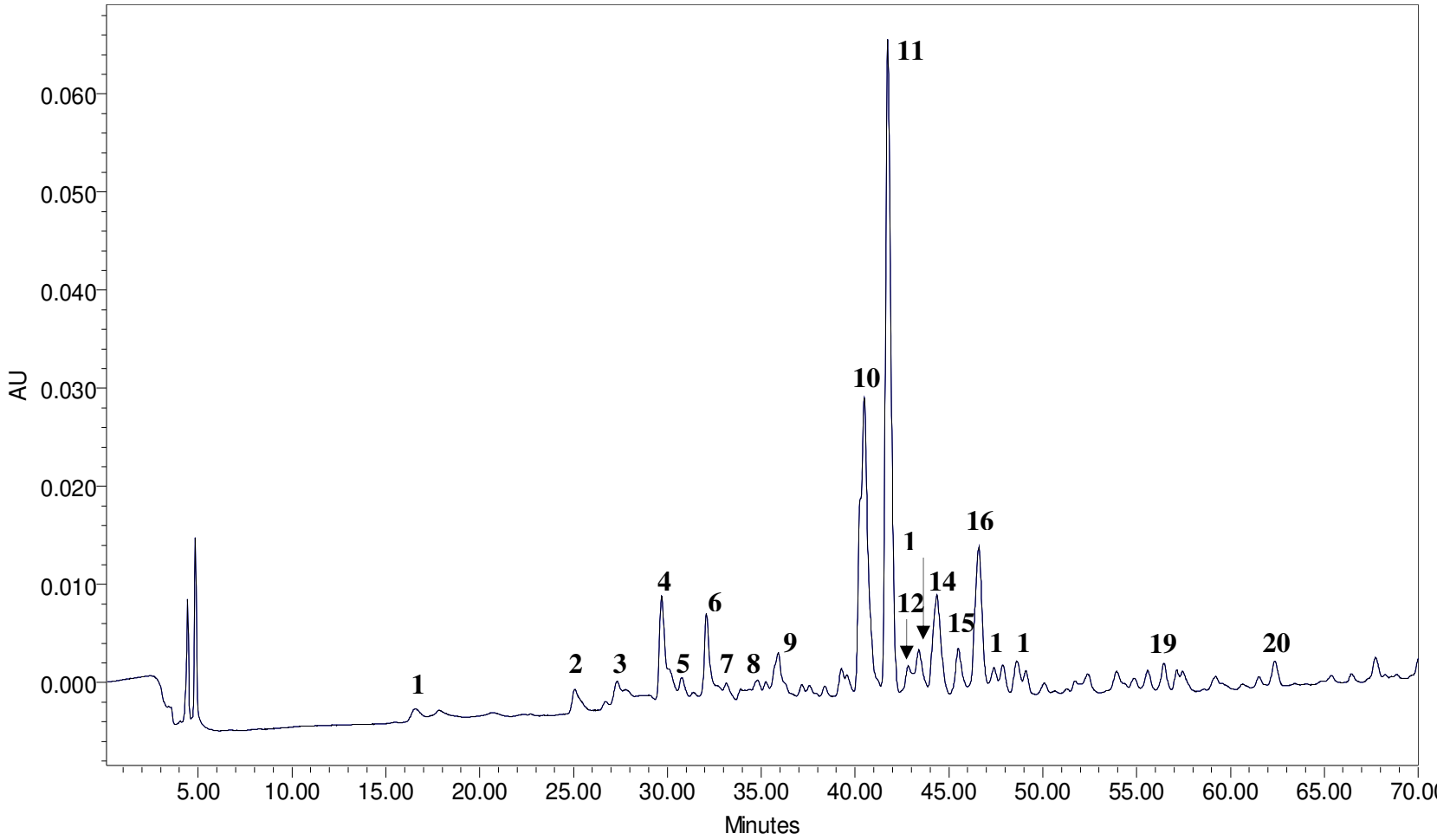


Fig. 3. HPLC chromatogram of non-anthocyanin polyphenolics in *E. oleracea* juice at 280 nm. Peak assignments are shown in Table 2.

Table 2. Characterization of non-anthocyanin polyphenolics present in *E. oleracea* fruits.

Peak No.	RT (min)	Compound	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)[1]	Concentration (mg/kg)
1	16.5	protocatechuic acid	263, 292	153.2	109.2, 91.0	1.77 ± 0.11
2	24.9	*p*-hydroxybenzoic acid	253.9	137.3	112.9	1.80 ± 0.13
3	26.7	(+)-catechin	277.5	289.2	245.2, 203.2, 187.2, 161.3	5.11 ± 0.22
4	29.5	vanillic acid	263, 291	167.3	140.9, 108.0, 95.2	5.05 ± 0.27
5	31.1	luteolin di-glycoside	266, 352	609.2	489.1, 369.2, 285.2	7.33 ± 0.68
6	33.1	syringic acid	271	196.9	182.2, 153.1, 138.1	4.02 ± 0.36
7	32.4	apigenin di-glycoside	266, 356	593.1	575.1, 502.9, 473.1, 353.1, 269.1	8.13 ± 0.68
8	34.1	(-)-epicatechin	277.5	289.2	245.2, 203.2, 187.2, 161.3	1.07 ± 0.10
9	36.3	taxifolin derivative	272, 356	449.4	327.1, 269.2, 225.1	7.89 ± 0.57
10	41.3	isoorientin	272, 352	447.2	393.1, 357.1, 327.2, 299.2, 285.1	34.8 ± 1.19
11	41.9	orientin	272, 348	447.2	429.2, 357.1, 327.3, 299.2, 285.1	53.1 ± 1.84
12	42.5	isovitexin derivative	267, 338	431.1	341.2, 311.1, 283.0, 269.1	3.71 ± 0.22
13	43.0	ferulic acid	323.7	193.2	149.1, 134.1, 117.0	0.98 ± 0.10
14	44.6	taxifolin deoxyhexose	295, 340	449.1	269.1	7.91 ± 0.19
15	45.7	procyanidin dimer	235, 282	577.1	425.0, 407.2, 289.1, 287.1	4.37 ± 0.47
16	47.1	isovitexin	267, 338	431.1	341.2, 311.1, 283.0, 269.1	10.6 ± 0.39
17	47.8	scoparin	257, 352	461.3	371.2, 341.1, 231.1	5.83 ± 0.23
18	48.7	procyanidin dimer	235, 282	577.1	425.0, 407.2, 289.1, 287.1	4.85 ± 0.61
19	56.5	procyanidin trimer	235, 291	865.1	577.2, 451.0, 425.0, 407.3	5.74 ± 0.48
20	63.7	procyanidin trimer	235, 291	865.1	577.2, 451.0, 425.0, 407.3	5.44 ± 0.36

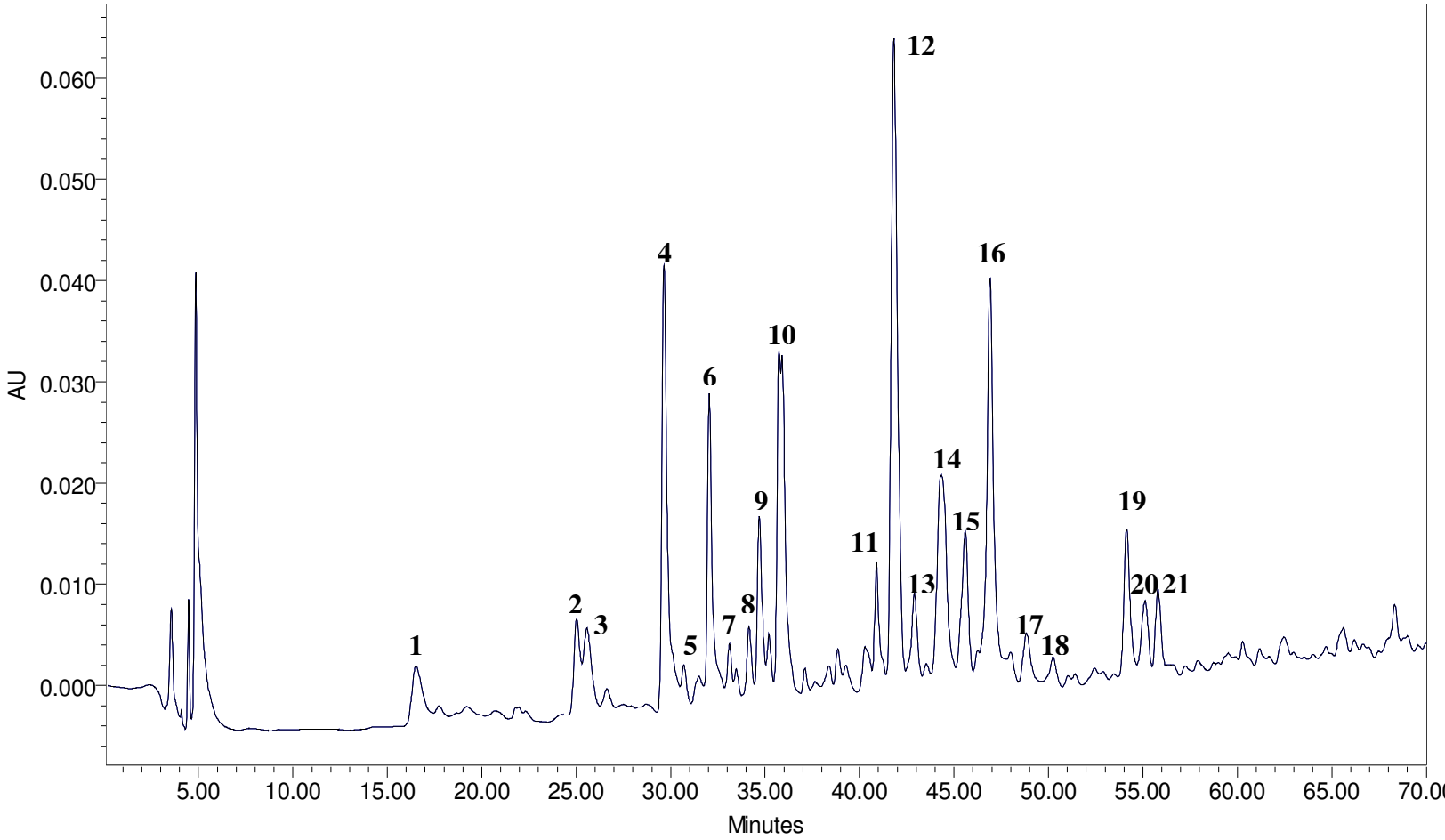


Fig. 4. HPLC chromatogram of non-anthocyanin polyphenolics present in *E. precatoria* juice at 280nm. Peak assignments are shown in Table 3.

Table 3. Characterization of non-anthocyanin polyphenolics present in *E. precatoria* fruits

Peak No.	RT (min)	Compound	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Concentration (mg/kg)
1	16.5	protocatechuic acid	263, 292	153.2	109.2, 91.0	2.38 ± 0.24
2	24.9	*p*-hydroxybenzoic acid	253.9	137.3	112.9	2.42 ± 0.44
3	26.7	(+)-catechin	277.5	289.2	245.2, 203.2, 187.2, 161.3	5.46 ± 0.57
4	29.7	vanillic acid	263, 291	167.3	140.9, 108.0, 95.2	13.4 ± 1.07
5	31.1	apigenin glycoside	258, 352	563.3	545.1, 473.2, 443.2, 353.1, 269.1	9.91 ± 0.84
6	33.1	syringic acid	271	196.9	182.2, 153.1, 138.1	10.1 ± 0.93
7	32.6	apigenin glycoside	258, 352	563.3	545.1, 473.2, 443.2, 353.1, 269.1	7.82 ± 0.61
8	34.1	(-)-epicatechin	277.5	289.2	245.2, 203.2, 187.2, 161.3	2.35 ± 0.28
9	34.7	unidentified flavone	272, 352	521.3	359.1, 344.2	5.11 ± 0.36
10	36.7	taxifolin derivative	272, 356	449.4	327.1, 269.2, 225.1	9.20 ± 0.72
11	41.3	isoorientin	272, 352	447.2	393.1, 357.1, 327.2, 299.2, 285.1	23.6 ± 1.07
12	41.9	orientin	272, 348	447.2	429.2, 357.1, 327.3, 299.2, 285.1	47.7 ± 2.04
13	43.0	ferulic acid	323.7	193.2	149.1, 134.1, 117.0	1.22 ± 0.13
14	44.7	taxifolin deoxyhexose	295, 340	449.1	269.1	7.50 ± 0.49
15	45.7	procyanidin dimer	235, 282	577.1	425.0, 407.2, 289.1, 287.1	52.9 ± 3.16
16	47.1	isovitexin	267, 338	431.1	341.2, 311.1, 283.0, 269.1	4.21 ± 0.18
17	49.2	apigenin glycoside	272, 352	533.4	443.2, 425.2, 383.1, 353.1	6.31 ± 0.57
18	50.4	apigenin glycoside	272, 352	533.4	443.2, 425.2, 383.1, 353.1	4.59 ± 0.41
19	53.2	procyanidin dimer	235, 282	577.1	425.0, 407.2, 289.1, 287.1	15.5 ± 1.22
20	54.9	procyanidin trimer	235, 291	865.1	577.2, 451.0, 425.0, 407.3	7.11 ± 0.44
21	56.3	procyanidin trimer	235, 291	865.1	577.2, 451.0, 425.0, 407.3	7.23 ± 0.38

Thus, isovitexin (apigenin-6-*C*-glucoside) identification was based on its distinctive molecular ion at *m/z*= 431.1, $[M-H]^-$, and subsequent fragmentation to product ions at *m/z*= 341.2, $[M-H-90]^-$, and *m/z*= 311.1, $[M-H-120]^-$ while scoparin (chrysoeriol 8-*C*-glucoside) was characterized by a precursor ion at *m/z*= 461.3, $[M-H]^-$, and fragment ions at *m/z*= 371.2, $[M-H-90]^-$, and *m/z*= 341.1, $[M-H-120]^-$.

Both orientin (luteolin-8-*C*-glucoside) and isoorientin (luteolin-6-*C*-glucoside) gave predominant molecular ions at *m/z*=447.2, $[M-H]^-$. Loss of water resulted in ion signals at *m/z*= 429.2, while fragmentation of the attached glycoside was likely responsible for ions at *m/z*=357.1, $[M-H-90]^-$, and *m/z*=327.2, $[M-H-120]^-$. Finally, cleavage of the *C*-sugar bond allowed the detection of the luteolin aglycone at *m/z*= 285.1. Identification of taxifolin deoxyhexose was based on spectral (λ_{max}= 295, 340) and mass spectrometric (*m/z*=449.1, $[M-H]^-$ and *m/z*=269.1, $[M-H-180]^-$) characteristics, as compared to previous reports using authentic standards (Schauss et al., 2006; Rijke et al., 2006). Additional luteolin and apigenin glycosides were also detected in both species, and tentative identifications were based on their typical spectral characteristics (absorption at 350-360 nm) and mass fragmentation patterns (ion signals at $[M-H-60]^-$, $[M-H-90]^-$, and $[M-H-120]^-$), along with fragment ions corresponding to luteolin (*m/z*=285.2, $[M-H]^-$) and apigenin (*m/z*=269.1, $[M-H]^-$) aglycones.

Orientin and isoorientin were the predominant flavonoids in both species, accounting for over 50% of the total flavonoid concentration. Isovitexin, scoparin, taxifolin deoxyhexose, two isovitexin and taxifolin derivatives, and two luteolin and apigenin glycosides were also present in *E. oleracea* fruits at concentrations between 3.7

and 10.6 mg rutin equivalents/kg. Of these, only isovitexin and taxifolin deoxyhexose were also detected in *E. precatoria* fruits (4.2 and 7.5 mg rutin equivalents/kg respectively) along with four apigenin glycosides, a taxifolin derivative, and an unidentified flavone, likely a glycoside, in concentrations ranging from 4.6 to 9.9 mg rutin equivalents/kg. Results were in agreement to previous HPLC-MS characterizations of flavonoids in *E. oleracea* (Gallori et al., 2004; Schauss et al., 2006), but this is the first report with quantitative information.

In addition to flavonoids, procyanidin dimers and trimers were among the most predominant non-anthocyanin polyphenolics in both açai species. Procyanidin dimers were identified based on precursor ion signals at *m/z*= 577.1, $[M-H]^-$, and fragments corresponding to (+)-catechin or (-)-epicatechin units (*m/z*= 289.1 and *m/z*= 287.1, $[M-H]^-$), probably resulting from cleavage of the interflavanoid bond, and characteristic of B-type procyanidin dimers (Friederich et al., 2000; Gu et al., 2003). Procyanidin trimers (*m/z*= 865.1, $[M-H]^-$) were characterized by predominant product ion signals at *m/z*=577.2, $[M-H-288]^-$, likely due to the loss of a (+)-catechin or (-)-epicatechin unit, yielding dimeric procyanidin fragment ions (Friedrich et al., 2000; Gu et al., 2003). Further fragmentation of ions at *m/z*=577.2 occurred in a similar manner as in the previously identified B-type procyanidins, confirming their identity. Procyanidin dimers were particularly abundant in *E. precatoria* fruits, accounting for more than 25% of the total non-anthocyanin polyphenolic content, while procyanidin trimers accounted for just over 5%. Conversely, procyanidin dimers and trimers accounted for only over 10% of the total non-anthocyanin polyphenolics in *E. oleracea*. Flavonol monomers such as (+)-

catechin and (-)-epicatechin also represented less than 5% of the total non-anthocyanin polyphenolic content for both species. Higher molecular weight compounds, likely polymeric procyanidins (Schauss et al., 2006), were also detected; however, quantification was not possible due to their poor resolution under these chromatographic conditions (Santos-Buelga & Williamson, 2003).

In relation to their antioxidant properties, both species were characterized by an initially high antioxidant capacity, 87.4 ± 4.4 µmol TE/g for *E. oleracea* and 114 ± 6.9 µmol TE/g for *E. precatoria* (Fig. 5).

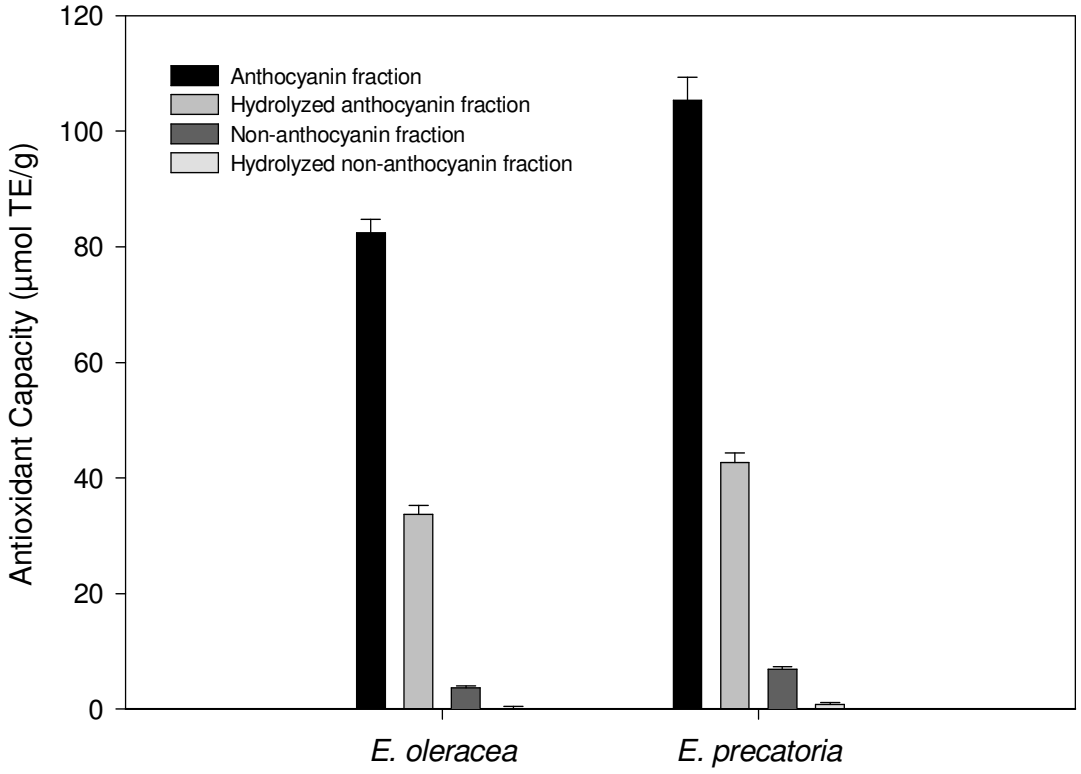


Fig. 5. Antioxidant capacity (µmol Trolox equivalents/mL) of non-hydrolyzed and hydrolyzed *E. oleracea* and *E. precatoria* phytochemical isolates. Bars represent the standard error of the mean (n=6).

Variations in antioxidant capacity between species were attributed to simple differences in phytochemical composition and concentration, primarily associated with the higher anthocyanin and procyanidin contents of *E. precatoria*. Fractionation of anthocyanins from remaining polyphenolics followed by acid hydrolysis provided additional detail that related to those compounds most responsible for radical scavenging contributions from each species. Anthocyanin-containing fractions from both species exhibited the highest antioxidant capacity, ranging from 82.4 ± 2.3 μmol TE/g in *E. oleracea* to 105.4 ± 3.9 μmol TE/g in *E. precatoria*, confirming that anthocyanins were the major contributors to antioxidant capacity at over 90% of the total. The remaining antioxidant capacity was due to non-anthocyanin compounds and represented from 3.7 ± 0.34 μmol TE/g in *E. oleracea* to 6.8 ± 0.52 μmol TE/mL in *E. precatoria*. Acid hydrolysis resulted in a significant reduction in the antioxidant capacity for both species, equivalent to 59.1 to 59.5% decrease for the anthocyanin isolate compared to 88.9 to 100% for the remaining polyphenolics (Fig. 5).

Chromatographic analyses of polyphenolics in the hydrolyzed, anthocyanin fractions revealed the presence of cyanidin (*m/z*=287.1, $[M-H]^-$, 99%) and peonidin (*m/z*=301.1, $[M-H]^-$, 1%) aglycones in *E. oleracea* and cyanidin (*m/z*=287.1, $[M-H]^-$, 94%) and pelargonidin (*m/z*=271.1, $[M-H]^-$, 6%) aglycones in *E. precatoria* fractions, further confirming their presence. Moreover, luteolin (*m/z*=285, $[M-H]^-$) and apigenin (*m/z*=269, $[M-H]^-$) aglycones were also detected in the hydrolyzed non-anthocyanin polyphenolic fraction of both species, along with trace concentrations of a cyanidin aglycone (<1 mg/kg), potentially derived from polymeric procyanidins.

Thermal stability of polyphenolics in açai was evaluated by holding açai pulps at 80ºC for 1, 5, 10, 30, and 60 min, in the presence and absence of oxygen, as compared to a non-heated control. No significant differences ($p<0.05$) were observed between the presence or absence of oxygen on polyphenolic degradation during heating. Non-anthocyanin polyphenolics, including flavone glycosides, flavonol derivatives, and phenolic acid concentrations remained constant during heating for up to 60 min, demonstrating appreciable thermal stability of these compounds in both açai species.

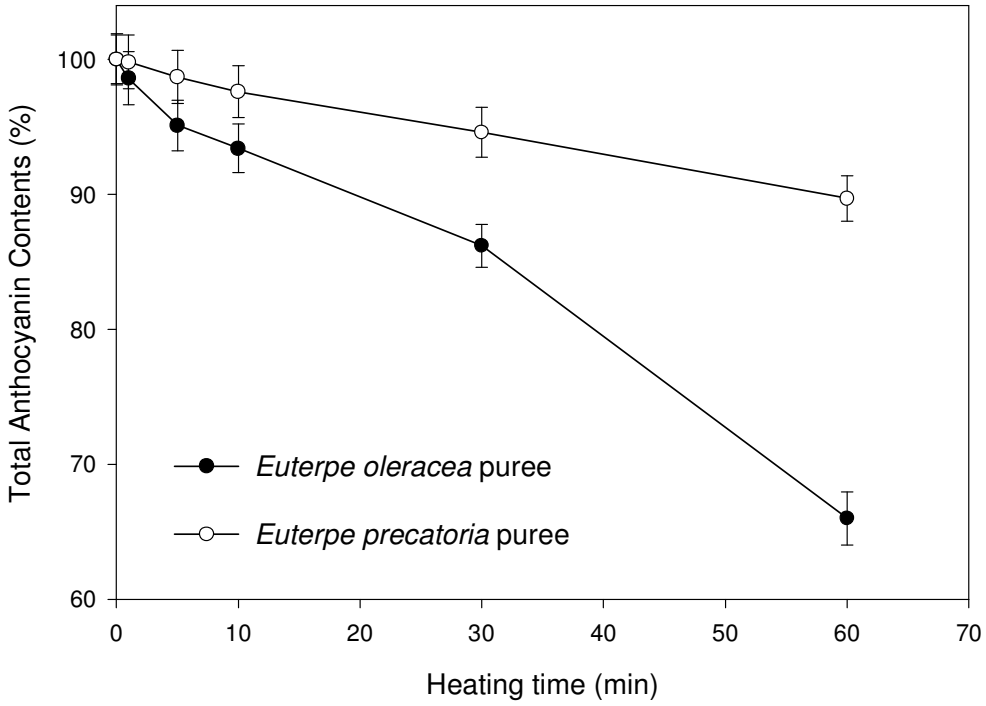


Fig. 6. Percent changes in total anthocyanin contents in *E. oleracea* and *E. precatoria* fruit purees following heating (80°C), as a function of heating time.

Yet extensive anthocyanin degradation occurred under similar heating conditions, likely due to accelerated chalcone formation with prolonged anthocyanin

exposure to high temperatures (Delgado-Vargas et al., 2002). Anthocyanin degradation rates were directly related to thermal exposure times (Fig. 6), yet highly variable between species, ranging from 10.3 ± 1.1% in *E. precatoria* to 34.0 ± 2.3% in *E. oleracea* purees. Variations in overall anthocyanin stability were attributed to differences in anthocyanin composition and variations in non-anthocyanin polyphenolics, which likely conferred additional stability. Cyanidin-3-rutinoside consistently showed a higher thermal stability (7.0 ± 0.6% loss following heating at 80°C for 1 h) than cyanidin-3-glucoside (up to 72 ± 5.3% loss under identical heating conditions) in both açai species. Therefore, an overall higher anthocyanin thermal stability in *E. precatoria* purees was likely attributed to higher concentrations of cyanidin-3-rutinoside (~90% of total anthocyanins, Table 1), compared to *E. oleracea* purees (~55% of total anthocyanins, Table 1).

Results were in agreement with previous investigations on the storage stability of anthocyanins in *E. oleracea* juice, where cyanidin-3-rutinoside half-lives doubled those of cyanidin-3-glucoside, both in the presence and absence of ascorbic acid (Pacheco-Palencia et al., 2007a). Similar observations have been also reported in other cyanidin-3-glucoside and cyanidin-3-rutinoside containing fruits, such as blackcurrants (Rubiskiene et al., 2005), where cyanidin-3-rutinoside was found to be the most thermally stable anthocyanin (35% loss after 150 min at 95°C). Variations in total anthocyanin contents during heating were highly correlated (r=0.98) to changes in total antioxidant capacity, which decreased by up to 10 ± 0.8% in *E. precatoria* and by 25 ± 2% in *E. oleracea*

purees, evidencing a major contribution of anthocyanins to the overall antioxidant capacity of both açai species.

Conclusion

Euterpe oleracea and *Euterpe precatoria* species shared similar polyphenolic profiles, characterized by the predominant presence of anthocyanins, which accounted for nearly 90% of the total antioxidant capacity in both açai fruits. Moreover, changes in antioxidant activity during heating were highly correlated to anthocyanin losses while phenolic acids, flavone glycosides, and flavanol derivatives present in both species, were not significantly altered by thermal exposure. Thus, both açai species are comparably suitable for food and beverage applications involving mild exposure to high temperatures.

CHAPTER IV

CHEMICAL COMPOSITION AND THERMAL STABILITY OF A PHYTOCHEMICAL-ENRICHED OIL FROM AÇAI

Introduction

Açai fruit (*Euterpe oleracea* Mart.) is currently among the most economically significant palm species in the Brazilian Amazon (Galotta & Boaventura, 2005), and has become one of the main export products of the Amazon estuary to other regions in the world. International growth of the açai trade in recent years has been attributed to the açai beverage industry and related products (Brondizio, Safar, & Siqueira, 2002), where much attention has been given to its antioxidant capacity and associated potential health benefits (Del Pozo, Brenes, & Talcott, 2004; Gallori et al., 2004; Lichtenthaler et al., 2005). As such, research efforts have focused on the study of açai pulps and juices, and factors affecting their stability and functional properties (Gallori et al., 2004; Lichtenthaler et al., 2005, Pacheco-Palencia et al., 2007a; Pacheco-Palencia et al., 2007b).

A distinguishing feature of açai fruit pulp is the presence of lipids that may account for up to 9% of the total fresh weight of the edible pulp (Clay & Clement, 1993; Lubrano, Robin, & Khaiat, 1994) and potentially represents a valuable by-product given its unique sensory characteristics, dark green color, and potential health benefits related to its traditional therapeutic uses by Amazonian inhabitants (Plotkin & Balick, 1984). A previous study on açai oil composition (Lubrano et al., 1994) reported 60% oleic acid,

22% palmitic acid, 12% linoleic acid, and 6% each of palmitoleic and stearic acids along with other fatty acids in trace amounts. At least five sterols were also identified including β-sitosterol (78%), stigmasterol (6.5%), δ5-avenasterol (6.5%), campesterol (6.0%), and cholesterol (2.0%). However, no reports on the polyphenolic and antioxidant composition of açai oil are available and its stability during processing and storage has not been previously assessed.

The present study was conducted to characterize the main polyphenolic compounds in oil extracts from açai fruit and to evaluate short and long-term stability of these compounds in terms of lipid oxidation and their impact on antioxidant capacity and total soluble phenolic contents. Based on these trials, the potential uses of açai oil extracts for food, supplement, and cosmetic applications were determined.

Materials and Methods

Açai oil was solvent extracted using a patent-pending process (Talcott, 2007) from a water-insoluble juice processing by-product obtained from the Bossa Nova Beverage Group (Los Angeles, CA) and solvent removed under vacuum to produce a crude oil for these trails. The resultant oil designated “high phenolics” was naturally enriched in polyphenolics present in the açai fruit itself, and was hypothesized to influence the oxidative stability of the oil. A second oil was prepared by repeatedly extracting the high phenolic oil with water and then extracting with 100% hexane to facilitate the removal of polyphenolics from the oil. Upon solvent removal under reduced pressure at <40°C an oil that was essentially free of phenolic acids (<5% of the

original oil) resulted and was designated “low phenolics”. An “intermediate phenolics” oil was then prepared as a 50:50 mixture of high and low phenolic oils.

Equal amounts of oils (5 mL) were loaded into screw-cap glass test tubes in triplicate, the headspace flushed with nitrogen, and stored at 20, 30, and 40°C in the dark for 10 weeks. Tubes were removed from storage periodically and treatments held at -20°C until analysis. The short-term thermal stability of polyphenolics in the high phenolic oil was also evaluated by loading 2 mL of oil into screw-cap glass test tube and heated to an internal temperature of 150 and 170°C for 0, 5, 10, and 20 min using a commercial vegetable oil as the heating medium. Samples were immediately cooled by immersion in cold water. For chemical analyses, oil samples (100 mg) were extracted with 4 mL a 1:1 hexane:methanol mixture, followed by an equivalent volume of water. The upper phase was discarded and the lower, hydrophilic phase was used for subsequent analyses. Phytochemical analyses on the enriched açai oil were compared to a polyphenolic extract from clarified açai juice, prepared by extensive liquid/liquid extraction with ethyl acetate to isolate non-anthocyanin polyphenolics from the aqueous juice. The solvent was evaporated under reduced pressure at <40°C and the isolate redissolved in a known volume of citric acid buffer (pH 3.5) and used for subsequent analyses.

Major polyphenolic compounds present in açai oil were analyzed by reversed phase HPLC with a Waters 2695 Alliance system (Waters Corp., Milford, MA), using water with 2% acetic acid (phase A) and a mixture of 68% acetonitrile, 30% water, and 2% acetic acid (phase B). The elution program ran phase B from 0 to 30% in 20 min, 30

to 50% from 20 to 50 min, and 50 to 90% in 75 min, at a flow rate of 0.8 mL/min. Initial conditions were then restored and kept constant for 10 min prior each injection.

Polyphenolics were identified and quantified based on their spectral characteristics and retention time, as compared to authentic standards (Sigma Chemical Co., St. Louis, MO). Further structural information was obtained by mass spectrometric analyses, performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher, San Jose, CA). Separations were conducted using the Phenomenex (Torrace, CA) Synergi 4µ Hydro-RP 80A (2 x 150 mm; 4 µm) with a C18 guard column. Mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in a mixture of 50% methanol, 50% acetonitrile (phase B) run at 0.25 mL/min. Polyphenolics were separated with a gradient elution program in which phase B changed from 5 to 30% in 5 min, from 30 to 65% in 70 min, and from 65 to 95% in 30 min and was held isocratic for 20 min. Electrospray ionization was conducted in the negative ion mode under the following conditions: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.3 kV; capillary temperature, 250°C; capillary voltage, 1.5 V; tube lens offset, 0 V.

Total soluble phenolics, measuring the metal reduction capacity of oil extractions, was analyzed by the Folin-Ciocalteu assay (Singleton & Rossi, 1965) and quantified in gallic acid equivalents (GAE). Antioxidant capacity was determined by the oxygen radical absorbance capacity (ORAC) assay in a BGM Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission), as previously described in chapter III. Results were quantified in µmol Trolox equivalents (TE) per

milliliter of oil. Overall oxidative stability of the oil was determined by measuring free fatty acids and peroxide value according to AOAC official methods (Horwitz, 2000).

Data from each chemical analysis was analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Mean separations were conducted using Tukey-Kramer HSD ($p<0.05$) as a post-hoc analysis. Linear and non-parametric correlations among chemical analyses were obtained and linear regression analyses were conducted using a significance level of 0.05.

Results and Discussion

Polyphenolic enriched oil from açai fruit was characterized by its dark green color and viscous appearance, along with a distinctive aroma, similar to that of açai fruit pulp. Further chemical tests were conducted by an independent contract laboratory, including specific gravity (0.9247 g/cm^3), refractive index (1.4685), iodine value (75.0 I_2/100g oil), peroxide value (5.71 meq/kg oil), and fatty acid composition (20.7% saturated, 69.6% monounsaturated, 9.7% polyunsaturated).

Major polyphenolic compounds (Fig. 7) present in oil extracts obtained from water-insoluble residues from açai pulp processing were identified based on their spectrophotometric characteristics and mass spectra and quantified against authentic standards when available. Açai pulp is a rich source of antioxidant polyphenolics that were co-extracted during the oil extraction process, and were likely concentrated in the resulting oil.

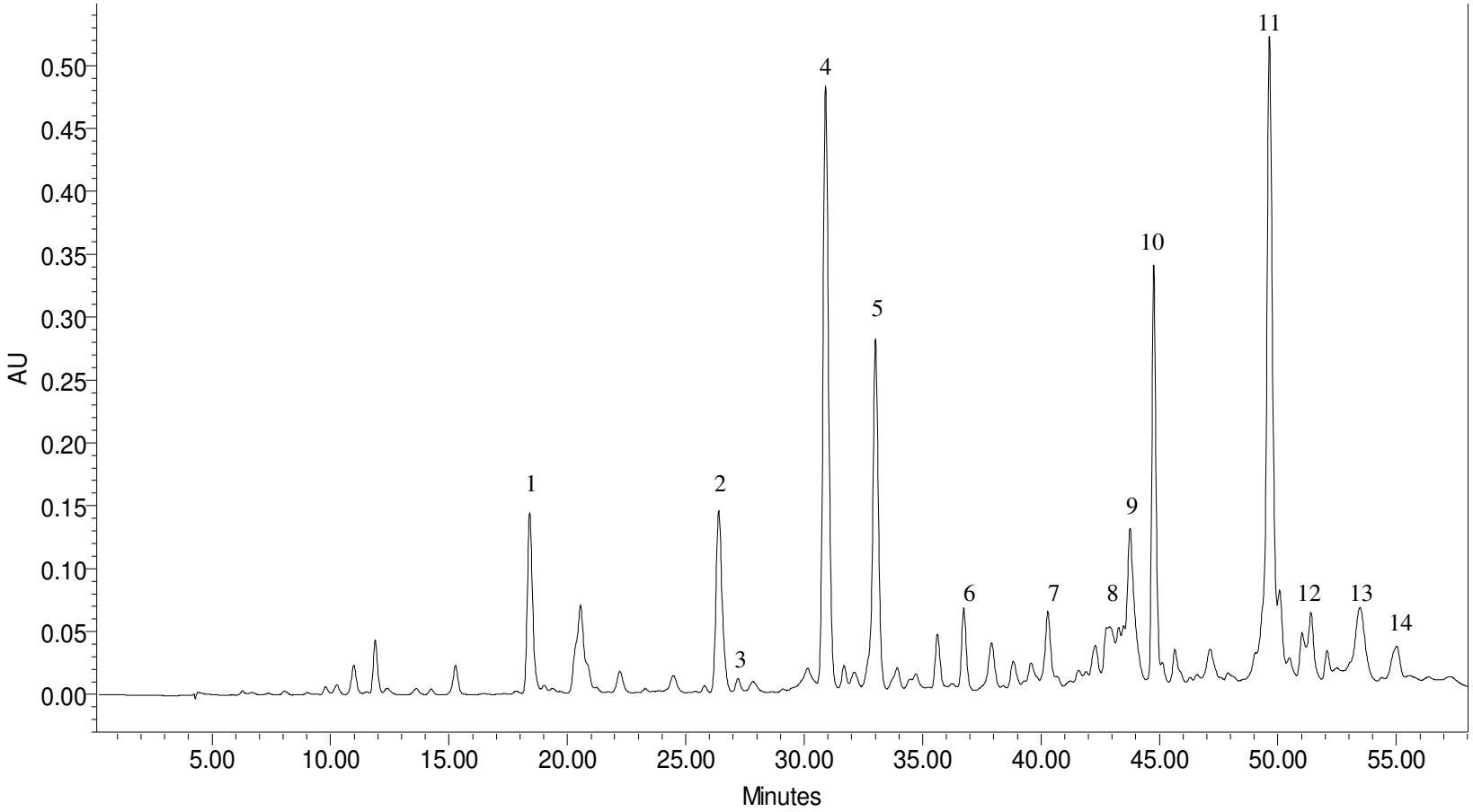


Fig. 7. HPLC chromatogram of polyphenolics present in a typical *E. oleracea* oil extract. Peak assignments: 1. protocatechuic acid; 2. *p*-hydroxybenzoic acid; 3. (+)-catechin; 4. vanillic acid; 5. syringic acid; 6-7. procyanidin dimers; 8. ferulic acid; 9-10 procyanidin dimers; 11-14 procyanidin trimers.

Table 4. HPLC-ESI-(-)MS^n analyses of polyphenolics in *E. oleracea* oil extracts.

Peak	RT (min)	λ_{max} (nm)	Compound	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)[1]	MS^3 (*m/z*)
1	18.5	263, 292	protocatechuic acid	153.2	109.2	91.0
2	26.4	253.9	*p*-hydroxybenzoic acid	137.3	112.9	
3	27.2	277.5	(+)-catechin	289.2	**245.2**, 205.2, 179.2	203.2, 187.2, 161.3
4	30.9	263, 291.7	vanillic acid	167.3	140.9, **108.0**	95.2
5	33.0	271	syringic acid	196.9	182.2, 153.1, **138.1**	123.1, 121.0, 106.0
6	37.9	235, 282	procyanidin dimer	577.1	425.0, **407.2**	289.2, 287.1
7	40.3	235, 282	procyanidin dimer	577.1	425.0, **407.2**	289.2, 287.1
8	42.8	324.9	ferulic acid	193.2	149.1, **134.1**	117.0
9	43.7	235, 282	procyanidin dimer	577.1	425.0, **407.2**	289.2, 287.1
10	44.8	235, 282	procyanidin dimer	577.1	425.0, **407.2**	289.2, 287.1
11	49.7	235, 291.7	procyanidin trimer	865.1	**577.2**, 559.1	451.0, 425.0,407.3, 287.1
12	51.6	235, 291.7	procyanidin trimer	865.1	**577.2**, 559.1	451.0, 425.0,407.3, 287.1
13	52.9	235, 291.7	procyanidin trimer	865.1	**577.2**, 559.1	451.0, 425.0,407.3, 287.1
14	55.0	235, 291.7	procyanidin trimer	865.1	**577.2**, 559.1	451.0, 425.0,407.3, 287.1

[1]Ions in bold indicate the most intense product ion, on which further MS analyses were conducted.

Table 5. Concentration (mg/L) and relative abundance (%) of non-anthocyanin polyphenolics present in *E. oleracea* clarified juice and oil extracts.

Polyphenolic	*E. oleracea* juice (mg/L)	Relative abundance (%)[1]	*E. oleracea* oil (mg/L)	Relative abundance (%)[1]
protocatechuic acid	1.8 ± 0.1	4.8 ± 0.3 [a]	630.8 ± 36	8.4 ± 0.5[b]
p-hydroxy benzoic acid	1.9 ± 0.2	5.1 ± 0.6 [a]	892 ± 52	11.9 ± 0.7[b]
(+)-catechin	5.3 ± 0.6	14.1 ± 1.6 [a]	66.7 ± 4.8	0.9 ± 0.1 [b]
vanillic acid	5.5 ± 0.2	14.6 ± 0.6 [a]	1,616 ± 94	21.6 ± 1.3 [b]
syringic acid	3.7 ± 0.4	9.8 ± 1.3 [a]	1,073 ± 62	14.3 ± 0.8 [a]
(-)-epicatechin	1.1 ± 0.1	2.9 ± 0.2	----	----
ferulic acid	1.1 ± 0.1	2.9 ± 0.3 [a]	101 ± 5.9	1.4 ± 0.1 [b]
procyanidin dimers	6.1 ± 0.7	16.1 ± 1.3 [a]	1,086 ± 121	14.5 ± 1.3 [a]
procyanidin trimers	11.2 ± 1.2	29.7 ± 3.1 [a]	2,016 ± 53	27.0 ± 2.4 [a]

[1]Values with different letters between columns represent a significant difference (paired samples t-test, $p<0.05$).

Phenolic acids such as vanillic acid (1,616 ± 94 mg/L), syringic acid (1,073 ± 62 mg/L), *p*-hydroxy benzoic acid (892 ± 52 mg/L), protocatechuic acid (629 ± 36 mg/L), and ferulic acid (101 ± 5.9 mg/L) were predominantly present in the oil while (+)-catechin (66.7 ± 4.8 mg/L), four procyanidin dimers (1,085.6 ± 121.3 mg (+)-catechin equivalents/L), and four procyanidin trimers (2,016.2 ± 53.2 mg (+)-catechin equivalents/L) were also detected in high concentrations (Tables 4 and 5).

In addition, five compounds exhibiting typical flavonoid spectral characteristics were detected at trace concentrations, from 0.58 to 4.21 mg rutin equivalents/kg in açai oil. Previous studies on polyphenolics in açai pulp (Gallori et al., 2004; Lichtenthaler et al., 2005; Pacheco-Palencia et al., 2007a; Pacheco-Palencia et al., 2007b), seeds (Rodrigues et al., 2006), and freeze-dried açai fruit (Schauss et al., 2006) reported the presence of phenolic acids such as vanillic, syringic, *p*-hydroxy benzoic, protocatechuic, and ferulic acid, as well as (+)-catechin, (-)-epicatechin, and B type procyanidins, from dimers to high molecular weight polymers that were not characterized. In this study, structural information was obtained by means of mass spectrometric analyses that confirmed the identities of the polyphenolics previously identified by HPLC and provided additional information on the identity and degree of polymerization of procyanidin dimers and trimers (Table 4). Both the catechin-like UV spectra and characteristic signals at *m/z* 577.1 ($[M-H]^-$) indicated the presence of procyanidin dimers while signals at *m/z* 865.1 ($[M-H]^-$) were attributed to procyanidin trimers.

Previous LC-ESI-MS^n studies on proanthocyanidins agreed that the *m/z* 577.1 ion is indicative of B-type procyanidin dimers (Friedrich et al., 2000) while fragmentation to

the *m/z* 425.0 ion was characteristic of the product obtained from Retro-Diels-Alder reaction of ring C and subsequent elimination of ring B from the flavan-3-ol (Gu et al., 2003). Finally, *m/z* 289.2 and 287.1 fragments, likely from cleavage of the interflavanoid bond (Friedrich et al., 2000; Gu et al., 2003; Pati et al., 2006) suggested that these procyanidin dimers consisted of two catechin units, although no differentiation between stereoisomers was possible. Similarly, procyanidin trimers (*m/z* 865.1) were characterized by a predominant product ion at *m/z* 577.2, likely corresponding to a dimeric fragment ion from cleavage of interflavanoid linkages, which has been recognized as the most important fragmentation mechanism in proanthocyanidin trimers (Friedrich et al., 2000). Further fragmentation of *m/z* 577.2 occurred in a similar manner as in the previously described procyanidin dimers.

The açai oil extracts used in these trials did not contain anthocyanins and were therefore compared with non-anthocyanin polyphenolics present in clarified açai juice by chemically contrasting content and concentrations under identical chromatographic conditions (Table 5). Similar polyphenolic profiles were observed in the açai oil extracts and non-anthocyanin polyphenolic extracts of açai juice, yet their concentrations differed markedly. Due to an enhanced extraction of compounds from water-insoluble residues from açai pulp processing, individual polyphenolic concentrations were significantly higher (from 12.6 to 469 times higher) in açai oil than in açai juice.

Dimeric and trimeric procyanidins were predominant in both açai oil extracts (3,102 ± 127 mg/L) and açai juice (17.2 ± 2.2 mg/L), and accounted for over 40% of the total polyphenolics present. Similarly, phenolic acids such as vanillic, syringic,

protocatechuic, *p*-hydroxybenzoic, and ferulic acids were predominant in both the oil extracts (101 to 1,616 mg/L) and açai juice (1.1 to 5.5 mg/L). However, their respective abundance differed significantly ($p<0.05$) between products and with the exception of ferulic acid, phenolic acids were appreciably enhanced in the oil (Table 5). Although both (+)-catechin and (-)-epicatechin were found in açai juice (1.1 and 5.3 mg/kg, respectively), only (+)-catechin (66.7 ± 4.8 mg/kg) was present in açai oil extracts. The similarities between the polyphenolic profiles of açai juice and açai oil extracts suggest that these polyphenolics have the ability to be extracted from açai by-product sources as free or bound compounds and deposited to a non-polar lipid phase which serves to appreciably enhance polyphenolic concentrations in the oil when compared to the fruit pulp from which they were derived.

The influence of naturally occurring polyphenolics on the phytochemical stability of extracted açai oil during storage was evaluated by monitoring changes to individual polyphenolics, total soluble phenolic content, and antioxidant capacity of oils stored at 20, 30, or 40°C for 10 weeks. Initial polyphenolic concentrations in the oil were adjusted to three concentration levels containing high, intermediate, and low polyphenolic concentrations by diluting original oil extracts with oil whose polyphenolics were removed by aqueous extraction. The açai oil containing the lowest polyphenolic concentration contained <5% of the original oil's concentration, whereas the intermediate oil was 50% of the original (Table 6).

Table 6. Major polyphenolics present in *E. oleracea* oil extracts (mg/L) adjusted to three different polyphenolic levels.

Polyphenolic	Polyphenolics (mg/L) in açai oil (High phenolics)	Polyphenolics (mg/L) in açai oil (Intermediate phenolics)	Polyphenolics (mg/L) in açai oil (Low phenolics)
Protocatechuic acid	630 ± 36	319 ± 18	11.8 ± 1.6
p-hydroxy benzoic acid	892 ± 527	578 ± 33	18.9 ± 1.2
(+)-catechin	66.7 ± 4.8	35.1 ± 2.4	2.2 ± 0.3
vanillic acid	1,616 ± 94	884 ± 51	31.4 ± 1.8
syringic acid	1,073 ± 62	602 ± 35	21.1 ± 1.4
ferulic acid	101 ± 5.9	57.7 ± 3.3	2.0 ± 0.2
procyanidin dimers	2,016 ± 53	633 ± 34	18.3 ± 1.1
procyanidin trimers	1,086 ± 121	1175 ± 72	33.9 ± 2.3

Table 7. Percent polyphenolic losses in *E. oleracea* oil extracts adjusted to different polyphenolic levels following storage at 20, 30, and 40ºC.

Polyphenolic	Polyphenolic losses in açai oil (High phenolics)			Polyphenolic losses in açai oil (Intermediate phenolics)		
	20°C[1]	30°C	40°C	20°C	30°C	40°C
	% Loss from Initial			% Loss from Initial		
protocatechuic acid	1.33 ± 0.4[a]	1.51 ± 0.6[a]	1.52 ± 0.3[a]	1.57 ± 0.2[a]	1.62 ± 0.4[a]	1.63 ± 0.4[a]
p-hydroxy benzoic acid	0.48 ± 0.2[a]	0.46 ± 0.2[a]	1.97 ± 0.2[b]	0.43 ± 0.2[a]	0.52 ± 0.8[a]	2.02 ± 0.5[b]
(+)-catechin	0.55 ± 0.1[a]	1.12 ± 0.3[b]	2.35 ± 0.6[c]	0.52 ± 0.1[a]	0.95 ± 0.2[b]	2.44 ± 0.5[c]
vanillic acid	0.33 ± 0.1[a]	1.03 ± 0.2[b]	2.55 ± 0.3[c]	0.32 ± 0.3[a]	1.17 ± 0.2[b]	2.65 ± 0.4[c]
syringic acid	0.86 ± 0.1e[a]	0.85 ± 0.2[a]	8.36 ± 1.0[b]	0.82 ± 0.2[a]	0.81 ± 0.1[a]	8.07 ± 1.0[b]
ferulic acid	14.8 ± 1.4[a]	16.3 ± 0.3[a]	32.2 ± 1.4[b]	13.6e ± 1.2[a]	15.2 ± 1.4[a]	33.1 ± 1.9e[b]
procyanidin dimers	9.27 ± 2.1[a]	20.3 ± 2.7[b]	33.2 ± 3.4[c]	12.1 ± 2.0[a]	20.9 ± 2.2[b]	29.3 ± 3.0[c]
procyanidin trimers	23.2 ± 1.8[a]	39.1 ± 3.3[b]	73.5 ± 4.4[c]	23.8 ± 2.2[a]	36.3 ± 4.0[b]	69.2 ± 5.0[c]

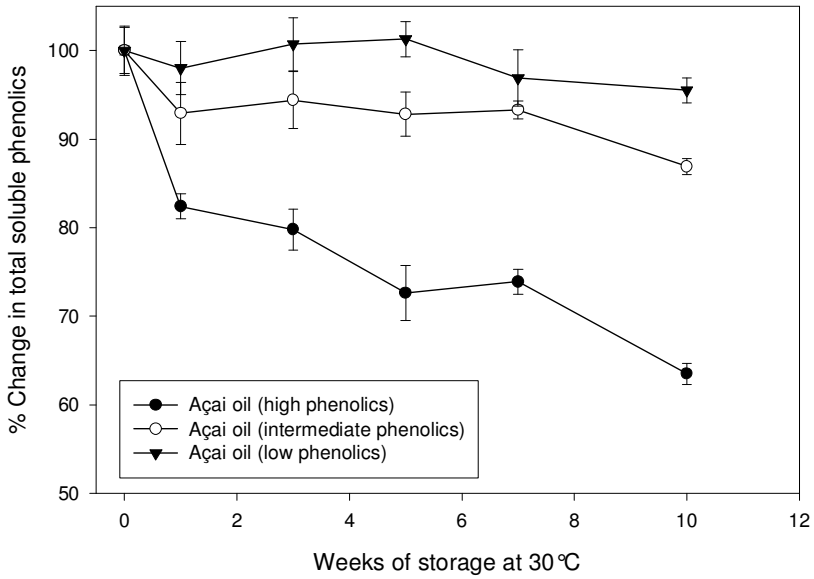
[1] Values with different letters within rows are significantly different (LSD test, $p<0.05$)

Individual polyphenolic concentrations were monitored periodically during storage, and no significant differences ($p<0.05$) were found between high and intermediate polyphenolic oils (Table 7) suggesting that polyphenolic losses were independent of initial polyphenolic contents. Moreover, no significant changes in (+)-catechin, protocatechuic acid, *p*-hydroxy benzoic acid, vanillic acid, or syringic acid concentrations were detected after 10 weeks of storage at 20 or 30°C and only minor changes (<10%) were observed following storage at 40°C indicating excellent storage stability of these compounds even under adverse handling conditions. Storage effects were more pronounced for ferulic acid and procyanidin dimers and trimers, as concentrations decreased by 9.3 and 23.2% respectively when stored at 20°C, by 20.3 and 39.1% when stored at 30°C and by 33.1 and 73.4% when stored at 40°C.

Due to the high stability of flavanol monomers during storage and earlier reports on the stable nature of procyanidins (Rios, Bennet, Lazarus, Remesy, Scalbert, & Williamson, 2002), it was hypothesized that decreased concentrations of procyanidin dimers and trimers during storage might be attributed to oil matrix effects on procyanidin extraction efficiency, such as the formation of complexes between procyanidins and proteins or other oil-soluble components over time, including phospholipids or other natural emulsifiers present in the oil. Evidence for complexation that decreased solubility of procyanidins was found upon further analyses of the oil following the initial polyphenolic extraction. Since phenolic acids and monomeric flavonoid concentrations remained constant during storage, total soluble phenolic contents were used as a potential indicator of the presence of residual procyanidins in the

polyphenolic-extracted oil. Results indicated that total soluble phenolic contents in the polyphenolic-extracted oil were 64.7 ± 3.9 mg GAE/L and thus reflected the presence of oil-bound procyanidins in the oil that was enhanced during oil storage.

Oil extracts were additionally evaluated for soluble polyphenolic contents, as a measure of total reducing capacity, and for changes in antioxidant capacity throughout the storage period. A statistically significant ($p<0.01$) correlation was found between total soluble phenolic content and antioxidant capacity by both linear ($r=0.94$) and non-parametric ($\rho=0.92$) methods during storage at 20, 30, and 40°C. The high polyphenolic oil had an initial antioxidant capacity of 21.5 ± 1.7 µmol TE/mL and a total soluble phenolic content of 1,252 ± 42 mg GAE/L whereas the intermediate polyphenolic oils were half these levels at 14.3 ± 1.2 µmol TE/mL and 695 ± 28 mg GAE/L, and low polyphenolic oils at 4.8 ± 0.3 µmol TE/mL and 192 ± 8.3 mg GAE/L, respectively. Total soluble phenolics expressed as GAE were found at appreciably lower concentrations than the sum of individual polyphenolic concentrations (Table 6) and is attributable to the high concentration of hydroxybenzoic acids in the oil that were previously shown to exhibit poor reducing capacity and radical scavenging activity (Rice-Evans et al., 1996; Kim & Lee, 2004). The antioxidant activity of phenolic acids is thought to depend on the number of hydroxy substitutions on their aromatic ring (Kim & Lee, 2004); however, the electron withdrawing properties of the carboxyl group in benzoic acids has a negative effect on hydrogen-donating abilities of hydroxybenzoic acids (Rice-Evans et al., 1996).



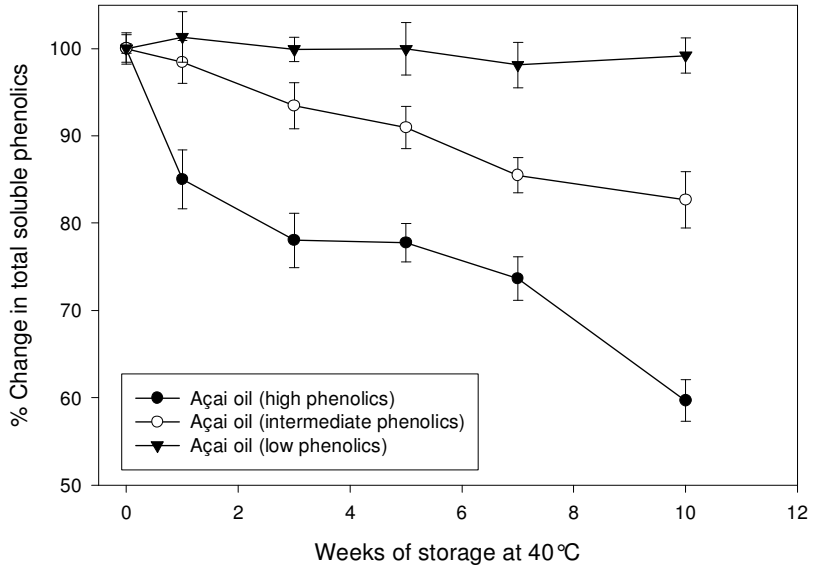


Fig. 8. Percent changes in total soluble phenolic contents during storage of *E. oleracea* oil extracts adjusted to different initial phenolic contents. Error bars represent the standard error of the mean (n=3).

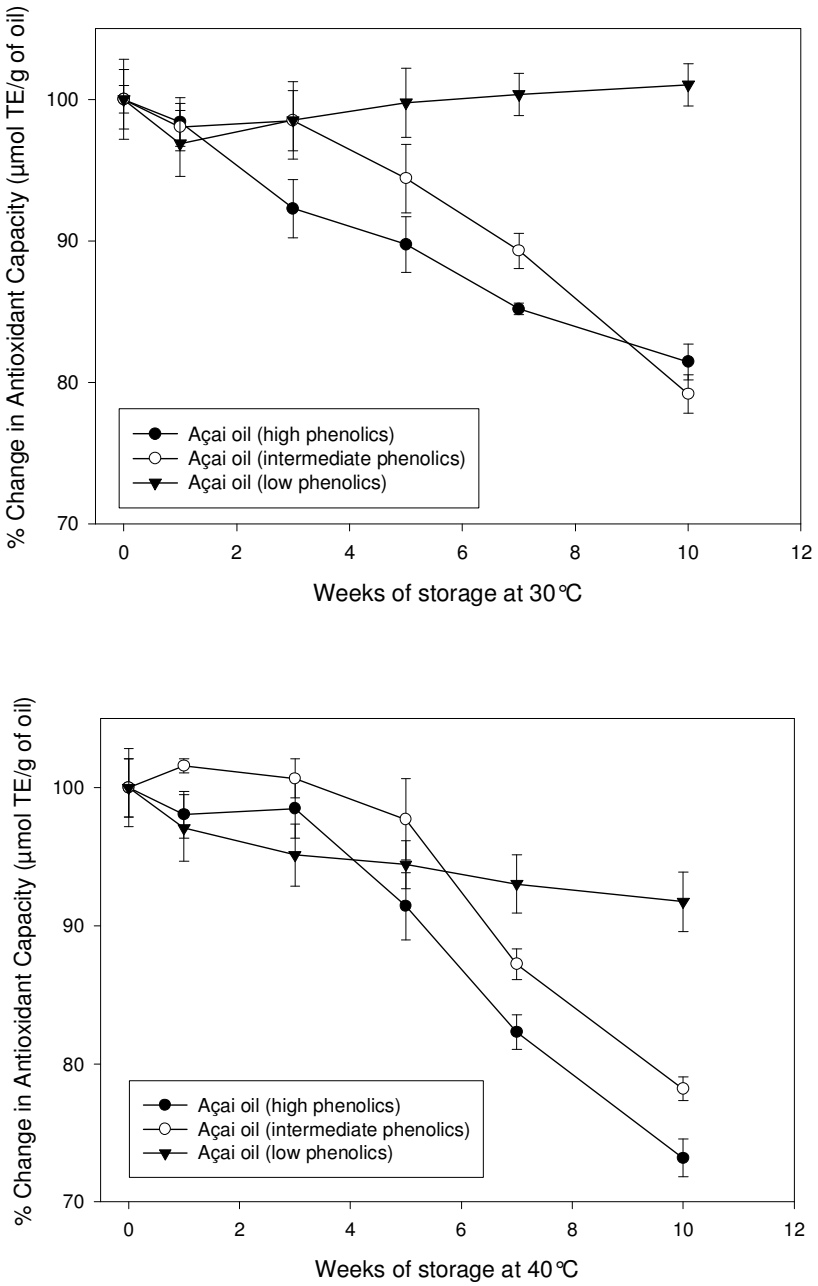


Fig. 9. Percent changes in antioxidant capacity during storage of *E. oleracea* oil extracts adjusted to different initial phenolic contents. Error bars represent the standard error of the mean (n=3).

During storage, total soluble phenolic content of the high polyphenolic oil decreased by 36.1 to 40.3% when stored at 20, 30, or 40°C (Fig. 8) corresponding to a 18.6 to 26.8% decrease in antioxidant capacity (Fig. 9). Both the intermediate and low polyphenolic oils experienced significantly ($p<0.05$) smaller losses for these attributes during storage ranging from minor losses (<10%) in the low polyphenolic oil at all storage temperatures and as high as 21.8% in the intermediate polyphenolic oil at 40°C. Linear regression analyses of antioxidant capacity and total soluble phenolic contents during storage further confirmed a significant ($p<0.01$) influence of oil polyphenolic concentration on retention of both total soluble phenolics and antioxidant capacity during storage, but no significant effect was attributed to storage temperature. Such differences might be attributed, at least partially, to previously observed differences in procyanidin concentrations (Table 6), which were more pronounced in high and intermediate polyphenolic oils.

The oxidative stability of açai oil extracts adjusted to three different polyphenolic concentrations was further assessed by monitoring changes in free fatty acid (% oleic acid) and peroxide values (meq/kg) following storage. Free fatty acid (<0.1%) and peroxide value (<10 meq/kg) were at threshold levels of detection prior to and after storage of the oils, indicating that lipids in the oil did not experience noticeable oxidative changes after 10 weeks of storage at 20, 30, or 40°C.

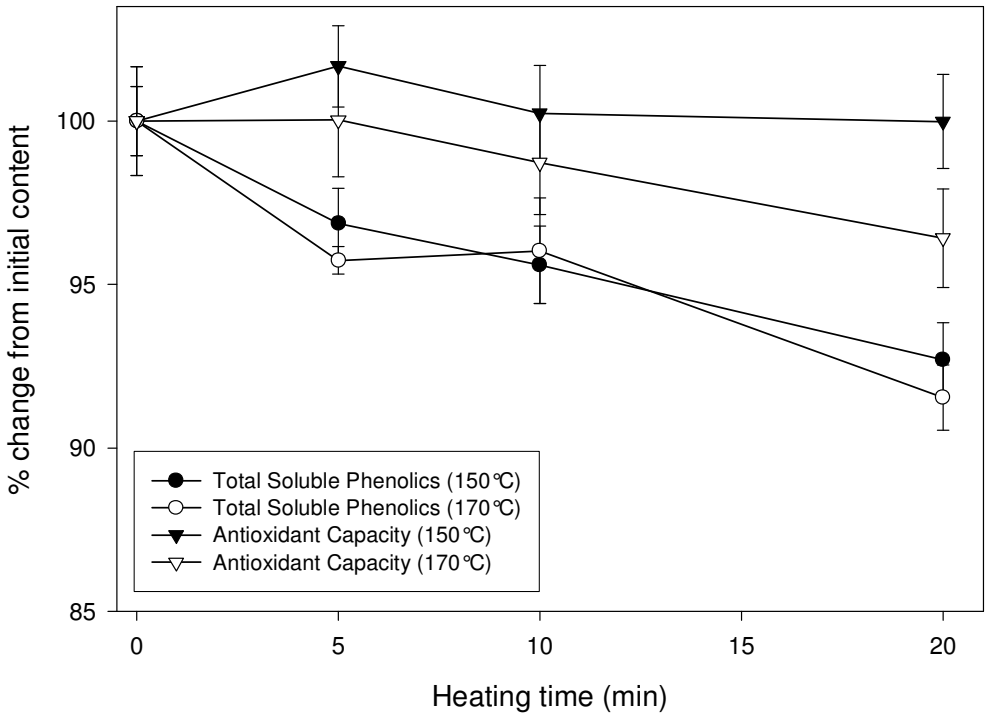


Fig. 10. Percent changes in total soluble phenolics and antioxidant capacity in *E. oleracea* oil extracts following heating. Error bars represent the standard error of the mean (n=3).

The short-term, high temperature storage stability of polyphenolics in açai oil extracts was evaluated by monitoring changes in total soluble phenolic contents, antioxidant capacity, and individual polyphenolic concentrations following heating of the high polyphenolic oil to a temperature of 150 or 170°C and holding for 5, 10, and 20 min. This short-term trial was to simulate cooking effects on the oil and to determine if the physical stability of the oil would degrade under moderate to high temperatures. However, even under the most extreme time and temperature combination, no changes

in the physical nature of the oil were observed such as smoke or color degradation and no significant ($p<0.05$) changes to individual polyphenolic concentrations were detected during this evaluation. Minor changes in overall antioxidant capacity (<5%) and soluble phenolic contents (<10%) were observed under these same conditions (Fig. 10) with slightly greater losses observed at 170°C compared to 150°C. Therefore, the extracted açai oil demonstrated excellent thermal stability for the polyphenolics present, and indicated its potential for culinary applications involving moderate exposure times to high temperatures.

Conclusion

The phytochemical composition of açai oil extracts from water-insoluble residues of açai pulp processing were characterized and found to be appreciably enhanced in non-anthocyanin polyphenolics such as phenolic acids and procyanidins. Individual polyphenolic contents were not significantly altered by long-term storage at temperature up to 40°C for 10 weeks nor by short-term heating at temperatures up to 170°C for 20 min indicating good stability of these compounds and their antioxidant properties. Due to its high polyphenolic content, storage stability, and unique sensory characteristics, açai oil is a promising new alternative to traditional oils for food, supplement, and cosmetic applications.

CHAPTER V

IN-VITRO ABSORPTION AND BIOLOGICAL ACTIVITY OF PHYTOCHEMICAL RICH EXTRACTS FROM AÇAI

Introduction

Intake of naturally occurring polyphenolics in fruits and vegetables has been associated with a reduced risk of developing various types of cancer in several epidemiological studies (Hertog, Bueno-de-Mesquita, & Fehily, 1996; Dragsted, Strube, & Leth, 1997; Abedamowo et al., 2004). In addition to their antioxidant properties and ability to protect vital cellular components from oxidative stress, polyphenolics have also been shown to inhibit cell proliferation, induce apoptosis, alter cell cycle kinetics, and interfere with intracellular signal transduction in several in-vitro cancer cell models (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999; Elattar & Viriji, 1999; Birviba, Pan, & Rechkemmer, 2002; Mertens-Talcott, Lee, & Talcott, 2006). Information on the inhibitory effects of individual polyphenolic compounds on carcinogenesis is abundant; however, it has been suggested that isolated compounds may not behave on the same manner as when present in whole foods (Liu, 2003). Thus, studies considering the unique combination of phytochemicals in specific commodities may provide information that more properly reflects the potential health benefits associated with their consumption.

Açai (*Euterpe oleracea* Mart.), a palm fruit native to the Amazon estuary, has received much attention in recent years, due to potential health benefits associated with

its high antioxidant capacity and phytochemical composition (Lichtenthaler et al., 2005; Rodrigues et al., 2006; Schauss et al., 2006, Pacheco-Palencia et al., 2007a). However, reports on its biological properties are limited (Del Pozo-Insfran et al., 2006) and its anticarcinogenic activity has yet to be investigated in more detail. The present study was conducted to evaluate the antiproliferative activities of phytochemical rich extracts from açai fruit in HT-29 human colon adenocarcinoma cells. Non-anthocyanin polyphenolic fractions from açai juice were contrasted against polyphenolic extracts from a phytochemical enriched açai oil, characterized previously in chapter IV, and found to contain concentrated amounts of phenolic acids and flavonoids naturally present in açai fruit. Moreover, potential relations between the magnitude of the observed antioxidant and antiproliferative activities and the polyphenolic composition of the extracts were determined.

Results from this study will provide additional evidence on the biological activity of açai polyphenolics in-vitro and offer new information on their bioavailability. In addition, a key factor to relate in-vitro study results to outcomes in-vivo is information regarding bioavailability. In the present study, polyphenolic absorption from açai extracts was evaluated using Caco-2 human intestinal cell monolayer models. Although some studies have been conducted to evaluate the bioavailability of certain polyphenolics in-vitro (Kobayashi et al., 2000; Konishi et al., 2003), information on the absorption of polyphenolics, particularly mixtures, is still very limited. As a result, polyphenolic absorption from açai extracts was additionally evaluated using Caco-2 cell monolayer models as an in-vitro model for intestinal absorption. Similarly to

proliferation studies, non-anthocyanin polyphenolic extracts from açai juice were contrasted against homologous extracts from a phytochemical enriched açai oil. Results from this study were aimed to asses the bioactive properties of açai polyphenolics and offer initial information on their bioavailability.

Materials and Methods

Frozen, pasteurized, açai pulp was kindly donated by Bossa Nova Beverage Group (Los Angeles, CA) and shipped overnight to the Department of Nutrition and Food Science at Texas A&M University. Prior to polyphenolic isolation, açai pulp was clarified according to a previously described procedure (Pacheco-Palencia et al., 2007b). Phenolic acids and non-anthocyanin flavonoids were isolated from açai juice by repeated liquid/liquid extraction with ethyl acetate (1:1 ratio). The upper ethyl acetate fraction was recovered, passed through a 5 cm bed of sodium sulfate to remove residual water, evaporated under vacuum (<40°C), and redissolved in dimethyl sulfoxide (DMSO). Açai oil was extracted using a patent-pending process (Talcott et al., 2007) from a water-insoluble juice processing by-product obtained from the Bossa Nova Beverage Group. Polyphenolics present in the resultant oil were extracted (3 times) by the addition of a methanol and water (80:20 v/v mixture) and centrifuged at 5,000*g* for 15 min. The methanolic extracts were then recovered, pooled and concentrated under vacuum at <40°C until complete solvent removal. The crude extract was reconstituted in DMSO and used for further analyses.

Polyphenolic isolates were standardized to a total soluble phenolic content of 1200 mg gallic acid equivalents/L, corresponding to the total soluble phenolic content of single-strength polyphenolic-enriched açai oil, and equivalent to a 300-fold concentrated açai juice. Total soluble phenolics were analyzed by the Folin-Ciocalteau assay, according to the procedure by Singleton & Rossi (1965) and quantified against a gallic acid standard curve. All polyphenolic isolates were sterile-filtered prior use in cell culture experiments and normalized to a final concentration of 0.1% DMSO when applied to the cells. A control with 0.1% DMSO was included in all assays.

Polyphenolic isolates were analyzed by reversed phase HPLC with a Waters 2695 Alliance system (Waters Corp., Milford, MA), according to previously described chromatographic conditions, detailed in chapter IV. Identification and quantitation of polyphenolics was based on their spectral characteristics and retention time, as compared to authentic standards (Sigma Chemical Co., St. Louis, MO). Compound identities were further confirmed by mass spectrometric analyses, performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA). Separations were conducted using the Phenomenex (Torrace, CA) Synergi 4µHydro-RP 80A (2 x 150 mm; 4 µm; S/N = 106273-106275) with a C18 guard column. Mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in 50:50 methanol and acetonitrile (phase B) run at 0.3 mL/min. Polyphenolics were separated with a gradient elution program in which phase B changed from 5 to 30% in 5 min, from 30 to 65% in 70 min, and from 65 to 95% in 30 min and was held isocratic for 20 min. Ionization was conducted in the negative ion

mode under the following conditions: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.5 kV; capillary temperature, 250 °C; capillary voltage, 1.5 V; tube lens offset, 0 V. Total soluble phenolic contents were determined using the Folin-Ciocalteu assay (described in chapter IV) and quantified in gallic acid equivalents (GAE), used to normalize total phenolic concentrations between açai juice and açai oil extracts. Antioxidant capacity was determined by the oxygen radical absorbance capacity assay using a BGM Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission), as described in chapter IV. Results were quantified in μmol Trolox equivalents (TE) per milliliter of extract.

Cell proliferation studies were conducted in HT-29 human colon adenocarcinoma cells obtained from ATCC (Manassas, VA), and cultured in Dulbecco's modified Eagle's medium (1X) (DMEM) containing 5% fetal bovine serum, 1% non-essential amino acids, 100 units/mL penicillin G, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, and 10 mM sodium pyruvate (Gibco BRL Life Technology, Grand Island, NY). Cells were incubated at 37 °C under 5% CO_2, and utilized between passages 10-20. Uniform amounts ($5x10^4$ cells/well) of cells were inoculated into each well of a 12-well tissue culture plate. After a 24 hr incubation the growth medium was replaced with 1000 μL of media containing different concentrations of polyphenolic extracts (from 0.04 to 12 μg gallic acid equivalents/mL). Following incubation for 48 hr, cell numbers were determined using a Beckham Coulter Particle Counter (Fullerton, CA). Cell numbers were expressed as a percentage of the DMSO control (0.1% DMSO). The extract concentration where cell proliferation was inhibited by 50% (IC_{50}) was

calculated by linear regression analyses on cell inhibition percentages as a ratio to the DMSO control.

Generation of reactive oxygen species (ROS) was determined by the dichlorofluorescein (DCF) assay, performed according to the procedures described by Mertens-Talcott, Bomser, Romero, Talcott, & Percival, 2005. Briefly, cells ($5x10^4$/mL) were passed into 96-well plates and incubated for 24 h. Cells were then washed twice with PBS and preloaded with dichlorofluorescein diacetate (DCFH-DA) substrate by incubating with 10 µmol DCFH-DA for 30 min at 37°C. Cells were subsequently washed and incubated with polyphenolic extracts, adjusted to concentrations ranging from 0.04 to 12 µg gallic acid equivalents/mL. Fluorescence was determined after 30 min incubation with polyphenolics using a BGM Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission).

In-vitro intestinal absorption was assessed using Caco-2 cell monolayer models. Caco-2 colon carcinoma cells obtained from American Type Culture Collection (ATCC, Manassas, VA), and cultured in Dulbecco's modified Eagle's medium (1X) high glucose (DMEM) containing 10% fetal bovine serum, 1% non-essential amino acids, 100 units/mL penicillin G, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate. Cells were incubated at 37 °C and 5% CO_2 (chemicals were obtained from Gibco BRL Life Technology, Grand Island, NY).

Cells between passages 10-20 were seeded in 12-mm transparent polyester cell culture insert well plates (Transwell, Corning Costar Corp., Cambridge, MA) at 1.0 x 10^5 cells per insert with 0.5 mL of medium in the apical side and 1.5 mL of medium in

the basolateral side. Cells were allowed to grow and differentiate to confluent monolayers for 21 days, as previously described (Hidalgo, Raub, & Borchardt, 1989). Transepithelial electrical resistance (TEER) values were monitored with an EndOhm Volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL) and monolayers with TEER values >350 Ω cm^2 after correction for the resistance in control wells were used for transport experiments. TEER values were also obtained at the conclusion of transport experiments to insure integrity of the monolayer.

For transport studies, media pH was adjusted to 6.0 on the apical side and 7.4 on the basolateral side using Hank's balanced salt solution (HBSS, Fischer Scientific, Pittsburgh, PA) containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid solution (MES), and HBSS containing *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] buffer solution (1 M) (HEPES) to create a pH gradient similar to the absorption sites in the small intestine environment (chemicals obtained from Gibco BRL Life Technology, Grand Island, NY). Polyphenolic extract solutions were diluted in HBSS (from 2.4 to 36 µg gallic acid equivalents/mL) and loaded into the apical side of the cells. Transepithelial transport was followed over time and sample aliquots (200 µL) were taken at 30, 60, and 120 min.

Data from in-vitro experiments were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Mean separations were conducted using post-hoc Tukey-Kramer HSD ($p<0.05$) pairwise comparisons. Correlation and linear regression analyses were conducted using a significance level of 0.05.

Results and Discussion

Characterization of polyphenolics present in açai juice and in the phytochemical enriched açai oil used in this study is detailed previously in chapters III and IV. Biological activities of phytochemical-rich extracts from açai oil and non-anthocyanin polyphenolic extracts from clarified açai juice were compared, and their respective chemical compositions were contrasted under identical chromatographic conditions. Similar to their respective original matrices, both extracts contained several phenolic acids, flavonoids, and procyanidins. Phenolic acids such as protocatechuic (*m/z* = 153.2), *p*-hydroxybenzoic (*m/z* = 137.3), vanillic (*m/z* = 167.3), syringic (*m/z*= 196.9), and ferulic (*m/z* = 193.2) acids, and the monomeric flavonols (+)-catechin (*m/z* = 289.2) and (-)-epicatechin (*m/z* = 289.2) were identified based on spectral and mass spectrometric characteristics as compared to authentic standards.

In addition, various procyanidin dimers and trimers were identified based on their distinctive fragmentation patterns and spectral similarities to (+)-catechin and (-)-epicatechin. Procyanidin dimers were characterized by signals at *m/z* 577.1, and major fragmentations to the *m/z* 425.0, typical of the Retro-Diels-Alder product of ring C, and *m/z* 289.2, likely corresponding to a monomeric (+)-catechin or (-)-epicatechin unit resulting from cleavage of the interflavanoid bond, as described in chapter IV. Fragmentations were characteristic of B-type procyanidins, composed exclusively by (+)-catechin and (-)-epicatechin units, although no differentiation between these isomers was possible by mass spectrometric means.

Table 8. Concentration (mg/L) and relative abundance of polyphenolics present in *E. oleracea* juice and oil extracts.

Polyphenolic	*E. oleracea* juice (mg/L)	Relative abundance (%)[1]	*E. oleracea* oil (mg/L)	Relative abundance (%)[1]
protocatechuic acid	159.1 ± 12.3	2.3 ± 0.1 [a]	540.2 ± 29.9	7.8 ± 0.4 [b]
p-hydroxy benzoic acid	172.4 ± 14.1	2.4 ± 0.3 [a]	570.3 ± 30.8	8.2 ± 0.9 [b]
(+)-catechin	491.4 ± 24.9	7.0 ± 0.8 [a]	66.8 ± 5.7	1.0 ± 0.1[b]
vanillic acid	577.1 ± 40.2	8.2 ± 0.5 [a]	1607.8 ± 64.4	23 ± 1.8 [b]
syringic acid	433.7 ± 23.4	6.1 ± 0.7 [a]	969.1 ± 47.1	14 ± 1.1 [a]
(-)-epicatechin	445.9 ± 37.4	6.3 ± 0.2	----	----
ferulic acid	121.9 ± 13.4	1.7 ± 0.3 [a]	109.3 ± 10.1	1.6 ± 0.2 [a]
Procyanidin dimers	2297.2 ± 148	33 ± 2.3 [a]	1028.6 ± 126	15 ± 1.2 [b]
procyanidin trimers	2366.9 ± 185	34 ± 3.1 [a]	2041.1 ± 58.9	29 ± 2.7 [a]

[1] Values with different letters between columns represent a significant difference (paired samples t-test, $p<0.05$).

Procyanidin trimers (*m/z* = 865.1) were additionally characterized by a predominant product ion at *m/z* 577.2, likely corresponding to a dimeric fragment ion from cleavage of interflavanoid linkages, and further fragmentation to ions *m/z* 425.0, and *m/z* 289.2, as previously discussed (chapter IV). Individual polyphenolic concentrations in açai juice and oil extracts are presented in Table 8.

Similar polyphenolic profiles were observed in the açai oil extracts and non-anthocyanin polyphenolic extracts of açai juice (Fig. 11); however, their absolute and relative ratios differed markedly. Individual phenolic acid concentrations were generally higher (by up to 3.4 fold) in açai oil extracts (540 to 1607 mg/L) than in juice extracts (159 to 577 mg/L), with the exception of ferulic acid (109 and 122 mg/L respectively). In contrast, flavanols such as (+)-catechin and (-)-epicatechin were abundantly present in juice extracts (492 and 446 mg/L respectively), yet no (-)-epicatechin and only limited (+)-catechin concentrations (67 mg/L) was detected in extracts from açai oil. Similarly, procyanidin dimer concentrations in açai juice extracts (2,297 mg (+)-catechin equivalents/L) doubled those present in oil extracts (1,029 mg (+)-catechin equivalents/L), yet equivalent concentrations of procyanidin trimers were present in both extracts (2,041-2,367 mg (+)-catechin equivalents/L).

Differences in composition among extracts were attributed not only to variations in polyphenolic extraction protocols, due to the different nature of the matrices (aqueous and oil), but also to initial differences in composition between extracts.

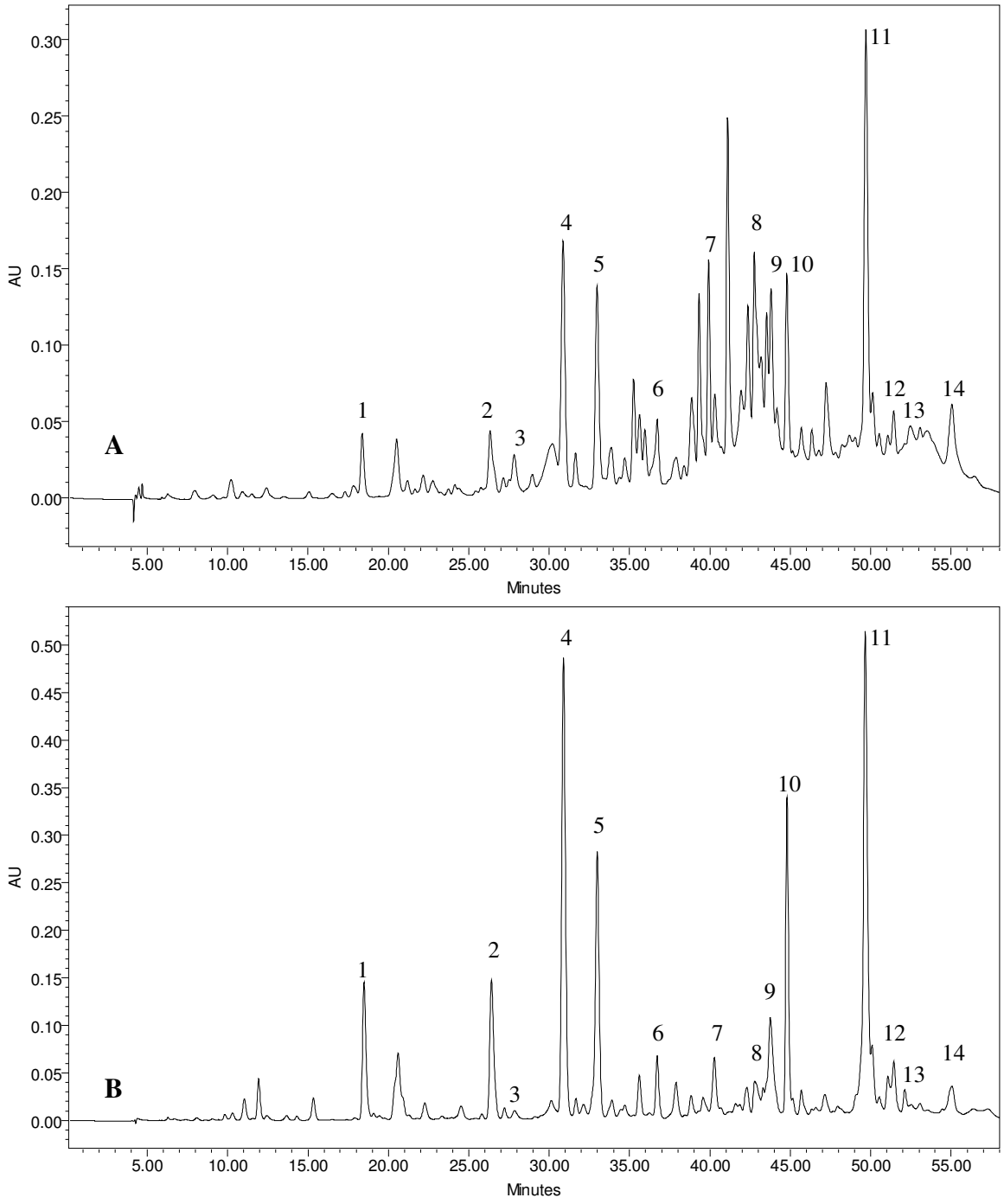


Fig. 11. HPLC chromatogram of polyphenolics in phytochemical-rich extracts from açai juice (A) and açai oil (B). Peak assignments: 1. protocatechuic acid; 2. *p*-hydroxybenzoic acid; 3. (+)-catechin; 4. vanillic acid; 5. syringic acid; 6-7. procyanidin dimers; 8. ferulic acid; 9-10 procyanidin dimers; 11-14 procyanidin trimers.

As previously shown in chapter IV, individual polyphenolics were significantly higher in açai oil than in açai juice by a factor ranging from 12.6 to 469.4 fold, owing to the selective, enhanced extraction of compounds from water-insoluble residues of açai pulp. Due to these initial differences in total polyphenolic concentrations between açai juice and the phytochemical enriched oil, both phytochemical extracts were normalized to an equivalent concentration of total soluble phenolics (1200 mg gallic acid equivalents/L) for their subsequent use in cell culture experiments. Such polyphenolic concentration was comparable to that originally present in the oil (single strength) and was also equivalent to 300 times the total soluble phenolic content of açai juice, following anthocyanin extraction. Thus, non-anthocyanin polyphenolic contents were originally 300-fold higher in the phytochemical enriched açai oil than in açai juice, which must be considered when making inferences from in-vitro biological activity outcomes. Resultant antioxidant capacity of both polyphenolic extracts following the normalization was 17.2 ± 0.16 µmol TE/mL for the açai juice extract and 15.3 ± 0.11 µmol TE/mL for the açai oil extract.

The antiproliferative activities of açai juice and oil extracts were evaluated in a cell culture model using HT-29 colon carcinoma cells. Total cell numbers were indicative of the proliferative activity of HT-29 cells and the corresponding cytotoxic effects of each extract. Both polyphenolic extracts caused significant ($p<0.01$) decreases in total cell numbers in a concentration-dependent manner (Fig. 12). However, polyphenolic extracts from açai oil were more than twice as effective in reducing total cell numbers across all dilutions, with an IC_{50} of 4.5 µg GAE/mL compared to 10.2 µg

GAE/mL for açai juice extracts. Previous investigations have reported synergistic effects among polyphenolics when present together using in vitro cell culture models (Katsube, Iwashita, Tsushida, Yamaki, & Kobori, 2003; Chang, Huang, Hsu, Yang, & Wang, 2005; Mertens-Talcott & Percival, 2005), and interactions among phenolic acids and flavanols are also hypothesized to play an important role in the inhibitory action of açai oil and juice extracts on cell proliferation observed in this study.

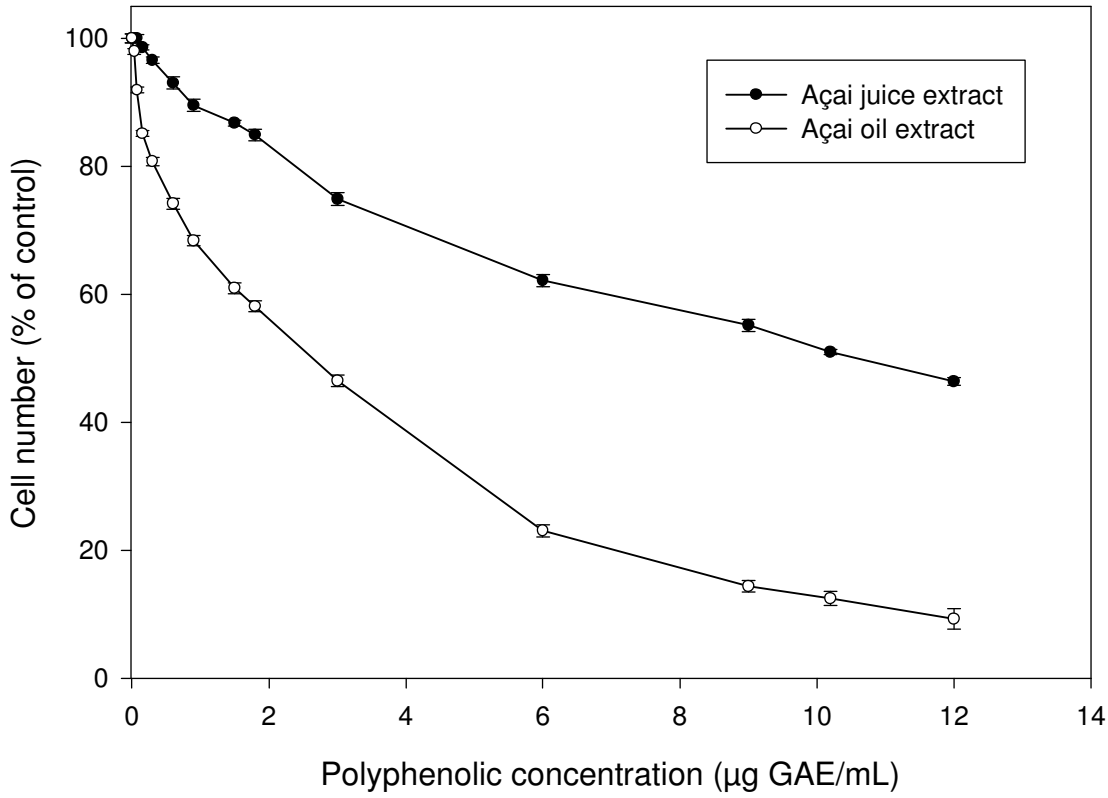


Fig. 12. Percent changes in total HT-29 cell numbers expressed as a ratio to control cells following treatment with juice or oil polyphenolic extracts for 48 h. Error bars represent the standard error of the mean (n=6).

Variations on the inhibitory effects by açai juice and oil extracts may be associated to their respective differences in polyphenolic composition. As observed earlier (Table 8), açai oil extracts were characterized by 3.4-fold higher phenolic acid, and 2 to 14-fold lower monomeric and dimeric flavanol concentrations than its juice counterparts. Thus, results from this study not only suggest a potential role of phenolic acids on cell growth inhibition, but also indicate that interactions with other polyphenolic components may play a role in the overall antiproliferative activity of complex phytochemical extracts. Previous studies on HT-29 cell models have suggested non-additive interactions involving flavanol derivatives (Seeram, Adams, Hardy, & Heber, 2004; Ferguson, Kurowska, Freeman, Chambers, & Koropatnick, 2004), while potent inhibitory effects of cloudberry, bilberry, raspberry, black currant, strawberry, and lingonberry phytochemical extracts have been attributed to synergistic interactions among non-anthocyanin polyphenolics, including phenolic acids and flavanols, and their potentiating effect on the expression of $p21^{WAF1}$, a key inhibitor of cell cycle arrest and apoptotic signaling pathways (Wu, Koponen, Mykkanen, & Torronen, 2007). However, the nature of such non-additive interactions among polyphenolics is still not clear and potential reaction mechanisms have yet to be elucidated.

Cell growth inhibition achieved by açai polyphenolic-rich extracts was related to soluble phenolic concentrations originally present in açai juice (4 µg GAE/mL) and in the phytochemical enriched açai oil (1200 µg GAE/mL), aimed to establish minimum inhibitory concentrations for both açai products. Thus, 10 µg GAE/mL of açai juice extract resulted in 50% reduction of cell proliferation while a similar reduction was

achieved by 3 µg GAE/mL of açai oil extract (Fig. 12). These concentrations may be directly related to the total polyphenolic amounts responsible for the observed inhibition, if the total volume (1 mL) used in cell culture studies is considered. Thus, 10 µg GAE of açai juice extract would correspond to the phenolic amount present in 2.5 mL of açai juice while 3 µg GAE of açai oil extract would be equivalent to the polyphenolic content of 2.5 µL of açai oil; both of which were equivalent in terms of their inhibitory effects on cell proliferation. These observations suggest polyphenolic-enriched açai oil was 1000 times more effective than non-anthocyanin açai juice polyphenolics for inhibition of colon carcinoma cell proliferation when present at equal concentrations. Thus, it would be necessary to consume 1000 mL of açai juice to equate the effects of 1 mL of açai oil, when only non-anthocyanin polyphenolics are considered.

Moreover, when effective açai juice and oil amounts (2.5 mL and 2.5 µL respectively) are related to their respective antioxidant activities (15.3 µmol TE/mL açai oil and 17.2 µmol TE/mL açai juice), major differences are observed in terms of their minimum inhibitory concentration in terms of antioxidant contents. Açai oil present in amounts (2.5 µL) equivalent to very low antioxidant activities (0.04 µmol TE) appeared to be as affective as higher amounts of açai juice (2.5 mL), equivalent to nearly 1000 times higher antioxidant activities (38.2 µmol TE). Therefore, while in-vitro antioxidant activity of polyphenolic compounds has been generally associated to their potential health benefits in-vivo (Hou, 2003), results from this study suggest the existence of additional non-antioxidant mechanisms by which polyphenolics, particularly phenolic acids and flavonols, inhibit cancer cell proliferation. According to Nichenametla,

Taruscio, Barney, & Exon (2006), potential non-antioxidant mechanisms for chemopreventive activity of phenolic acids and flavonols include inhibition of carcinogen formation or activation, deactivation or detoxification of the carcinogen, prevention of carcinogen binding to DNA, and enhancement of DNA repair levels.

The generation of ROS was evaluated by the DCF assay, and was conducted to assess the role of ROS generation on the cytotoxic effects of açai polyphenolic extracts in HT-29 cells. Both açai extracts induced a significant increase on the generation of reactive oxygen species in a concentration-dependent manner (Fig. 13).

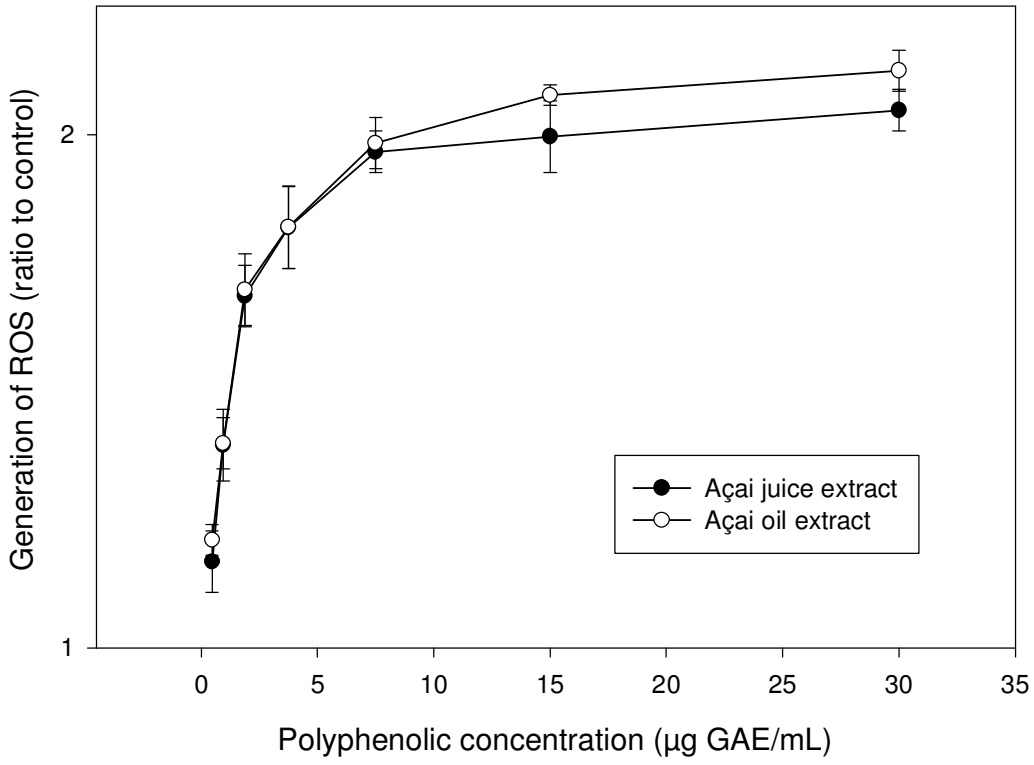


Fig. 13. Intracellular levels of ROS in HT-29 cells following treatment with açai juice or oil polyphenolic extracts. Error bars represent the standard error of the mean (n=6).

No significant differences between açai juice or oil polyphenolic extracts were detected at any of the concentrations tested, while both extracts induced the generation of ROS at concentrations between 0.4 and 30 µg GAE/mL. However, their inductive effect was more pronounced at lower concentrations (<5 µg GAE/mL), and decreased markedly above 7.5 µg GAE/mL. Similarly, low polyphenolic concentrations (<5 µg GAE/mL) of açai juice or oil extracts were effective at increasing average ROS generation rates (Fig. 14).

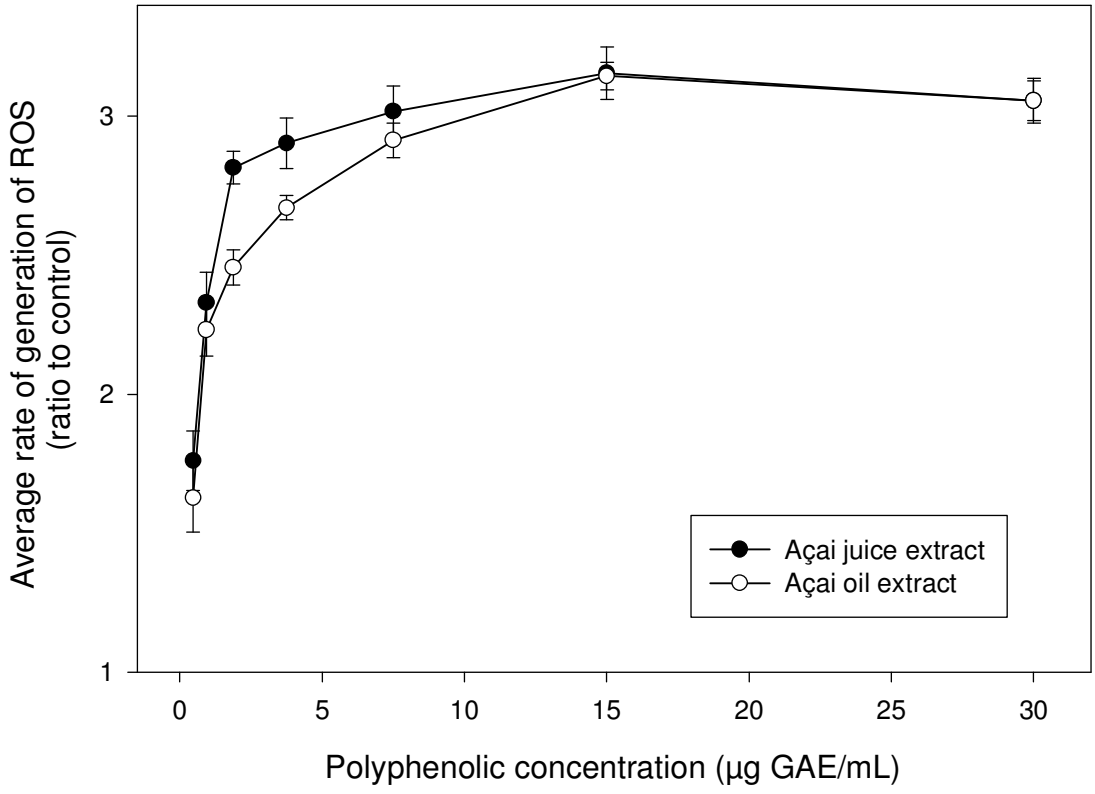


Fig. 14. Intracellular rate of generation of ROS in HT-29 cells following treatment with açai juice or oil polyphenolic extracts. Error bars represent the standard error of the mean (n=6).

Decreased generation of ROS by higher polyphenolic extract concentrations may be at least partially attributed to the presence of polyphenolics in sufficient amounts to reduce the generation of reactive oxygen species through their antioxidant potential; however, due to the complex nature of these reactions, their influence can only be hypothesized. Moreover, previously observed differences between açai juice and oil extracts in terms of their inhibitory effects in HT-29 cell proliferation, do not appear to be explained by their similarities on the rate and degree of generation of ROS, suggesting that induction of reactive oxygen species is likely not the main mechanism responsible for the previously observed cell growth inhibition in HT-29 cells.

Transport of polyphenolics from açai juice and oil extracts was evaluated using Caco-2 cell monolayers as an in vitro intestinal absorption model. Caco-2 cells have been the most extensively characterized and functional model in the field of drug absorption and permeability (Balimane, Chong, & Morrison, 2000) and have been previously used for evaluating intestinal absorption and transport of various phenolic acids (Kobayashi et al., 2000; Konishi et al., 2003), flavonoids (Walgren et al., 1998; Vaidyanathan & Walle, 2001), and procyanidins (Deprez et al., 2001). Transport of polyphenolics across the Caco-2 monolayers was studied in the apical to basolateral direction. Polyphenolic extracts were loaded into the apical side of the cell monolayers and individual polyphenolic concentrations appearing in the basolateral side were evaluated over time, after 0.5, 1, and 2 h incubation. Analytical HPLC chromatograms of polyphenolics present in the basolateral solutions after 2h incubation are presented in Fig. 15.

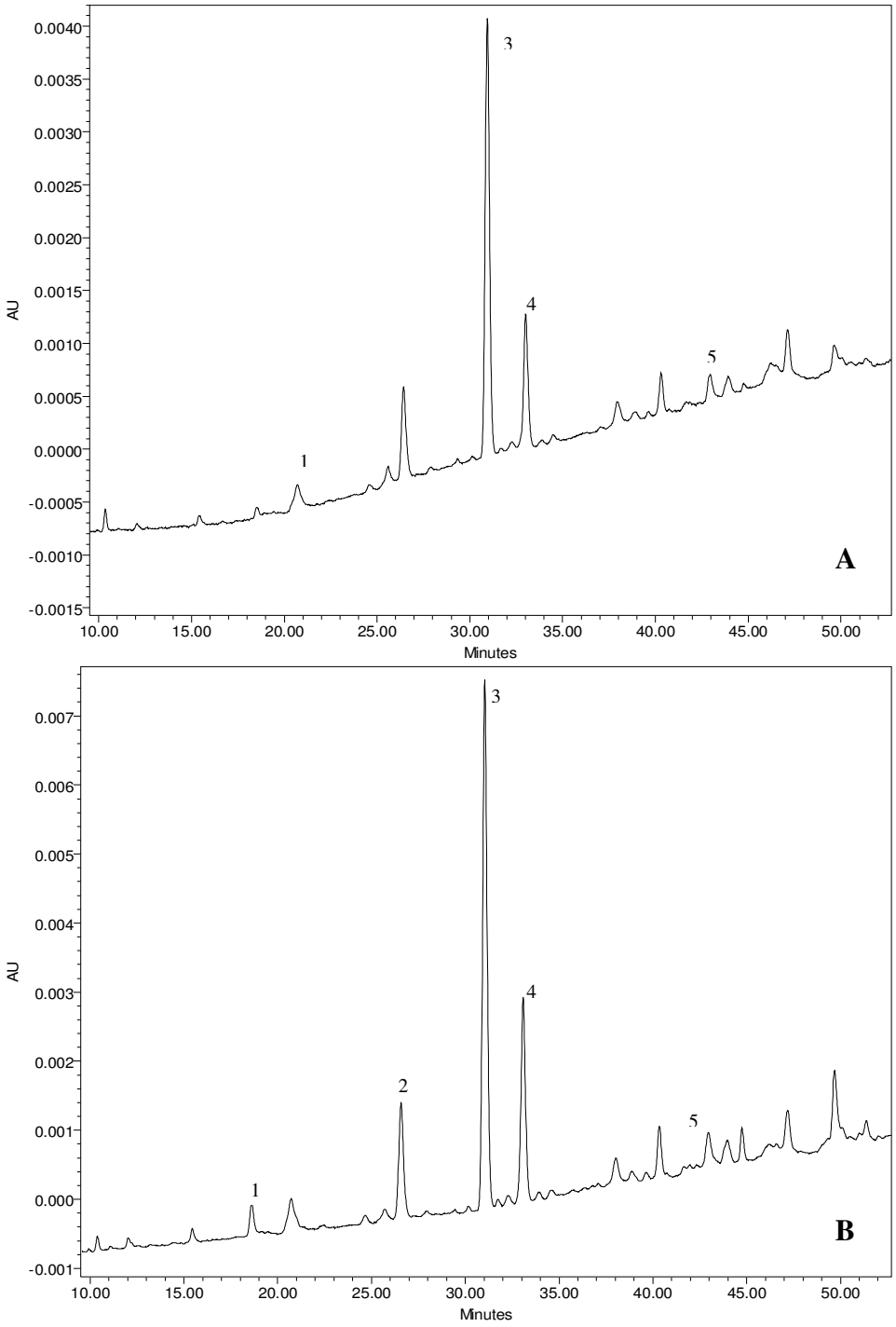


Fig. 15. Typical HPLC chromatogram of polyphenolics present in the basolateral side of Caco-2 cell monolayers following incubation with açai juice (A) or oil (B) polyphenolic extracts for 2h. Peak assignments: 1. protocatechuic acid; 2. *p*-hydroxybenzoic acid; 3. vanillic acid; 4. syringic acid; 5. ferulic acid.

Table 9. Average transport rates of polyphenolics from açai juice and oil extracts from the apical to the basolateral side of Caco-2 cell monolayers.

Polyphenolic	Transport (µg/mL·h) of polyphenolics from açai juice					Transport (µg/mL·h) of polyphenolics from açai oil				
	12 µg[1,2]	**30 µg**	**60 µg**	**120 µg**	**180 µg**	**12 µg[1,2]**	**30 µg**	**60 µg**	**120 µg**	**180 µg**
p-hydroxy benzoic acid	0.020 [a]	0.236 [c]	0.355 [d]	0.495 [e]	0.730 [f]	0.060 [b]	0.771 [f]	1.774 [g]	3.416 [h]	4.038 [h]
vanillic acid	0.047 [a]	0.500 [c]	0.635 [c]	1.286 [d]	1.962 [e]	0.109 [b]	2.066 [e]	5.750 [f]	8.228 [g]	10.42 [h]
syringic acid	0.030 [a]	0.161 [b]	0.276 [c]	0.572 [d]	0.763 [e]	0.132 [b]	0.617 [d]	1.438 [f]	4.911 [g]	7.385 [h]
ferulic acid	---	0.027 [a]	0.100 [c]	0.140 [d]	0.152 [d]	---	---	0.021 [a]	0.065 [b]	0.112 [c]
(+)-catechin	0.022 [a]	0.267 [d]	0.289 [d]	0.503 [e]	0.913 [f]	---	---	0.019 [a]	0.044 [b]	0.091 [c]
(-)-epicatechin	0.034 [a]	0.355 [b]	0.372 [b]	0.660 [c]	1.050 [d]	---	---	---	---	---

[1] Total soluble phenolic contents (µg gallic acid equivalents), which represent the absolute polyphenolic amount loaded into the apical side of cell monolayers. These amounts are equivalent to 3, 7.5, 15, 30, and 45 mL of juice and to 10, 25, 50, 100, and 150 µL of oil, respectively.[2] Values with different letters within rows are significantly different (LSD test, $p<0.05$)

The phytochemical composition of each açai extract (Fig. 1) and their corresponding polyphenolic concentrations (Table 8) were previously discussed. Initial observations indicated that phenolic acids such as *p*-hydroxy benzoic, vanillic, syringic, and ferulic acids can be transported from the apical to the basolateral side of Caco-2 cell monolayers, along with monomeric flavanols such as (+)-catechin and (-)-epicatechin, when present in complex polyphenolic mixtures, such as those employed herein. Average transport rates (µg/mL·h) of polyphenolics from açai juice and oil extracts adjusted to different concentrations (µg gallic acid equivalents/mL), from the apical to the basolateral side of Caco-2 cell monolayers were monitored over time (Table 9).

Individual polyphenolic transport rates (0.02 to 10.4 µg/mL·h) increased in a concentration-dependent (24-360 µg GAE/mL) manner for both extracts; however, absolute phenolic acid transport rates were significantly higher ($p<0.05$) for açai oil extracts than for their juice counterparts at equivalent total phenolic concentrations while the opposite was true for (+)-catechin and (-)-epicatechin. These results are in good agreement with the corresponding polyphenolic profiles of açai juice and oil extracts, since phenolic acid concentrations in açai oil extracts were up to 3.4-fold higher than in açai juice extracts while the latter contained 14-fold higher flavanol concentrations than their oil equivalents.

Variations on individual polyphenolic transport rates were minor at low phenolic extract concentrations (~24 µg/mL·h); however, transport efficiency of vanillic and syringic acids was enhanced at higher extract concentrations. Higher transport efficiency of these phenolic acids might be related to their methylated structures, which have

shown to enhance the transport of anthocyanins (Yi et al., 2006) and other flavonoids (Ollila, Halling, Vuorela, Vuorela, & Slotte, 2002; Tammela et al., 2004) in similar Caco-2 cell models. Thus, a higher number of methyl groups has shown to increase polyphenolic transport through cell monolayers, while free hydroxyl groups have been associated with longer retention delays in membranes, likely due to hydrogen-bond formation between phenolic hydroxyl groups and polar groups of the lipid molecules at the lipid/water interface (Ollila et al., 2002).

Moreover, methoxylated phenolic derivatives have been found to exert a potent inhibitory effect on the fluorescein transport in Caco-2 monolayers, which is indicative of a higher structural affinity to the monocarboxylic acid transporter (MCT), previously shown to play a role in the active transport of several phenolic acids (Konishi et al., 2003; Konishi & Shimizu, 2003*)*. Flavanols, on the other hand, have been shown to be transported mainly via paracellular diffusion (Konishi et al., 2003); therefore, higher (+)-catechin and (-)-epicatechin transport rates in cells loaded with juice polyphenolic extracts are likely associated to their respective higher concentrations initially present in juice extracts when compared to their oil counterparts.

Relative transport efficiency of açai polyphenolics from juice and oil extracts by the end of the incubation period (2h) is summarized in Table 10. Transport efficiencies were expressed as the percentage of the initial polyphenolic concentration (loaded in the apical side) detected on the basolateral side of Caco-2 cell monolayers following incubation for 2h.

Table 10. Transport efficiency (%) of polyphenolics from açai juice and oil extracts, from the apical to the basolateral side of Caco-2 cell monolayers following incubation for 2h at 37°C.

Polyphenolic	% Transport efficiency of polyphenolics from açai juice					% Transport efficiency of polyphenolics from açai oil				
	12 µg[1,2]	30 µg	60 µg	120 µg	180 µg	12 µg	30 µg	60 µg	120 µg	180 µg
p-hydroxybenzoic acid	1.51 [a]	1.94 [b]	2.01 [b]	1.98 [b]	2.04 [b]	1.95 [b]	5.67 [c]	6.85 [d]	6.44 [d]	6.45 [d]
vanillic acid	1.07 [a]	1.13 [a]	1.10 [a]	1.11 [a]	1.13 [a]	1.14 [a]	5.18 [b]	6.31 [c]	8.11 [d]	7.91 [d]
syringic acid	0.55 [a]	0.61 [a]	0.64 [a]	0.63 [a]	0.62 [a]	1.02 [b]	2.47 [c]	3.21 [d]	5.51 [e]	5.50 [e]
ferulic acid	---	0.21 [b]	0.52 [c]	0.62 [c]	0.59 [c]	---	---	0.11 [a]	0.10 [a]	0.13 [a]
(+)-catechin	0.16 [a]	0.67 [b]	0.69 [b]	0.72 [b]	0.69 [b]	---	---	0.11 [a]	0.14 [a]	0.15 [a]
(-)-epicatechin	0.18 [a]	0.91 [b]	1.06 [b]	1.15 [b]	1.01 [b]	---	---	---	---	---

[1] Total soluble phenolic contents (µg gallic acid equivalents), which represent the absolute polyphenolic amount loaded into the apical side of cell monolayers. These amounts are equivalent to 3, 7.5, 15, 30, and 45 mL of juice and to 10, 25, 50, 100, and 150 µL of oil, respectively. [2] Values with different letters within rows are significantly different (LSD test, $p<0.05$).

Contrary to previous observations on polyphenolic transport rates, relative transport efficiencies of individual polyphenolics did not present systematic variations as a function of initial polyphenolic concentrations. In fact, transport efficiencies for *p*-hydroxy benzoic (~2.0%), vanillic (~1.0%), and syringic (~0.6%) acids were not affected ($p<0.05$) by initial polyphenolic concentrations in açai juice extracts while the same was true for ferulic acid (~0.1%) and (+)-catechin (~0.1%) in açai oil extracts. Similarly, equal transport efficiencies for ferulic acid, (+)-catechin, and (-)-epicatechin were observed at initial juice extract polyphenolic concentrations above 60 µg/mL. However, transport of phenolic acids such as *p*-hydroxy benzoic, vanillic, and syringic from açai oil extracts increased proportionally to the amount originally loaded into the apical compartment, up to a certain concentration (<240 µg/mL), after which no further changes in transport efficiency were observed. This effect might be partially attributed to the presence of concentrated amounts of other polyphenolic components at higher extract concentrations, which may interfere with both active and passive absorption mechanisms (Konishi et al., 2003; Yi et al., 2006).

Conclusion

Results from this study suggest polyphenolic extracts from açai juice and from a recently characterized phytochemical enriched açai oil are rich sources of phenolic acids, including vanillic, syringic, *p*-hydroxy benzoic, protocatechuic, and ferulic acid, and flavan-3-ols such as (+)-catechin, (-)-epicatechin, and procyanidin dimers and trimers. Both polyphenolic extracts were found to significantly inhibit cell proliferation and

increase the generation of reactive oxygen species (ROS) in a concentration-dependent manner. Additional to the generation of ROS, further cytotoxic mechanisms are likely responsible for the potent antiproliferative effects of açai extracts on HT-29 colon cancer cells. In addition, the bioavailability of polyphenolic compounds present in açai extracts were evaluated using Caco-2 monolayers as a model for intestinal absorption. It was demonstrated that polyphenolic mixtures containing phenolic acids such as *p*-hydroxy benzoic, vanillic, syringic, and ferulic acids, and monomeric flavanols such as (+)-catechin and (-)-epicatechin can be transported from the apical to the basolateral side of Caco-2 cell monolayers. Results from this study provide further evidence on the antiproliferative properties of açai polyphenolics in cancer cells and offer new information on their bioavailability.

CHAPTER VI

CHEMICAL STABILITY OF AÇAI ANTHOCYANINS AS INFLUENCED BY NATURAL AND ADDED POLYPHENOLIC COFACTORS IN MODEL JUICE SYSTEMS

Introduction

Anthocyanins have been categorized as the most important group of water-soluble pigments in plants, and are responsible for most blue, red, and related colors in flowers and fruits (Clifford, 2000). Anthocyanin color is an important sensory characteristic and often a major quality parameter for a variety of fruit products. Açai (*Euterpe oleracea* Mart.), a palm fruit native to the Brazilian Amazon, has been the focus of increased international attention as a functional ingredient (Del Pozo, Brenes, & Talcott, 2004; Schauss et al., 2006) and is a potential rich source of anthocyanins (Gallori et al., 2004; Lichtenthaler et al., 2005; Pacheco-Palencia et al., 2007a; Pacheco-Palencia et al., 2007b). Two predominant anthocyanins, cyanidin-3-rutinoside and cyanidin-3-glucoside, are responsible for most of its characteristic dark purple color, and are often a major source of color in açai-containing juices and beverages (Schauss et al., 2006; Lichtenthaler et al., 2005; Pacheco-Palencia et al., 2007a). However, anthocyanins are highly reactive and generally experience extensive degradation during long-term storage, leading to dark, dull, brown hues (Jurd, 1972).

Anthocyanin color changes are known to be influenced by several factors, including pH, temperature, light, and the presence of enzymes, sugars, metals, and

phenolic cofactors (Markakis, 1982). Among these, the presence of non-anthocyanin polyphenolics may significantly affect anthocyanin color, as they participate in copigmentation reactions in enhanced color and increased stability during storage (Singleton, 1972). Intermolecular copigmentation reactions are common in nature and occur when colorless phenolic cofactors are attracted to anthocyanins via weak hydrophobic forces (Mazza & Brouillard, 1990). Anthocyanin copigmentation reactions are detected by both a hyperchromic shift, where absorbance at the λ_{max} of the absorption spectrum increases, and by a bathochromic shift, where a change toward higher wavelengths (nm) at the λ_{max} of the absorption spectrum occurs (Baranac, Petranovic, & Dimitric-Markovic, 1996; Malien-Aubert, Dangles, & Amiot, 2001).

Previous studies have evaluated the use of externally added polyphenolic cofactors, particularly phenolic acids, to enhance and stabilize anthocyanin color in berry juices (Eiro & Heinonen, 2002). External addition of commercial polyphenolic cofactors from rosemary (*Rosmarinus officinalis*) has also shown to result in enhanced anthocyanin color and increased stability in grape juices (Talcott, Brenes, Pires, & Del Pozo-Insfran, 2003; Brenes, Del Pozo-Insfran, & Talcott, 2005). Thus, the natural occurrence and predominant presence of certain groups of polyphenolic cofactors in açai fruit, including phenolic acids, flavone-*C*-glycosides, and procyanidins (Gallori et al., 2004; Pacheco-Palencia et al., 2007b; Schauss et al., 2006), may play a significant role in the structural and pigment stability of anthocyanins in açai fruit juices.

The role of flavone-*C*-glycosides is of particular interest, as their presence in other anthocyanin-rich fruits has not been previously reported. Moreover, the potential use of

flavone-*C*-glycoside-rich extracts as anthocyanin color enhancers and stabilizing agents has not been evaluated. Extracts rich in flavone-*C*-glycosides may be obtained from various botanical sources, among which, rooibos (*Aspalathus linearis)* tea, a leguminous shrub native to South Africa, is among the highest, most widely available, and easily extractable sources (Krafczyk & Glomb, 2008). This study was conducted to evaluate the influence of different groups of naturally occurring polyphenolic cofactors on the stability of anthocyanins in açai fruit juice models stored under different pH (3.0, 3.5, and 4.0) and temperature (5, 20, and 30°C) conditions and assess the influence of externally added polyphenolic cofactors from rooibos tea, rich in flavone-*C*-glycosides, and from commercial rosemary extracts on the stability of açai juice models. Relations between anthocyanin degradation and sulfite bleaching resistance, reducing capacity, and antioxidant activity were additionally determined.

Materials and Methods

Polyphenolic cofactors were isolated from various açai fruit products prior use in juice models. Phenolic acids and procyanidins were extracted from a previously characterized, polyphenolic-enriched açai oil (chapter IV), which contained concentrated amounts of phenolic acids and procyanidins originally present in açai fruit pulp. Açai oil was extracted from a semi-solid filter cake obtained from Bossa Nova Beverage Group (Los Angeles, CA), commercially used for açai pulp clarification, using a hydro-alcoholic solution, as described in a patent-pending process (Talcott et al., 2007). Following solvent removal, the resultant oil was kept frozen (-20°C) until use. Açai oil

was extensively extracted with an equal volume of a 1:1 (v/v) hexane:methanol solution until complete dissolution. A known volume of 0.1 M aqueous citric acid buffer at pH 3.0 was added to the mixture, to form a bi-layer, from which the hydrophilic phase was retained. Residual methanol in the hydrophilic phase was evaporated under reduced pressure (<40°C) and the resulting aqueous solution was recovered, adjusted to pH 7.0, and loaded onto C18 Sep-Pak Vac 20 cm³ mini-columns (Waters Corporation, Milford, MA). Phenolic acids were eluted in the unbound fraction, while procyanidins were eluted with acidified methanol (0.01% HCl). The aqueous, phenolic acid-containing fraction was then acidified to pH 3.0, loaded onto a second Sep-Pak column, and eluted with acidified methanol (0.01% HCl). Methanol from each extraction was then evaporated under vacuum (<40°C) and each fraction was re-dissolved in a known volume of the citric acid buffer.

In a second extraction, anthocyanins and naturally occurring flavone-*C*-glycosides in açai fruit were extracted from clarified açai pulp (Bossa Nova Beverage Group, Los Angeles, CA). Clarified açai pulp was likewise loaded onto Sep-Pak mini-columns (Waters Corporation, Milford, MA), and sequentially eluted with acidified water (0.01% HCl) to remove metals, sugars and other water soluble components, followed by ethyl acetate to remove phenolic acids, and finally acidified methanol (0.01% HCl), to elute both anthocyanins and flavone-*C*-glycosides. Following solvent evaporation and re-dissolution in the citric acid buffer, the methanolic fraction was loaded onto manually packed, lipophilic Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO) 40 cm³ mini-columns, previously equilibrated with water. Columns were washed

with 30% aqueous methanol (v/v) to elute anthocyanins, followed by 60% aqueous methanol (v/v) to elute flavone-*C*-glycosides, and with 100% methanol to elute procyanidins. These procyanidins were combined with the procyanidin fraction previously obtained from açai oil. All solvents were finally evaporated under reduced pressure (<40°C) and each resulting isolate was redissolved in the citric acid buffer (pH 3.0).

Açai fruit models were created based on the predominant anthocyanins present in the fruit (cyanidin-3-rutinoside and cyanidin-3-glucoside), adjusted to a total concentration ~500 mg/L in all models. Four different anthocyanin-based models were prepared by combining anthocyanins with a major group of naturally occurring polyphenolic cofactors in açai fruit, which included: phenolic acids, procyanidins, flavone-*C*-glycosides, and an anthocyanin isolate from açai fruit as a control. Cofactor concentrations on each model were adjusted according to the original ratio of total phenolic cofactors to anthocyanins (1:10) naturally present in 100% clarified açai pulp. Each model was adjusted to three different pH levels (3.0, 3.5, and 4.0) and equal amounts of each anthocyanin model were loaded into screw-cap glass test tubes in triplicate. Treatments were stored in the dark at 5, 20, and 30°C for up to 60 days. Sodium azide (50 mg/L) was added to all treatments to prevent microbial growth during storage. Individual tubes were removed from storage at predetermined time intervals and held at -20°C until analysis.

Additional anthocyanin-based models containing externally added polyphenolic cofactors were prepared by copigmenting anthocyanin isolates from açai fruit (~500

mg/L) with a rooibos tea extract, rich in flavone-*C*-glycosides, or with a commercial rosemary extract (ColorEnhance, 3.5% rosmarinic acid, Naturex, South Hackensack, NJ), and compared to an uncopigmented anthocyanin control. Rooibos tea extracts were obtained from rooibos tea, prepared by brewing 50 g of loose rooibos tea obtained from a local market in 50 mL boiling water for 30 min. Both rooibos tea and commercial rosemary extracts were subsequently diluted in citric acid buffer (0.1 M, pH 3.0) and further purified using Sep-Pak columns. Sugars, organic acids, and other water soluble components were removed with water, and polyphenolic components were recovered with ethyl acetate. Following solvent removal, compounds were redissolved in a known volume of citric acid buffer (0.1 M, pH 3.0), adjusted to equal soluble phenolic contents (10,000 mg gallic acid equivalents/L, corresponding to single-strength rosemary extract) and added to anthocyanin isolates to contain final concentrations of 0.2% v/v, based on previous reports using similar rosemary extracts (Talcott et al., 2003; Brenes et al., 2005). All models were finally adjusted to pH 3.0, 3.5, or 4.0, loaded into screw-cap glass tubes in triplicate, and stored at 30°C for up to 30 days. Sodium azide (50 mg/L) was added to all treatments to retard microbial growth and pH was measured every other day to confirm its consistency during storage. Individual tubes were removed from storage every 3 days and held at -20°C until analysis.

Polyphenolic compounds were analyzed by reversed phase HPLC with a Waters 2695 Alliance system (Waters Corporation, Milford, MA), using previously described chromatographic conditions (Pacheco-Palencia et al., 2007a). Polyphenolic identification and quantification was based on spectral properties, retention time, and comparison to

authentic standards. Phenolic acid, flavonol, and anthocyanin standards were obtained from Sigma Chemical Company (St. Louis, MO), and flavone-*C*-glycoside standards were obtained from Indofine Chemical Company (Hillsborough, NJ). Mass spectrometric analyses were additionally performed on a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (ThermoFisher, San Jose, CA). Separations were conducted using a Dionex (Dionex Corporation, Sunnyvale, CA), Acclaim 120 column (4.6 x 250 mm; 5 µm) with a C18 guard column. Mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in methanol (phase B) run at 0.30 mL/min. A gradient elution program in which phase B changed from 5 to 30% in 10 min, from 30 to 65% in 70 min, and from 65 to 95% in 30 min was used and held isocratic for 20 min prior each injection. Electrospray ionization in the negative ion mode was conducted under the following conditions: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 5 units/min; spray voltage, 3.5 kV; capillary temperature, 250°C; capillary voltage, 1.5 V; tube lens offset, 0 V.

Total anthocyanin contents were determined spectrophotometrically at 520 nm, according to the pH differential method described by Wrolstad, Durst, & Lee (2005) and quantified using equivalents of cyanidin-3-glucoside (mg/L) with a molar extinction coefficient of 29,600 (Jurd & Asen, 1966). Anthocyanin sulfite bleaching resistance was also determined spectrophotometrically, based on color retention in the presence of sodium sulfite (Rodriguez-Saona et al., 1999). Total soluble phenolic contents were estimated from the total metal reducing capacity of each model in the Folin-Ciocalteu assay, as described in chapter IV, and quantified in gallic acid equivalents (GAE).

Antioxidant capacity was determined by the oxygen radical absorbance capacity assay (ORAC) described in chapter III, using fluorescein as the fluorescent probe, and measured on a BMG Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission). Results were quantified and expressed as μmol Trolox equivalents (TE) per milliliter.

A 4 x 3 x 3 factorial design that included four anthocyanin-based models, three pH levels, and three storage temperatures was employed. Data from each chemical analysis was analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL) and mean separations were conducted using Tukey-Kramer HSD ($p<0.05$) as a post-hoc analysis. Linear regression analyses and parametric correlations were conducted using a 0.05 significance level.

Results and Discussion

Anthocyanins, phenolic acids, procyanidins, and flavone-*C*-glycosides present in açai juice models were characterized based on their spectral characteristics and mass spectrometric fragmentation patterns and quantified against authentic standards when available (Table 11). All models were adjusted to equal cyanidin-3-glucoside and cyanidin-3-rutinoside concentrations (519 ± 48 mg/L, Table 11). In addition, a particular group of naturally occurring phenolic cofactors in açai fruit was included on each model: phenolic acids (protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, and ferulic acids), procyanidins (dimers and trimers), and flavone-*C*-glycosides, including luteolin 6-*C*-glucoside (isoorientin), luteolin 8-*C*-glucoside (orientin), apigenin-6-C-glucoside

(isovitexin), and two additional flavone *C*-glycosides, tentatively identified as apigenin 6,8-di-*C*-glycosides (Fig. 16). Phenolic cofactor concentrations were adjusted (48.9 to 56.1 mg/L, Table 11) to yield a phenolic cofactor-to-anthocyanin ratio of 1:10, corresponding to the ratio of total phenolic cofactors to anthocyanins originally present in clarified açai pulps (Pacheco-Palencia et al., 2007a; Pacheco-Palencia et al., 2008).

Table 11. Polyphenolic composition of *E. oleracea* juice models.

Juice model	Polyphenolic	$[M-H]^-$ (*m/z*)	Concentration (mg/L)
All anthocyanin models	cyanidin-3-glucoside	447.0	208 ± 18
	cyanidin-3-rutinoside	593.1	311 ± 27
	Total anthocyanins		***519 ± 48***
Anthocyanin-phenolic acid models	protocatechuic acid	153.1	6.15 ± 0.7
	p-hydroxy benzoic acid	137.2	7.74 ± 0.6
	vanillic acid	167.1	19.5 ± 1.3
	syringic acid	197.0	13.8 ± 1.1
	ferulic acid	193.1	1.67 ± 0.2
	Total phenolic acids		***48.9 ± 4.1***
Anthocyanin-flavone-*C*-glycoside models	apigenin 6-*C*-glucosyl-8-*C*-arabinoside (schaftoside)	563.1	5.84 ± 0.3 [b]
	apigenin 6,8-di-*C*-glucoside (vicenin-2)	593.5	5.70 ± 0.4 [b]
	luteolin 6-*C*-glucoside (isoorientin)	447.1	16.4 ± 1.5 [b]
	luteolin 8-*C*-glucoside (orientin)	447.1	23.2 ± 2.2 [b]
	apigenin 6-*C*-glucoside (isovitexin)	431.0	4.95 ± 0.5 [b]
	Total flavone-C-glycosides		***56.1 ± 4.9***
Anthocyanin-procyanidin models	(+)-catechin	289.0	2.33 ± 0.2
	procyanidin dimers	577.2	18.8 ± 1.4 [a]
	procyanidin trimers	865.1	34.4 ± 3.5 [a]
	Total procyanidins		***55.5 ± 5.6***

[a] (+)-catechin equivalents, [b] rutin equivalents.

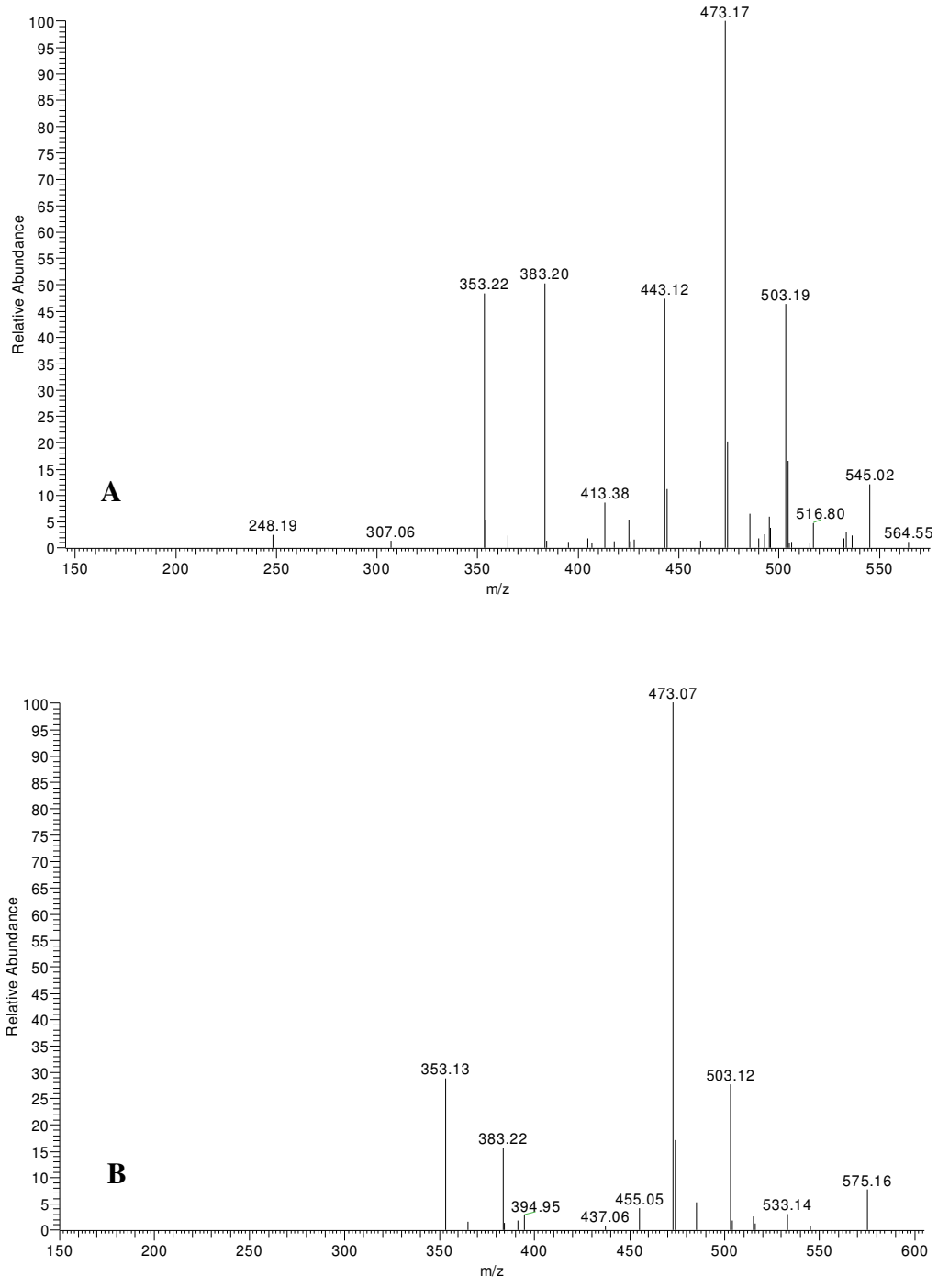


Fig. 16. ESI-MS negative product ion spectra of (A) apigenin-6-*C*-glucosyl-8-*C*-arabinoside (shaftoside, 563.1, $[M-H]^-$), and (B) apigenin 6,8-di-*C*-glucoside (vicenin-2, 593.5, $[M-H]^-$).

Identification of flavone-*C*-glycosides was based on their characteristic MS patterns, as preferential fragmentation of the glycosidic moiety occurs, yielding typical product ions at $[M-H-60]^-$, $[M-H-90]^-$, and $[M-H-120]^-$ (Ferreres, Silva, Andrade, Seabra, & Ferreira, 2003; Gattuso, Barreca, Gargiulli, Leuzzi, & Caristi, 2007; Pereira, Yariwake, & McCullagh, 2005). Thus, tentative identification of apigenin-6-*C*-glucosyl-8-*C*-arabinoside was based on precursor ion signals at *m/z*= 563.1 ($[M-H]^-$), and respective product ions at *m/z*= 503.1 ($[M-H-60]^-$), *m/z*= 473.2 ($[M-H-90]^-$); and *m/z*= 443.1 ($[M-H-120]^-$), as noted in Fig. 16. Exact fragmentation patterns corresponded to those previously reported for apigenin-6-*C*-glucosyl-8-*C*-arabinoside (shaftoside, *m/z*= 563.1, $[M-H]^-$) in quince seeds (Ferreres et al., 2003) and in *Scutellaria baicalensis* roots (Han, Ye, Xu, Sun, Wang, & Guo, 2007). Tentative identification of apigenin 6,8-di-*C*-glucoside was based on ion signals at *m/z*= 593.5, $[M-H]^-$, and similar neutral losses yielding ions at *m/z*= 533.1 ($[M-H-60]^-$); *m/z*= 503.1 ($[M-H-90]^-$); and *m/z*= 473.1 ($[M-H-120]^-$) (Fig. 16). Mass fragmentations were identical to those of apigenin 6,8-di-*C*-glucoside (vicenin-2, *m/z*= 593.5, $[M-H]^-$), previously reported in wild basil leaves (Grayer, Kite, Abou-Zaid, & Archer, 2000) and quince seeds (Ferreres et al., 2003).

The influence of naturally occurring polyphenolic cofactors on the stability of anthocyanins in açai fruit was evaluated by monitoring changes in anthocyanin concentrations of model juice systems adjusted to different conditions of temperature (5, 20, or 30°C for up to 12 weeks) and pH (3.0, 3.5, and 4.0). Initial spectrophotometric determinations of total anthocyanin contents were consistent among anthocyanin controls and models combined with phenolic acids or procyanidins (522.9 ± 27.3 mg

cyanidin-3-glucoside equivalents/L). However, models containing flavone-*C*-glycosides, were 18.0% higher in initial absorbance values (617.1± 30.1 mg/L), attributed to hyperchromic shifts resulting from anthocyanin copigmentation reactions. Molecular associations between anthocyanins and other phenolics are commonly associated with enhanced anthocyanin color, despite equivalent individual anthocyanin concentrations (Boulton, 2001). Copigmentation reactions are often accompanied by a bathochromic shift in the wavelength of maximum absorbance (Talcott et al., 2003); however, no change was observed in the presence of flavone-*C*-glycosides in these models.

Degradation rates for cyanidin-3-glucoside, cyanidin-3-rutinoside, and total anthocyanins followed first-order ($p<0.01$) degradation kinetics. Rate constants (β_1, in $days^{-1}$), half-lives (t½, time, in days, to achieve a 50% reduction on initial concentrations), and temperature quotients (Q_{10}, fold increase in degradation rate when temperature was increased from 20 to 30°C) were calculated as previously described (Pacheco-Palencia et al., 2007b) and are shown in Tables 12-14. Anthocyanin models experienced significant pH- and temperature-dependent total anthocyanin losses, with half-lives ranging from 4.01 to 23.2 days for models stored at 30°C, from 6.94 to 47.0 days when stored at 20°C, and from 48.2 to 259 days for models kept at 5°C (Table 12). Total anthocyanin losses were also influenced by pH variations, and increased by 45-60% when the pH was raised from 3.0 to 3.5 and by 90-100% when raised from 3.5 to 4.0, regardless of storage temperature or cofactor presence.

Table 12. Kinetic parameters of anthocyanin pigment degradation during storage of açai models.

Juice model	pH	β_1[a]			$t_{1/2}$[b]			Q_{10}[c]
		5°C	20°C	30°C	5°C	20°C	30°C	20-30°C
Anthocyanin control models	3.0	4.62	23.3	49.3	150 b[d]	29.7 b	14.1 b	2.10
	3.5	7.19	43.2	89.7	96.4 c	16.0 c	7.73 cd	2.07
	4.0	11.4	69.1	132	60.8 e	10.0 d	5.25 d	1.91
Anthocyanin-phenolic acid models	3.0	3.80	21.4	38.8	182 b	32.4 b	17.9 ab	1.81
	3.5	6.27	37.9	69.8	111 c	18.3 c	9.94 c	1.84
	4.0	11.8	68.9	136	58.9 e	10.1 d	5.11d	1.97
Anthocyanin-flavone-*C*-glycoside models	3.0	2.98	14.8	29.9	233 a	47.0 a	23.2 a	2.03
	3.5	4.61	25.2	50.8	150 b	27.5 b	13.6 b	2.02
	4.0	9.23	48.0	102	75.1 d	14.4 c	6.82 d	2.12
Anthocyanin-procyanidin models	3.0	2.68	20.8	40.2	259 a	33.3 b	17.2 b	1.93
	3.5	4.41	36.9	68.4	157 b	18.8 c	10.1 c	1.85
	4.0	8.29	67.0	133	83.6 d	10.3 d	5.22 d	1.98

[a] Reaction rate constant (β_1 x 10^3 $days^{-1}$). [b] Half-life (days) of initial absorbance for each juice model. [c] Temperature coefficient of anthocyanin pigment degradation rate as influenced by a 10°C increase in storage temperature. [d] Values with different letters within columns are significantly different (student's t test, $p<0.05$).

Table 13. Kinetic parameters of cyanidin-3-glucoside degradation during storage of açai models.

Juice model	pH	β_1[a]			$t_{1/2}$[b]			Q_{10}[c]
		5°C	20°C	30°C	5°C	20°C	30°C	20-30°C
Anthocyanin control models	3.0	8.05	57.6	179	86.1 b[d]	12.0 b	3.87 b	3.11
	3.5	11.4	111	334	61.0 d	6.24 c	2.08 c	3.01
	4.0	20.4	189	612	33.9 f	3.67 d	1.13 d	3.24
Anthocyanin-phenolic acid models	3.0	5.42	42.8	128	128 a	16.2 a	5.42 a	2.98
	3.5	9.64	73.0	232	71.9 c	9.50 b	2.99 b	3.18
	4.0	17.3	136	411	40.0 e	5.10 c	1.68 c	3.03
Anthocyanin-flavone-*C*-glycoside models	3.0	4.82	41.8	124	144 a	16.6 a	5.57 a	2.98
	3.5	8.53	72.0	223	81.3 b	9.63 b	3.11 b	3.09
	4.0	15.4	128	394	45.1 e	5.40 c	1.76 c	3.07
Anthocyanin-procyanidin models	3.0	5.12	48.7	141	136 a	14.2 a	4.91 a	2.90
	3.5	9.01	84.5	259	77.0 bc	8.21 bc	2.68 b	3.06
	4.0	16.4	158	467	42.3 e	4.39 cd	1.48 cd	2.96

[a] Reaction rate constant (β_1 x 10^3 $days^{-1}$). [b] Half-life (days) of initial absorbance for each juice model. [c] Temperature coefficient of cyanidin-3-glucoside degradation rate as influenced by a 10°C increase in storage temperature. [d] Values with different letters within columns are significantly different (student's t test, $p<0.05$).

Table 14. Kinetic parameters of cyanidin-3-rutinoside degradation during storage of açai models.

Juice model	pH	β_1 [a]			$t_{1/2}$ [b]			Q_{10} [c]
		5°C	20°C	30°C	5°C	20°C	30°C	20-30°C
Anthocyanin control models	3.0	4.06	24.4	66.3	171 a[d]	28.4 a	10.7 a	2.72
	3.5	7.80	42.1	119	88.9 b	16.4 b	5.81 b	2.83
	4.0	12.0	64.8	185	57.8 c	10.7 c	3.75 c	2.85
Anthocyanin-phenolic acid models	3.0	3.72	20.4	56.8	186 a	34.0 a	12.4 a	2.78
	3.5	6.62	34.3	101	105 b	20.2 b	6.87 b	2.95
	4.0	11.9	63.2	179	58.2 c	11.0 c	3.87 c	2.83
Anthocyanin-flavone-*C*-glycoside models	3.0	3.31	20.9	55.3	210 a	33.2 a	12.8 a	2.65
	3.5	5.85	33.8	96.8	118 b	20.5 b	7.16 b	2.86
	4.0	10.6	59.7	172	65.7 c	11.6 c	4.04 c	2.87
Anthocyanin-procyanidin models	3.0	3.51	23.2	58.8	197 a	29.9 a	12.0 a	2.53
	3.5	6.18	39.7	103	112 b	17.5 b	6.70 b	2.61
	4.0	11.2	73.5	187	61.7 c	9.43 c	3.71 c	2.54

[a] Reaction rate constant (β_1 x 10^3 $days^{-1}$). [b] Half-life (days) of initial absorbance for each juice model. [c] Temperature coefficient of cyanidin-3-rutinoside degradation rate as influenced by a 10°C increase in storage temperature. [d] Values with different letters within columns are significantly different (student's t test, $p<0.05$).

Similar pH and temperature-dependent degradation patterns were observed when individual cyanidin-3-glucoside and cyanidin-3-rutinoside concentrations were monitored (Tables 13, 14) and both cyanidin-3-glucoside ($r=0.94$) and cyanidin-3-rutinoside ($r=0.93$) concentrations were highly correlated ($p<0.001$) to total anthocyanin losses in all models throughout storage. However, temperature quotients (Q_{10}, Tables 12-14) indicated cyanidin-3-glucoside was less stable to temperature changes (Q_{10}=2.9-3.2, Table 13) compared to cyanidin-3-rutinoside (Q_{10}=2.5-2.9, Table 14), and that individual anthocyanin concentrations were significantly less stable to temperature changes compared to total anthocyanin contents (Q_{10}=1.8-2.1, Table 13). Observed differences between total anthocyanin contents and changes in individual anthocyanin concentrations were likely due to color contributions from polymeric anthocyanins formed during storage, and to the potential influence of other matrix components in the juice models (Wrolstad et al., 2005).

Similar detrimental effects were observed at increasing pH values, and changes from pH 3.0 to 3.5 nearly doubled cyanidin-3-glucoside and cyanidin-3-rutinoside degradation rates (Tables 13, 14) and increased total anthocyanin degradation rates by ~50% in all juice models. Detrimental effects of both high temperature and pH on anthocyanin stability and color were attributed to shifts in the anthocyanin equilibrium toward the colorless pseudobase and chalcone forms, expected to occur under these conditions (Markakis, 1982; Clifford, 2000).

Structural differences also had a significant influence on anthocyanin stability, with cyanidin-3-rutinoside being consistently more stable ($t_{½}$=2.67 to 210 days, Table

14) than cyanidin-3-glucoside (t½=1.13 to 144 days, Table 13) in all models, regardless of variations in temperature, pH, or cofactor composition; and were in agreement with our previous observations in ascorbic acid-fortified açai juices (Pacheco-Palencia et al., 2007a). Additional models based on a cyanidin-3-rutinoside standard in aqueous buffer (pH 3.5) revealed a gradual conversion to cyanidin-3-glucoside over time, at up to 12.5% after 20 days of storage at 30°C, indicating potential hydrolysis of anthocyanin glycosidic bonds during storage. Gradual hydrolysis of glycosidic bonds would lead to increased cyanidin-3-glucoside and decreased cyanidin-3-rutinoside concentrations during storage of açai fruit-containing juices, resulting in higher proportions of the more labile cyanidin-3-glucoside, and leading to increasingly higher anthocyanin degradation rates. Moreover, hydrolysis of the glycosidic bond has been proposed as one of the early steps in the degradation of anthocyanins, as the resulting aglycone is unstable and undergoes spontaneous ring fission and subsequent degradation (Adams, 1972; Adams, 1973; Markakis, 1974; Clifford, 2000).

The presence of phenolic cofactors had also a significant ($p=0.006$) influence on the stability of açai fruit anthocyanins in juice models. Overall, the presence of flavone-*C*-glycosides resulted in increased total anthocyanin stability at all temperature and pH combinations in relation to the anthocyanin control, while no significant effects were attributed to the presence of phenolic acids or procyanidins (Table 12). Protective effects were more pronounced for cyanidin-3-glucoside, where addition of flavone-*C*-glycosides resulted in higher anthocyanin stability in all models (Table 13). By contrast, cyanidin-3-rutinoside degradation rates were not significantly affected by the presence of flavone-*C*-

glycosides (Table 14), likely due to its inherently higher stability in all models. Thus, the positive influence of flavone-*C*-glycosides on anthocyanin stability in açai fruit models was likely a result of their protective effect on cyanidin-3-glucoside, the most labile of açai fruit anthocyanins. Further models using authentic cyanidin-3-glucoside standards (205 ± 14 mg/L) adjusted to pH 3.5 and to which phenolic acid, flavone-*C*-glycoside, and procyanidin isolates from açai fruit were added confirmed the ability of flavone-*C*-glycosides to act as effective copigments and resulted in increased ($p<0.01$) anthocyanin stability during storage (Fig. 17). Stable anthocyanin copigment complexes with flavone-*C*-glycosides were reported to occur naturally in Iris flowers (Asen, Stewart, Norris, & Massie, 1970); however, this is the first report on the ability of flavone-*C*-glycosides to act as copigments in fruit juice systems.

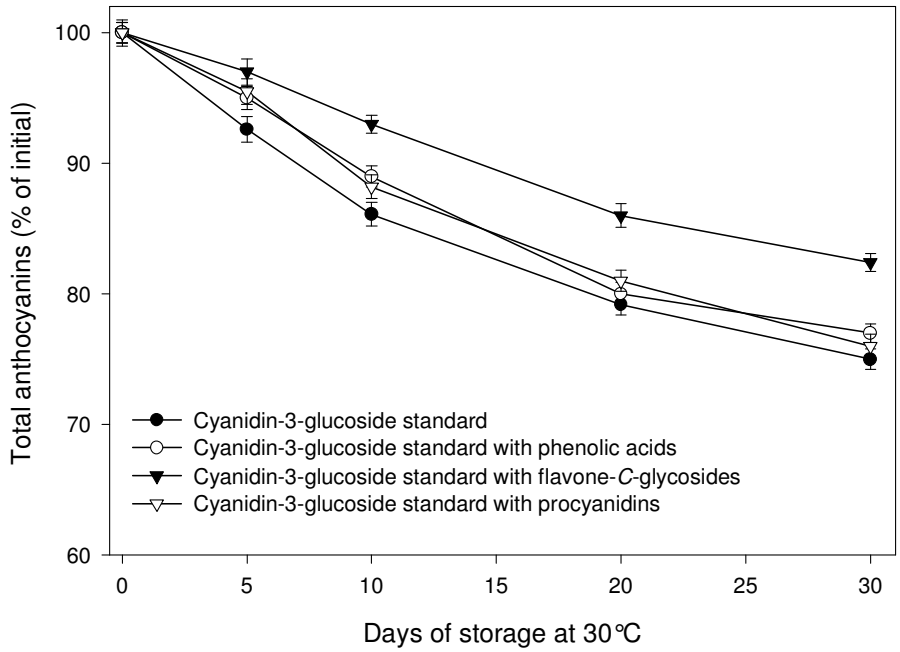


Fig. 17. Percent changes in total anthocyanin contents during storage (30°C) of cyanidin-3-glucoside standard models adjusted to pH 3.0. Error bars represent the standard error of the mean (n=3).

Sulfite bleaching resistance of anthocyanins in açai models was also correlated ($p<0.01$) to total anthocyanin contents ($r = 0.77$), and was indicative of hue changes in color from dark red to brown. Temperature, pH, and phytochemical composition significantly ($p<0.03$) influenced anthocyanin bleaching resistance, expressed as percent polymeric anthocyanin contents during storage. All phenolic cofactors decreased anthocyanin polymerization rates throughout storage with effects generally more pronounced in models adjusted to pH 3.0 or 3.5, regardless of storage temperature (Fig. 18).

Açai anthocyanin models were also evaluated for pH variations, soluble phenolic contents, as a measure of total reducing capacity, and for changes in antioxidant capacity throughout storage (Fig. 19). No significant pH variations were detected in any of the models following addition of phenolic cofactors or throughout storage. However, addition of phenolic copigments resulted in increased total soluble phenolic contents (23.1 ± 1.8% increase) and antioxidant capacity (30.2 ± 2.6% increase) in relation to uncopigmented anthocyanin controls that contained 974 ± 79 mg gallic acid equivalents/L and an antioxidant capacity of 23.8 ± 1.15 µmol TE/mL. All phenolic cofactors resulted in higher soluble phenolic contents and antioxidant capacity throughout storage when compared to uncopigmented anthocyanins, at all temperature and pH conditions (Fig. 19). Moreover, changes in soluble phenolic contents and antioxidant capacity were correlated ($r=0.47$ and 0.68, respectively) to variations in total cyanidin-3-glucoside and cyanidin-3-rutinoside concentrations during storage of açai juice models.

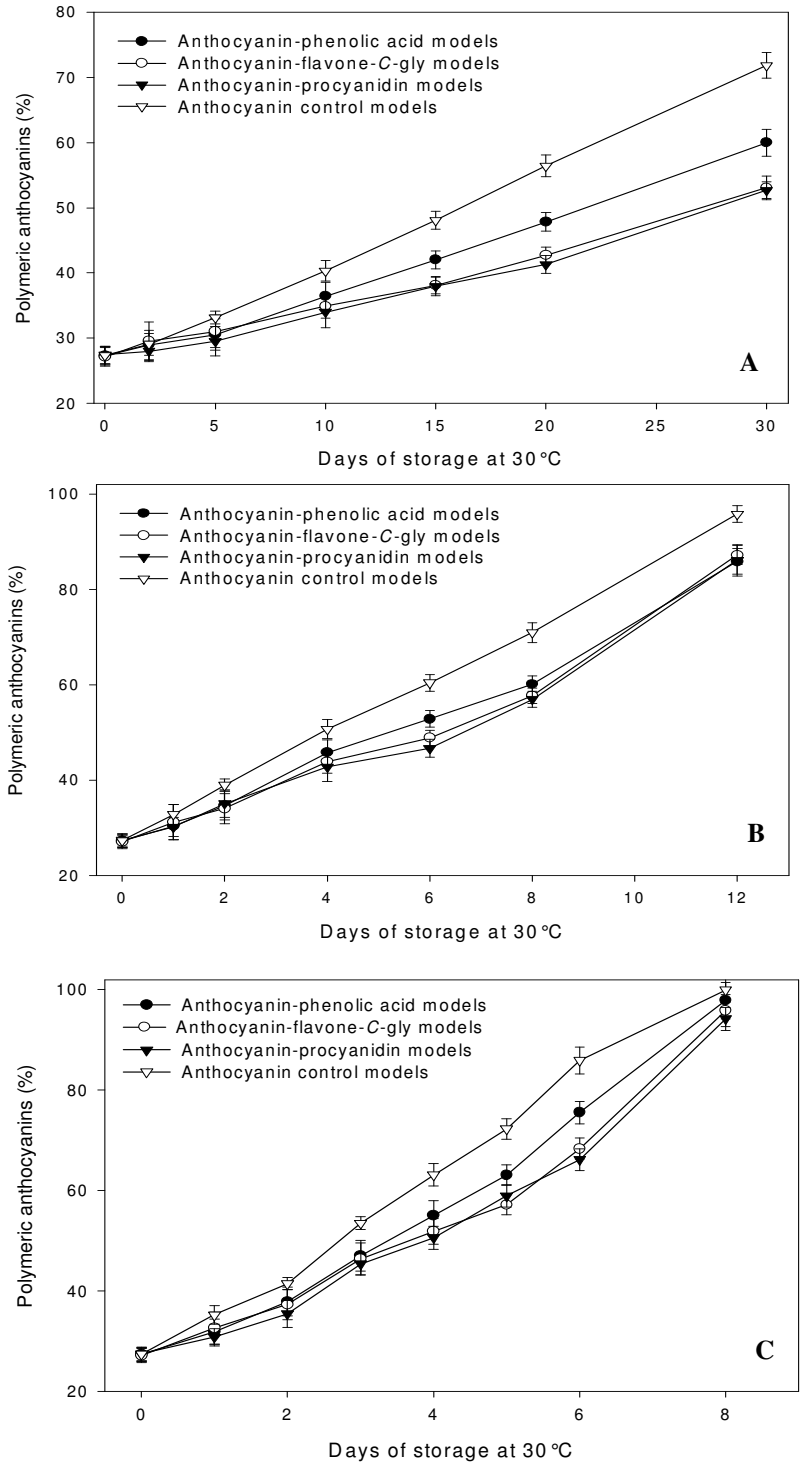


Fig. 18. Percent polymeric anthocyanins during storage (30°C) of açai models adjusted to pH 3.0 (A), 3.5 (B), and 4.0 (C). Error bars represent the standard error of the mean (n=3).

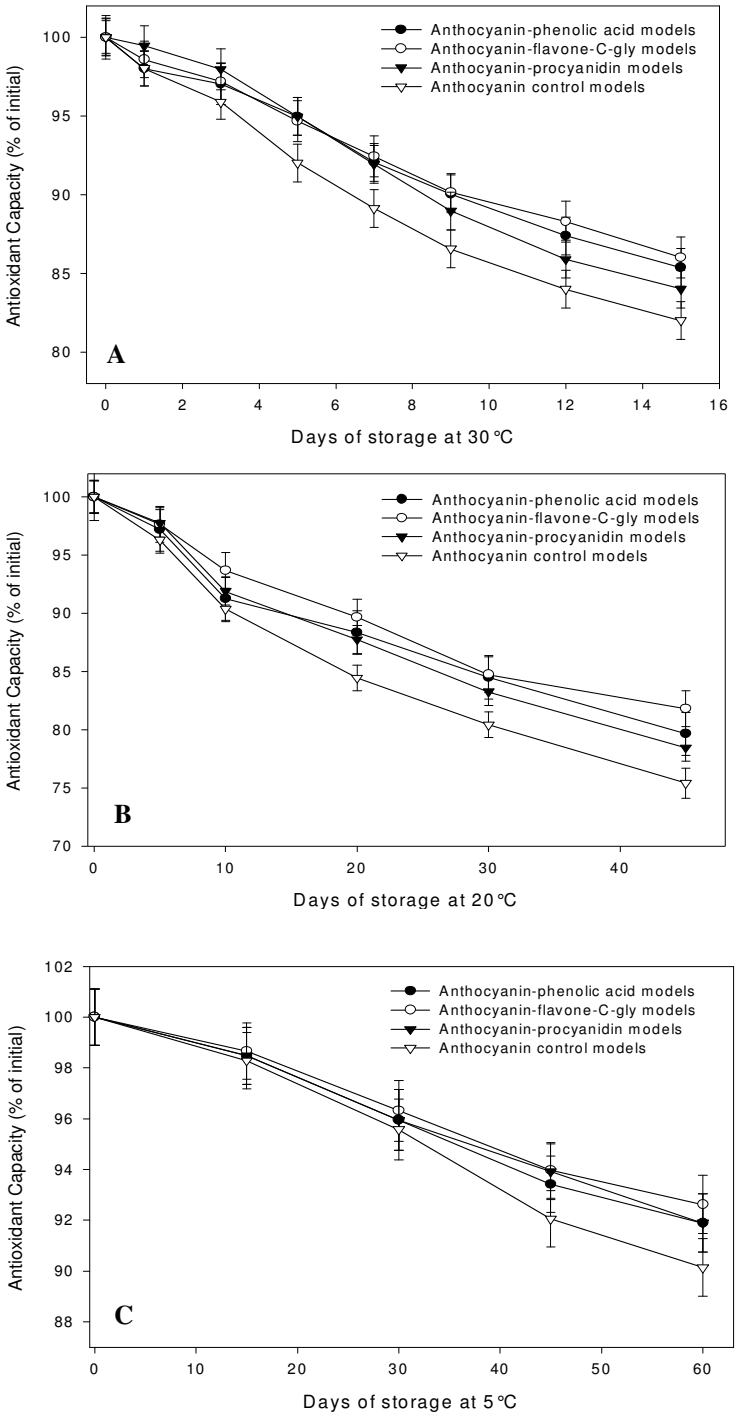


Fig. 19. Percent changes in antioxidant capacity of açai models adjusted to pH 3.0 during storage at 30°C (A), 20°C (B), and 5°C (C). Error bars represent the standard error of the mean (n=3).

The overall stability of naturally occurring polyphenolic cofactors during storage of açai anthocyanin models was additionally evaluated. Phenolic acid concentrations remained unchanged ($p<0.05$) in models stored at up to 20°C, yet losses were <10% at 30°C. Similarly, flavonol derivatives were stable in models stored at 5°C but experienced temperature-dependent losses following storage at both 20 and 30°C, of up to 12.1 ± 1.3% after 30 days, regardless of pH differences. Specifically, the predominant flavone *C*-glycosides, orientin and isoorientin, experienced temperature-dependent losses of up to 11.1% during storage at 5 to 30°C for up to 60 days, while no changes occurred for the flavone *C*-glycosides luteolin 6,8-di-*C*-glucoside, apigenin 6,8-di-*C*-glucoside, isovitexin, and scoparin.

The influence of externally added flavone-*C*-glycoside cofactors on the stability of anthocyanin isolates from açai fruit was additionally evaluated and compared to a commercially available anthocyanin color enhancer isolated from rosemary. Extracts rich in flavone-*C*-glycosides were obtained from rooibos tea (*Aspalathus linearis*), which was previously identified to contain high amounts of flavone-*C*-glycosides (Bramati, Minoggio, Gardana, Simonetti, Mauri, & Pietta, 2002; Marnewick, Joubert, Joseph, Swanevelder, Swart, & Gelderblom, 2005; Krafczyk & Glomb, 2008). Chemical analyses of rooibos extracts used in these trials revealed the presence of orientin (39.9 ± 2.8%), isoorientin (48.1 ± 3.1%), vitexin (19.9 ± 1.4%), and isovitexin (19.7 ± 1.3%), along with lower concentrations of *C*-linked dehydrochalcone glycosides (12.3 ± 1.1%), likely aspalathin and nothofagin (Marnewick et al., 2005; Krafczyk & Glomb, 2008). Copigmentation of anthocyanin isolates with rooibos extracts (0.2% v/v) resulted in

immediate hyperchromic shifts of up to 45.5 ± 3.8% at pH 3.0, 30.2 ± 2.3% at pH 3.5, and 14.7 ± 1.3% at pH 4.0, that related to proportionally higher increases in visual red color at all pH levels. A less pronounced hyperchromic shift was observed with the commercial rosemary extracts (0.2% v/v), which resulted in 27.5 ± 2.2%, 21.1 ± 1.3%, and 10.7 ± 0.9% higher absorbance at pH 3.0, 3.5, and pH 4.0 respectively.

Table 15. Kinetic parameters of total anthocyanin degradation during storage of açai models with externally added polyphenolic cofactors.

Juice model	β_1 [a]			$t_{1/2}$ [b]		
	pH 3.0	pH 3.5	pH 4.0	pH 3.0	pH 3.5	pH 4.0
Anthocyanin control models	64.5	120	294	10.7 c[c]	5.79 b	2.36 b
Anthocyanin-rooibos models	53.6	80.0	209	12.9 a	8.67 a	3.32 a
Anthocyanin-rosemary models	59.8	83.4	214	11.6 b	8.30 a	3.24 a

[a] Reaction rate constant (β_1 x 10^3 $days^{-1}$). [b] Half-life (days) of initial anthocyanin content for each juice model. [c] Values with different letters within columns are significantly different (student's t test, $p<0.05$).

Kinetic parameters of total anthocyanin degradation, expressed as the sum of cyanidin-3-glucoside and cyanidin-3-rutinoside concentrations, revealed consistently higher ($p<0.05$) anthocyanin half-lives for anthocyanin models containing rooibos ($t_{1/2}$= 3.32 to 12.9 days) or rosemary extracts ($t_{1/2}$= 3.24 to 11.6 days) as compared to an anthocyanin isolate control ($t_{1/2}$= 2.36 to 10.7 days) (Table 15). Changes in total anthocyanin contents were inversely correlated ($p<0.05$) to anthocyanin bleaching

resistance (r=0.72) and directly correlated to antioxidant capacity (r=0.65). Thus, addition of rooibos extracts, rich in flavone-*C*-glycosides, not only resulted in higher color intensities but also increased anthocyanin stability during long-term storage (30°C) of juice models, in a comparable manner to a known copigment source such as rosemary extract, indicating its potential for anthocyanin stabilization in açai-containing foods, juice blends and beverages.

Conclusion

The influence of naturally occurring and externally added polyphenolic cofactors on the chemical and color stability of predominant anthocyanins in açai fruit (*Euterpe oleracea*), cyanidin-3-glucoside and cyanidin-3-rutinoside, was investigated. Overall, the presence of flavone-*C*-glycosides induced an increase in anthocyanin color and enhanced total anthocyanin stability, regardless of pH (3.0, 3.5, or 4.0) or storage temperature (5, 20, or 30°C), while no significant effects were attributed to the presence of phenolic acids or procyanidins. External addition of flavone-*C*-glycoside-rich extracts from rooibos tea also resulted in up to ~45% higher anthocyanin color and up to ~40% higher anthocyanin stability compared to uncopigmented anthocyanin isolates, and had similar copigmentation effects to a commercial anthocyanin color enhancer extracted from rosemary. Results suggest flavone-*C*-glycosides are a potential alternative for the food and beverage industry for their use as color enhancers and stabilizing agents in products containing non-acylated cyanidin glycosides, particularly açai fruit juice blends and beverages.

CHAPTER VII

PHYTOCHEMICAL MODELS FOR ANTHOCYANIN POLYMERIZATION REACTIONS IN AÇAI JUICE SYSTEMS

Introduction

Anthocyanins are among the most widely distributed naturally occurring pigments in plants, and represent a major source of red, blue, and purple hues in many fruit products. Anthocyanin color is often a major quality parameter in many fruit juices and functional beverages, influencing consumer's preference, acceptability, and ultimately choice of a particular product. American consumers spend over $4.5 billion annually in natural fruit juices, and an additional $10 billion in functional beverages (Mintel International U.S. Functional Beverage Report, 2008), with nearly 20% of products containing anthocyanins. However, anthocyanin pigment stability during processing and storage remains a major problem facing beverage manufacturers. Anthocyanin stability during processing and storage is known to be influenced by a wide variety of factors, including anthocyanin structure and concentration, pH, temperature, light, and the presence of enzymes, oxygen, metal ions, ascorbic acid, sugar and their degradation products (Rodriguez-Saona et al., 1999; Stingzing et al., 2002). Moreover, anthocyanin polymerization reactions during storage negatively impact appearance and quality attributes, as hue transformations occur, from bright red to dark, dull, brick-red colors (Baranac et al., 1996; Monagas et al., 2006).

Polymerization reactions in anthocyanin-containing juices and their effects on sensory properties are generally acknowledged, yet reaction mechanisms and nature of the products formed are still poorly understood. Anthocyanin polymerization reactions have been particularly studied in aged red wines, where three basic types of anthocyanin-derived pigments have been described: ethyl-linked condensed products resulting from aldehyde-mediated reactions between anthocyanins and flavanols (Berg & Akiyoshi, 1975; Timberlake & Bridle, 1976; Rivas-Gonzalo, Bravo-Haro, & Santos-Buelga, 1995; Dallas, Ricardo-da-Silva, & Laureano, 1996; Francia-Aricha, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1997; Es-Safi et al., 2000; Es-Safi et al., 2002), 4-substituted anthocyanin-vinylphenol adducts (pyranoanthocyanins), containing additional pyrano rings (Fulcrand, Cameira do Santos, Sarni-Manchado, Cheynier, & Bonvin, 1996; Bakker & Timberlake, 1997; Francia-Aricha et al., 1997; Swarz, Wabnitz, & Winterhalter, 2003; Rein, Ollilainen, Vahermo, Yli-Kauhaluoma, & Heinonen, 2005; Oliveira et al., 2006), and anthocyanin-flavanol condensed products (Jurd, 1967; Mateus, Silva, Santos-Buelga, Rivas-Gonzalo, & De Freitas, 2002; Salas, Fulcrand, Meudec, & Cheynier, 2003; Remy-Tanneau, Le Guerneve, Meudec, & Cheynier, 2003; Salas, Atanasova, Poncet-Legrand, Meudec, Mazauric, & Cheynier, 2004a; Gonzalez-Paramas et al., 2006). However, reactions involved in the formation of anthocyanin polymers in fruit juices are largely unknown. Based on our previous reports (Pacheco-Palencia et al., 2007a; Pacheco-Palencia et al., 2007b), açai (*Euterpe oleracea* Mart.) fruit juices seem to be particularly prone to accelerated anthocyanin polymerization reactions during storage, giving rise to undesirable brown hues in açai-containing products. As a result,

the aim of this work was to elucidate potential precursors and mechanisms for anthocyanin polymerization reactions in açai fruit juice systems, provide structural information on the products formed, and evaluate their ability to occur in açai fruit juices. Results from these investigations may assist in the development of strategies to enhance color and quality stability of similar anthocyanin-containing products.

Materials and Methods

Anthocyanin models were based on isolates extracted from 100% açai fruit pulp. Pasteurized açai pulp was kindly donated by Bossa Nova Beverage Group (Los Angeles, CA) and shipped frozen to the Department of Nutrition and Food Science at Texas A&M University. Prior to anthocyanin isolation, açai pulp was clarified according to a previously described procedure (Pacheco-Palencia et al., 2007b) to remove lipids and insoluble solids. Clarified açai pulp was then loaded onto activated C18 Sep-Pak 6 cc cartridges (Waters Corporation, Milford, MA), and eluted repeatedly with water, to remove sugars, organic acids, metals, and proteins. Elution with ethyl acetate followed, removing phenolic acids and most non-anthocyanin flavonoids (Pacheco-Palencia et al., 2007a), and anthocyanins were finally recovered with acidified methanol (0.01% HCl). Methanol was evaporated under vacuum (<40°C), and the resulting anthocyanin isolate redissolved in a 0.1M citric acid buffer (pH 3.0) and adsorbed onto a second C18 Sep-Pak cartridge (Waters Corporation, Milford, MA). A protocol adapted from the separation of anthocyanins containing *o*-diphenolic systems, in which anthocyanins containing an *o*-diphenolic system form negatively charged complexes in alkaline borate

solutions (Hong & Wrolstad, 1990), was employed. Briefly, the loaded C18 cartridge was continuously rinsed with an alkaline borate solution (0.1 N, pH 9.0), until a colorless eluent was obtained. Eluted anthocyanins (monomers) were recovered and immediately reconverted into their red oxonium salt forms by dilution with 4N HCl. Anthocyanins remaining on the cartridge (polymers) were finally eluted with acidified methanol (0.01% HCl), concentrated under vacuum at <40°C until complete solvent removal, and reconstituted in a 0.1 M citric acid buffer (pH 3.0) for use in model systems. Anthocyanin monomers were further concentrated using a third C18 Sep-Pak cartridge, from which anthocyanins were recovered with 0.01% HCl in methanol, subjected to complete solvent evaporation under vacuum (<40°C), and likewise reconstituted in a 0.1 M citric acid buffer (pH 3.0) for use in model systems. Anthocyanin monomer and polymer isolates were finally standardized to 500 mg cyanidin-3-glucoside equivalents/L, determined spectrophotometrically, as previously described in chapter VI.

Anthocyanin model systems consisted of mixtures of anthocyanin monomer and polymer fractions combined in varying proportions (10:90, 20:80, 30:70, and 50:50 polymer-to-monomer fraction ratio), along with a 100% anthocyanin monomer control. Each model was adjusted to pH 3.5, loaded into screw-cap glass test tubes in triplicate, and stored at 35°C for up to 20 days. Sodium azide (50 mg/L) was added to all treatments to retard microbial growth and pH was maintained constant throughout storage. Models were sampled every 2-3 days and samples were held at -20°C until analysis.

A similar approach was used to assess the influence of açai polymers on authentic cyanidin-3-glucoside standards and on blackberry anthocyanin isolates, which were independently combined with açai polymers in 1:9 (10%), 1:4 (20%), 1:2 (33%), and 1:1 (50%) polymer-to-anthocyanin ratios, in addition to an anthocyanin control. Models were likewise adjusted to pH 3.5, stored at 35°C for up to 20 days, sampled every 2-3 days, and immediately analyzed or held at -20°C until analysis. Blackberry anthocyanins were extracted from a commercial blackberry concentrate (Glcc Co., Paw Paw, MI), which were diluted with water, loaded onto C18 Sep-Pak cartridges, eluted with ethyl acetate (for non-anthocyanin polyphenolic removal), and recovered with acidified methanol (0.01% HCl). The solvent was evaporated under vacuum (<40°C) and the resulting isolate redissolved in a known volume of citric acid buffer (0.1M, pH 3.5) prior use in anthocyanin models. Anthocyanin monomer and polymer fractions were subjected to acid hydrolysis (90°C, 2N HCl) to assist their characterization. Sample aliquots were loaded onto tightly sealed screw-cap glass test tubes, heated in a water bath at 90°C for 30 min, and immediately analyzed on the HPLC-ESI-MS^n system.

Polymeric anthocyanin fractions were thiolyzed to provide further structural data. Thiolysis of polymeric anthocyanin fractions was based on the method by Guyot, Marnet, & Drilleau (2001) and Gu et al. (2002). Briefly, 100 µL of sample (adjusted to ~2,000 mg/L in methanol) was mixed with 100 µL of acidified methanol (3.3% v/v HCl) and 200 µL of benzyl mercaptan (5% v/v in methanol), in a 2.0 mL glass vial (National Scientific, Rockwood, TN). The mixture was reacted for 30 min at 40°C, kept at room temperature for 12 h to ensure complete degradation, and kept frozen (-18°C) until direct

HPLC analysis. Pure (+)-catechin and (-)-epicatechin solutions were also thiolyzed in each batch to obtain their respective epimerization rates.

Polyphenolics present in monomeric and polymeric anthocyanin fractions were analyzed by HPLC-ESI-MS^n. Tandem reversed phase HPLC and mass spectrometry analyses were conducted using a Thermo Finnigan LCQ Deca XP Max MS^n ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA). Separations were performed on an Acclaim 120 5µm 120Å (4.6 x 250 mm) column and mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in methanol (phase B) run at 0.6 mL/min. Polyphenolics were separated with a gradient elution program in which phase B changed from 5 to 10% in 3 min, from 10 to 30% in the following 17 min, from 30 to 50% in the following 20 min, from 50 to 70% in the following 15 min, and from 70 to 100% in the following 10 min. Initial conditions were then restored within 1 min, and held constant for 20 min prior each injection. Ionization was conducted in the negative ion mode under the following conditions: sheath gas (N_2), 60 units/min; auxiliary gas (N_2), 10 units/min; spray voltage, 3.5 kV; capillary temperature, 350 °C; capillary voltage, 1.5 V; tube lens offset, 0 V. Identification and quantitation of polyphenolics was based on their spectral characteristics, retention time, and mass spectrometric properties, which were compared to authentic standards when available (Indofine Chemical Company, Hillsborough, NJ, and Sigma Chemical Co., St. Louis, MO).

Antioxidant capacity was determined by the oxygen radical absorbance capacity assay using a BMG Labtech FLUOstar fluorescent microplate reader (485 nm excitation

and 538 nm emission), as previously described in chapter III, and results were quantified in μmol Trolox equivalents (TE)/mL. Total anthocyanin contents and anthocyanin sulfite bleaching resistance were determined spectrophotometrically at 520 nm, according to the procedures described in chapter VI, and quantified using equivalents of cyanidin-3-glucoside (mg/L).

Statistical analyses were conducted independently for each anthocyanin source (açai, blackberry, or anthocyanin standard). Data from each analysis was analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL), conducting post-hoc Tukey-Kramer HSD mean separations ($p<0.05$). A significance level of 0.05 was used for parametric correlations and linear stepwise regression models.

Results and Discussion

Polyphenolic composition of monomeric and polymeric anthocyanin fractions from açai was assessed by HPLC-ESI-MS^n analyses and characterized according to elution time, spectral properties, and MS fragmentation patterns. The term "monomeric" was used for fractions rich in anthocyanin glycosides, eluting as individual, resolved peaks, while the term "polymeric" referred to those fractions characterized by a prolonged, large, unresolved baseline, in the 500-530 nm range of HPLC chromatograms. Polyphenolics present in monomeric anthocyanin fractions (Table 16) were characterized as follow:

Peaks 1, 2, 4, and 5 were tentatively assigned to flavonol glycosides, potentially kaempferol (*m/z* 609.1, $[M-H]^-$) and quercetin (*m/z* 609.2, 463.2, 609.1, $[M-H]^-$) mono

and di-glycosides. Typical glycoside losses (*m/z* 162, eg. *m/z* 609.1 to *m/z* 447.0) were observed in all cases, and the detection of two independent losses of a sugar moiety from the molecular ion ($[M-H]^-$) was likely indicative of two separate glycosylations (di-*O*-glycosides), as the loss of inner sugar from a two-ring sequence of *O*-diglycosides does not occur in the negative ion mode (Cuyckens & Claeys, 2004). Fragmentation to *m/z* 447.0 and 285.3 were characteristic of a kaempferol glycoside (Petsalo, Jalonen, & Tolonen, 2006) and ions at *m/z* 301.0, 283.1, 255.2, and 151.0 were distinctive of quercetin glycosides (Gil-Izquierdo & Mellenthin, 2001).

Table 16. Mass spectrometric characteristics of polyphenolics present in monomeric anthocyanin fractions from açai fruit pulp.

Peak No.	RT (min)	Tentative Identification	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)[1]
1	18.4	Kaempferol di-glycoside	609.1	447.0, 285.3
2	19.8	Quercetin di-glycoside	609.2	301.0, 283.1, 255.2, 151.0
3	20.4	Apigenin derivative	451.0	269.2, 225.1, 149.1
4	21.2	Quercetin glycoside	463.2	301.0, 283.1, 255.2, 151.1
5	21.5	Quercetin di-glycoside	609.1	301.0, 283.1, 255.2, 151.0
6	23.0	Apigenin derivative	485.0	448.9, 269.0, 225.1, 149.1
7	30.3	Cyanidin-3-glucoside	447.0	285.0, 257.0, 183.1
8	31.5	Cyanidin-3-rutinoside	593.0	285.0, 257.0, 183.1
9	34.5	Isoorientin	447.1	357.1, 327.0, 299.1, 285.0
10	35.7	Orientin	447.1	357.1, 327.0, 299.1, 285.0

Peaks 3 and 6 were tentatively identified as apigenin derivatives, yielding ion signals at *m/z* 451.0 and *m/z* 485.0, [M-H$^-$]. Neutral losses could not be attributed to any particular

acylation or sugar substitution; however, predominant ions at *m/z* 269.2, 225.1, and 149.1 corresponded to the fragmentation pattern of apigenin.

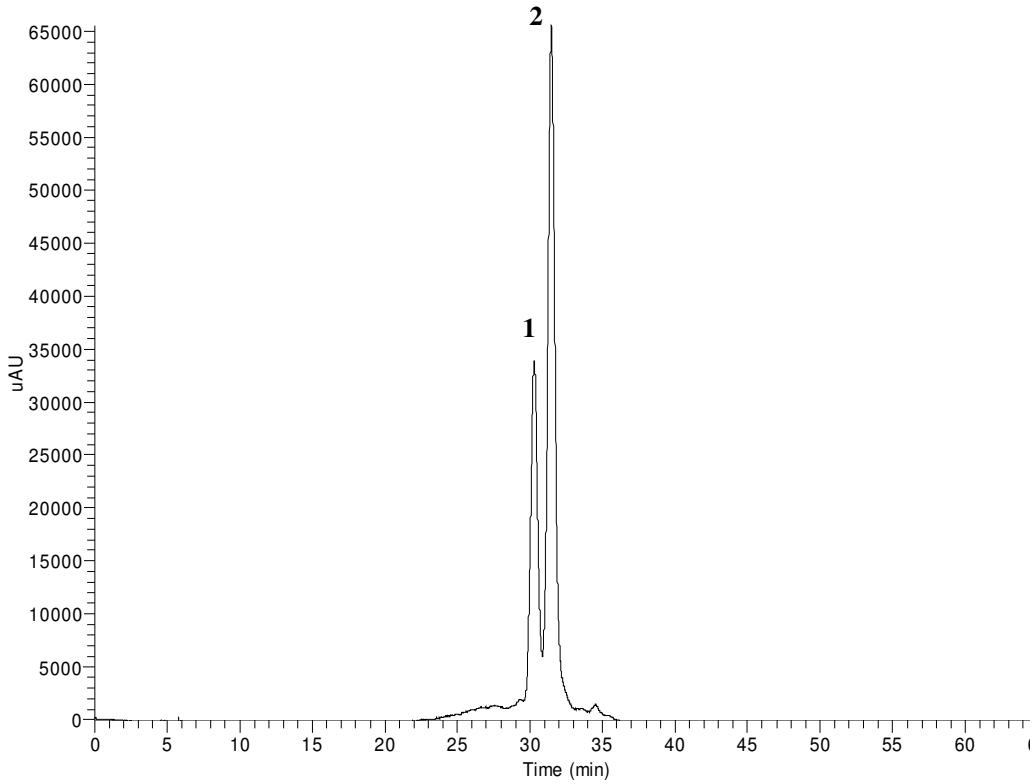


Fig. 20. Chromatographic profile (520 nm) of monomeric anthocyanin fractions from açai pulp. Peak assignments: 1. cyanidin-3-glucoside; 2. cyanidin-3-rutinoside.

Peaks 7 and 8 were respectively identified as cyanidin-3-glucoside (*m/z* 447.0, [M-H$^-$]), and cyanidin-3-rutinoside (*m/z* 593.0, [M-H$^-$]), yielding major fragments at *m/z* 285.0, 257.0, and 183.1, corresponding to a cyanidin aglycone. Identities were confirmed by comparison to authentic standards. A typical HPLC chromatogram is shown in Fig. 20.

Peaks 9 and 10 corresponded to isoorientin (luteolin-6-*C*-glucoside, *m/z* 447.1, [M-H$^-$]), and orientin (luteolin-8-*C*-glucoside, *m/z* 447.1, [M-H$^-$]), both of which fragmented to

m/z 357.1, 327.0, 299.1, and 285.0. Neutral losses of 90 and 120 Da were characteristic of *C*-glycosides, as fragmentation in the *C*-glycosidic unit is generally favored in these compounds (Li et al., 1991; Waridel, Wolfender, Ndjoko, Hobby, Major & Hostettmann, 2001; Pereira, Yariwake, & McCullagh, 2005). Identities were confirmed by comparison to authentic standards.

Chromatographic (Fig. 20) and mass spectrometric (Table 16) data indicated monomeric anthocyanin fractions from açai fruit pulp were composed of two predominant anthocyanins (530.4 ± 33.2 mg/L), cyanidin-3-glucoside (31.4 ± 2.9%) and cyanidin-3-rutinoside (68.6 ± 5.8%), along with minor amounts of various flavonol glycosides (11.6 ± 0.9 mg rutin equivalents/L), flavone-*C*-glycosides (43.8 ± 3.8 mg isoorientin equivalents/L), and other flavones, tentatively identified as apigenin derivatives (below quantitation limits).

Characterization of polyphenolics present in polymeric anthocyanin fractions from açai fruit pulp was primarily based on their mass spectrometric fragmentation patterns, as anthocyanin polymers appeared to elute as a wide, unresolved peak in HPLC chromatograms (Fig. 21), difficulting individual UV/Vis spectral assessments. Details on their mass spectrometric characteristics and tentative identities are shown in Table 17.

Table 17. Mass spectrometric characteristics of polyphenolics present in polymeric anthocyanin fractions from açai pulp.

Peak No.	RT (min)	Tentative Identification	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)[1]
11	17.9	Pelargonidin glycoside-(epi)(+)-catechin adduct	721.3	702.9, 685.4, 523.7,449.1, **431.3**, 269.2, 225.0, 183.0
12	18.6	Cyanidin glycoside adduct	611.1	**593.1**, 449.1, 431.0, 285.3, 257.1, 183.0
13	20.2	Pelargonidin glycoside-(epi)(+)-catechin adduct	721.2	703.1**,** 685.1, 523.9, 449.1, **431.0**, 269.2, 225.0, 183.0
14	21.2	Cyanidin glycoside-(epi)(+)-catechin adduct	883.1	**720.9**, 703.0, 685.0, 541.0, 523.0, 505.1, 449.0, 431.0, 285.0, 183.0
15	21.9	Cyanidin glycoside-(epi)(+)-catechin adduct	864.9	703.0, 685.0, 540.9, **430.9**, 285.0, 183.0
16	22.8	Cyanidin glycoside adduct	611.1	**593.0**, 449.0, 430.9, 284.9, 257.1
17	23.2	Pelargonidin-glycoside adduct	485.1	431.2, **269.1**, 225.0, 183.0
18	23.9	Pelargonidin glycoside adduct	793.0	757.0, **595.0**, 448.9, 430.9, 269.0, 225.0, 183.1
19	26.4	Cyanidin glycoside adduct	610.9	**448.9**, 431.0, 284.9, 257.1
20	28.4	Pelargonidin glycoside adduct	792.9	757.1, **594.9**, 449.0, 431.1, 269.2, 225.1, 183.1
21	29.8	Cyanidin-3-glucoside	447.1	**285.1**, 257.0, 183.0
22	31.0	Cyanidin-3-rutinoside	593.0	**285.1**, 257.1, 183.1
23	33.5	Pelargonidin-3-glucoside	431.0	**269.1**, 225.0, 183.1
24	34.4	Peonidin-3-glucoside	461.0	**299.2**, 284.0, 240.0

[1] Ions in bold indicate predominant fragments, on which further MS analyses were conducted.

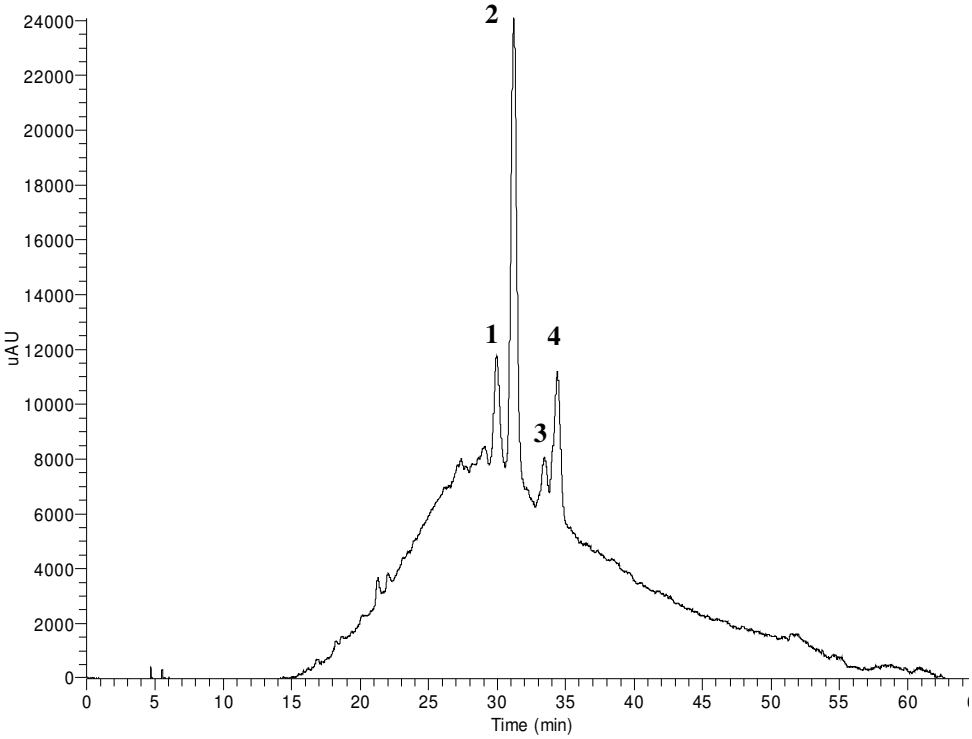


Fig. 21. Chromatographic profile (520 nm) of polymeric anthocyanin fractions from açai pulp. Peak assignments: 1. cyanidin-3-glucoside; 2. cyanidin-3-rutinoside; 3. pelargonidin-3-glucoside; and 4. peonidin-3-glucoside.

Peaks 11 and 13 were tentatively identified as a pelargonidin-3-glucoside-(+)-catechin adducts, yielding predominant ions at *m/z* 766.3 (formate adduct, $[M-H+HCOO]^-$) and *m/z* 721.1 ($[M-H]^-$). Subsequent water losses yielded ions at *m/z* 703.1 and *m/z* 685.4 respectively, followed by a neutral loss of 161 Da, possibly due to retro-diels-alder cleavage of a phenolic C-ring, and subsequent loss of B and C rings. This fragmentation pathway was consistent with that observed for a (+)-catechin standard, where retro-diels-alder cleavage of the C-ring resulted in signals at *m/z* 161.0, most likely a fragment of B and C rings, and *m/z* 125.0, probably due to the unmodified A ring of the (+)-catechin structure, in agreement with previous reports on (+)-catechin and (-)-epicatechin

fragmentation pathways (Miketova et al., 2000; Benavides, Montoro, Bassarello, Piacente, & Pizza, 2006). Moreover, a neutral loss of 290 Da from *m/z* 721.1 ($[M-H]^-$) to yield a signal at *m/z* 431.3 was also indicative of a (epi)catechin loss. Additional MS analyses on *m/z* 431.3 ($[M-H]^-$) and *m/z* 449.1 ($[M-H+H_2O]^-$), produced a major product signal at *m/z* 269.2 ($[M-H-162]^-$, glycoside loss), fragmenting to *m/z* 225.0 and *m/z* 183.0, corresponding to a pelargonidin aglycone (Lopes da Silva, De Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2002; Tian, Giusti, Stoner, & Shwartz, 2005).

Peaks 12, 16, and 19, likely cyanidin glycoside derivatives, were characterized by intensive ion signals at *m/z* 646.2 (formate adduct, $[M-H+HCOO]^-$) and *m/z* 611.1 ($[M-H]^-$). Tandem MS analyses yielded product ions at *m/z* 593.1 ($[M-H-H_2O]^-$) and 449.1 ($[M-H-162]^-$, possibly indicating loss of a glycosidic unit. Further fragmentation of *m/z* 449.1 produced signals at *m/z* 431.0 ($[M-H-H_2O]^-$) and *m/z* 285.3 ($[M-H-146]^-$, potentially due to loss of a deoxyhexose sugar), which was tentatively identified as a cyanidin aglycone, yielding ions at *m/z* 257.1 and *m/z* 125.0, in agreement with those of authentic cyanidin standards.

Peaks 14 and 15 were tentatively identified as a cyanidin glycoside adducts, yielding predominant ion signals at *m/z* 883.1 and *m/z* 864.9 ($[M-H]^-$), respectively. Subsequent neutral losses (162 Da) were likely due to the loss of a glycosidic unit, and resulted in strong ion signals at *m/z* 720.9, *m/z* 703.1, and *m/z* 685.0 ($[M-H-H_2O]^-$). Additional fragmentation on *m/z* 703.1 produced *m/z* 541.0 $[M-H-H_2O-162]^-$, probably due to the loss of a second glycosidic unit, and *m/z* 523.0 and *m/z* 505.1, resulting from neutral water losses (18 Da). Finally, a loss of 290 Da (from *m/z* 720.9), coincided with the mass

of (epi)catechin, and yielded fragments at 431.3 ($[M-H]^-$) and *m/z* 449.1 ($[M-H+H_2O]^-$), with a major product ion signal at *m/z* 285.0 ($[M-H-146]^-$, potentially following loss of a deoxyhexose), and corresponding to a cyanidin aglycone.

Peak 17 was tentatively assigned to a pelargonidin-glycoside derivative, characterized by a precursor ion at *m/z* 485.1 ($[M-H]^-$) and product ions at *m/z* 431.2 ($[M-H-36]^-$), 269.1 ($[M-H-162]^-$, glycoside loss), and *m/z* 225.0 and 183.0, corresponding to the fragmentation of a pelargonidin glycoside, most likely an hexose.

Peaks 18 and 20 were tentatively identified as a pelargonidin glycoside adducts, likely containing both an hexose and a deoxyhexose. A parent ion signal was observed at *m/z* 793.0 ($[M-H]^-$), with fragment ions at *m/z* 757.0 ($[M-H-36]^-$), *m/z* 595.0 ($[M-H-162]^-$, possibly due to an hexose loss) and *m/z* 448.9 ($[M-H-146]^-$, most likely due to loss of a deoxyhexose). Additional MS analyses on *m/z* 448.9 yielded ions at *m/z* 430.1 ($[M-H-H_2O]^-$), which fragmented to *m/z* 269.0 ($[M-H-162]^-$), and *m/z* 225.0 and 183.1 characteristic of a pelargonidin aglycone, as previously discussed.

Peaks 21 and 22 were respectively assigned to cyanidin-3-glucoside (*m/z* 447.1, $[M-H]^-$) and cyanidin-3-rutinoside (*m/z* 593.0, $[M-H]^-$), yielding typical cyanidin aglycone signals at *m/z* 285.1, *m/z* 257.0, and *m/z* 183.0. Identities were confirmed by comparison with authentic standards.

Peak 23 corresponded to pelargonidin-3-glucoside (*m/z* 431.0, $[M-H]^-$), fragmenting to 269.1 ($[M-H-162]^-$, following a glycoside loss), *m/z* 225.0, and *m/z* 183.1, typical of a pelargonidin aglycone, as previously established.

Peak 24 was identified as peonidin-3-glucoside (*m/z* 461.0, $[M-H]^-$). A neutral loss of 162 Da suggested the presence of an hexose, and resulted in a signal at *m/z* 299.2. Further fragmentation lead to signals at *m/z* 284.0, and *m/z* 240.0, characteristic of a peonidin aglycone (De Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2002; Kammerer, Carle, & Schieber, 2003; Tian et al., 2005).

In addition to mass spectral data, acid hydrolysis (2 N HCl, 90°C) of polymeric anthocyanin fractions confirmed the tentative identities previously assigned to anthocyanin aglycones (Fig. 22), and supported the presence of cyanidin (79.1 ± 5.1%), pelargonidin (16.6 ± 1.4%), and peonidin (4.3 ± 0.5%) aglycones in these fractions.

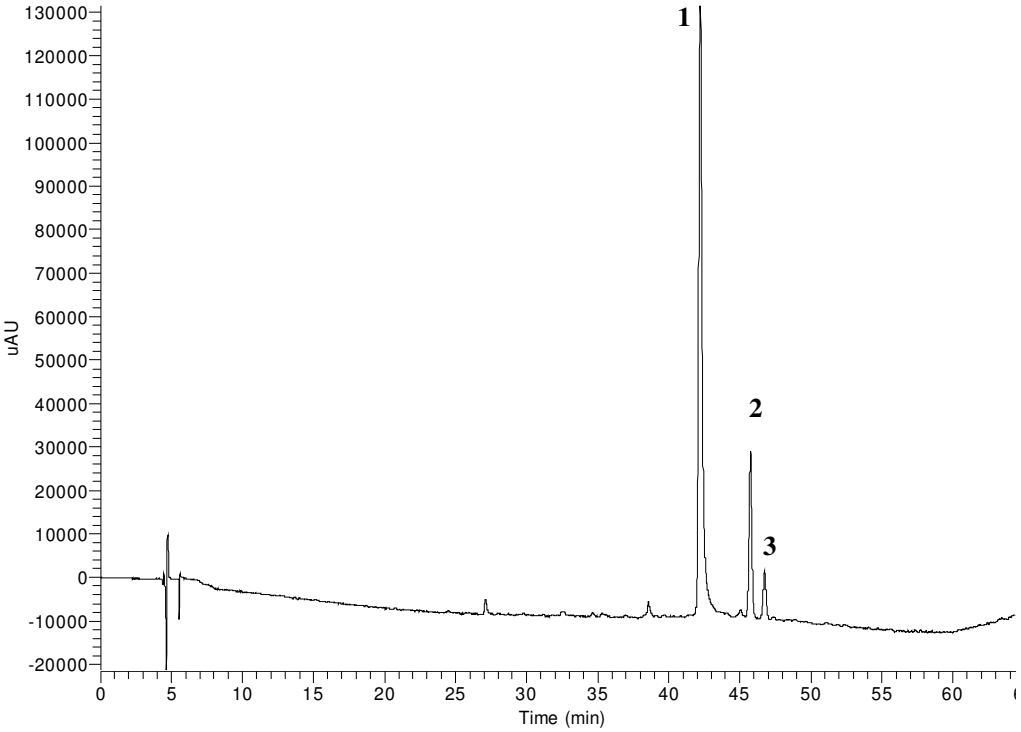


Fig. 22. Chromatographic profile (520 nm) of polymeric anthocyanin fractions from açai pulp following acid hydrolysis (2N HCl, 90°C) for 30 min. Peaks correspond to cyanidin (1), pelargonidin (2), and peonidin (3) aglycones.

As previously discussed, results from mass spectrometric analyses (Table 17) on polymeric anthocyanin fractions from açai pulp indicated the presence of a series of ions corresponding to masses consistent with direct condensation products of cyanidin and pelargonidin glycosides with flavanol derivatives. Increases on the magnitude of these ion signals were associated to higher, unresolved UV absorption at ~520 nm (Fig. 23). Moreover, higher ion signals were translated to increased UV signals, giving rise to new "peaks" (Table 17, peaks 1-7; Fig. 23), exhibiting absorption at 510-520 nm. These new signals generally preceded those for anthocyanin glycosides in HPLC chromatograms, indicating the relatively more polar character of the newly formed anthocyanin adducts.

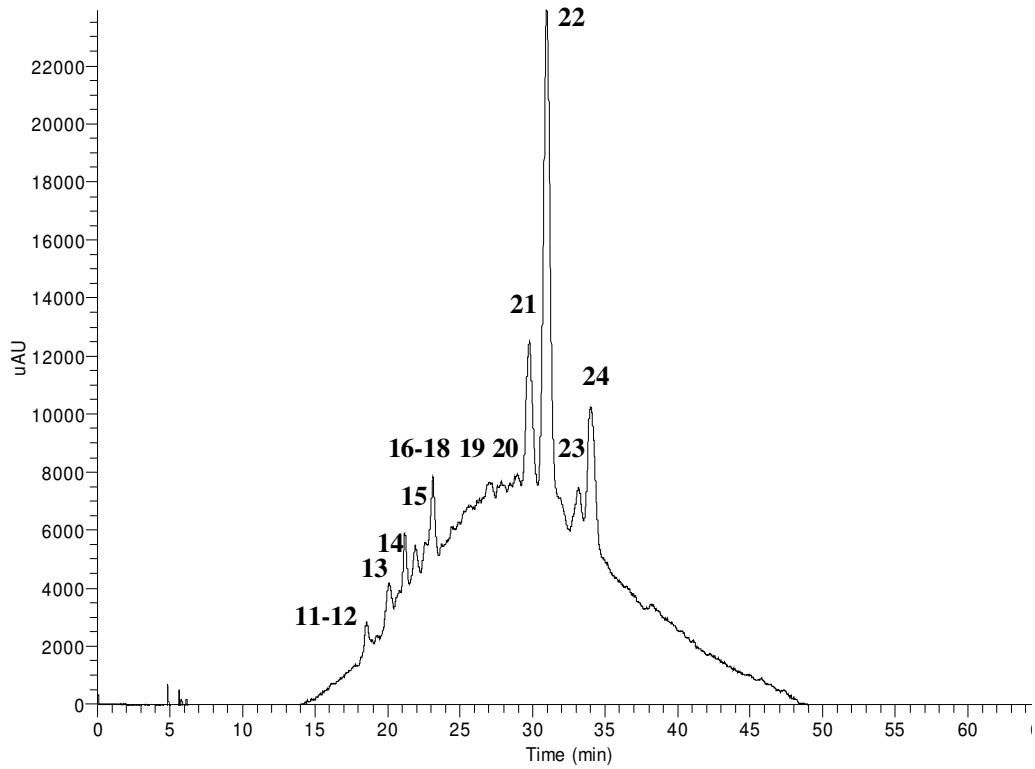


Fig. 23. Chromatographic profile (520 nm) of polymeric anthocyanin fractions from açai pulp following storage for 12 days at 35°C. Peak identities are summarized in Table 17.

In addition, polymeric anthocyanin fractions had higher sulfite bleaching resistance (60.4 ± 4.9% color retention) than their anthocyanin glycoside counterparts (9.5 ± 1.1% color retention), when total absorbance following sulfite addition was measured. These potential anthocyanin-flavanol adducts also resisted thiolysis, as (+)-catechin or (-)-epicatechin benzylthioethers were absent following thiolysis. However, both (+)-catechin (13.8 ± 0.4 mg/L) and (-)-epicatechin (161.3 ± 6.6 mg/L) were detected following thiolysis of polymeric anthocyanin fractions. Epimerization of flavanol standards under the thiolysis conditions used in these trials indicated conversion to (-)-epicatechin (29.9 ± 0.2%) was favored over epimerization to (+)-catechin (11.5 ± 0.7%), while similar recovery rates (65.8 ± 3.9%) were observed for both compounds. Thus, while no evidence on the occurrence of procyanidins or related flavanol derivatives in polymeric anthocyanin fractions was found, detection of (+)-catechin and (-)-epicatechin in polymeric anthocyanin fractions following thiolysis was consistent with their potential presence as structural components in anthocyanin adducts.

The influence of previously characterized “polymeric” anthocyanin fractions (Table 17), on anthocyanin stability and the formation of anthocyanin-based adducts during accelerated storage (35°C) was evaluated in model anthocyanin systems. Models were based on anthocyanin “monomer” isolates (550 mg cyanidin-3-glucoside equivalents/L), rich in cyanidin-3-glucoside and cyanidin-3-rutinoside (Table 16), to which “polymeric” anthocyanin fractions were added in varying proportions (0, 10, 20, 30, and 50%). Kinetic parameters of cyanidin-3-glucoside and cyanidin-3-rutinoside degradation, including first-order ($p<0.05$) degradation rates (β_1, in $days^{-1}$) and half-lives

(t½, or days to achieve a 50% reduction on initial anthocyanin concentrations) were calculated as described in the previous chapter and are shown in Table 18.

Table 18. Kinetic parameters of cyanidin-3-glucoside and cyanidin-3-rutinoside degradation during storage (35°C) of açai anthocyanin models.

Anthocyanin model	Cyanidin-3-glucoside		Cyanidin-3-rutinoside	
	β_1 [a]	$t_{1/2}$ [b]	β_1	$t_{1/2}$
100% monomers	20.8	33.3 a [c]	18.7	37.1 a
90% monomers, 10% polymers	48.1	14.4 b	20.7	33.5 a
80% monomers, 20% polymers	65.6	10.6 b	22.3	31.1 ab
70% monomers, 30% polymers	91.0	7.62 c	24.8	27.9 b
50% monomers, 50% polymers	96.8	7.16 c	28.2	24.6 bc
100% polymers	113	6.14 c	34.8	19.9 c

[a] Anthocyanin degradation rate constant (β_1 x 10^3 $days^{-1}$). [b] Half-life (days) of initial absorbance for each juice model. [c] Values with different letters within the same column are significantly different (student's t test, $p<0.05$).

Kinetic parameters of cyanidin-3-glucoside and cyanidin-3-rutinoside degradation during storage (Table 18) were significantly influenced ($p<0.01$) by the presence of polymeric anthocyanin fractions, and resulted in reduced anthocyanin half-lives ($t_{1/2}$) for models with polymeric anthocyanins added. Anthocyanin losses were proportional ($p<0.05$) to the relative amount of polymeric anthocyanins present and were correlated to increased anthocyanin sulfite bleaching resistance ($p<0.05$, $r=0.71$, initially

22.3 ± 1.8%) and to decreased antioxidant capacity ($p<0.05$, $r=0.49$, initially 36.3 ± 1.9 µmol TE/mL). Moreover, anthocyanin losses induced by the addition of anthocyanin polymers were related to higher, unresolved UV absorption at ~520 nm and to an apparent increase in the magnitude of MS signals at 18-24 min, consistent with the fragmentation patterns of previously identified (Table 17) anthocyanin-flavanol adducts, as shown in Table 19.

Table 19. Percent increase in MS ion signals of anthocyanin-based adducts following storage (35°C for 12 days) of açai anthocyanin models.

Peak No.	RT (min)	$[M-H]^-$ (*m/z*)	% Increase in Ion Signal			
			Models with 10% polymers	Models with 20% polymers	Models with 30% polymers	Models with 50% polymers
11	17.9	721.3	10.6 c[1]	24.5 b	38.4 a	40.1 a
12	18.6	611.1	12.2 c	26.0 b	39.9 a	43.3 a
13	20.2	721.2	9.81 c	21.5 b	33.1 a	36.3 a
14	21.2	883.1	8.90 c	20.2 b	32.8 a	34.8 a
15	21.9	864.9	7.90 c	19.5 b	28.6 a	31.2 a
16	22.8	611.1	9.91 c	16.3 b	24.0 a	28.1 a
17	23.2	485.1	9.42 c	15.7 b	23.5 a	24.8 a
18	23.9	793.0	9.35 c	15.4 b	23.1 a	25.2 a

[1] Values with different letters within the same row are significantly different (student's t test, $p<0.05$).

Detrimental effects of polymeric anthocyanin fractions on anthocyanin stability were consistently more pronounced for cyanidin-3-glucoside, where addition of 10% anthocyanin polymers resulted in a 2.3-fold decrease in anthocyanin half-life, but did not

alter ($p<0.05$) the stability of cyanidin-3-rutinoside (Table 18). Additional studies using authentic cyanidin-3-glucoside standards confirmed these observations, as half-lives were reduced by 1.5 to 2.1-fold following addition (10 to 50% v/v) of polymeric anthocyanin fractions from açai (Table 20).

Table 20. Kinetic parameters of cyanidin-3-glucoside degradation during storage (35°C) of models based on anthocyanin standards.

Anthocyanin model	β_1 [a]	$t_{1/2}$ [b]
Cyanidin-3-glucoside standard	44.7	15.5 a [c]
Cyanidin-3-glucoside with 10% açai polymers	65.2	10.6 b
Cyanidin-3-glucoside with 20% açai polymers	72.8	9.52 b
Cyanidin-3-glucoside with 30% açai polymers	78.8	8.79 bc
Cyanidin-3-glucoside with 50% açai polymers	94.2	7.36 c

[a] Reaction rate constant ($\beta_1 \times 10^3$ days^{-1}). [b] Half-life (days) of initial absorbance for each juice model. [c] Values with different letters within the same column are significantly different (student's t test, $p<0.05$).

Minor reductions in anthocyanin half-lives might be attributed to the lower stability of pure cyanidin-3-glucoside standards ($t_{1/2}$= 15.5 days, Table 20) when compared to analogous isolates from açai ($t_{1/2}$= 33.3 days, Table 18), likely due to the stabilizing effect of additional phenolic components (Table 16) on anthocyanins, as discussed in chapter VI.

Additional studies were conducted to assess the influence of polymeric anthocyanin fractions from açai on the stability of anthocyanin isolates from an external source, namely blackberry (508 ± 31 mg cyanidin-3-glucoside equivalents/L), containing high proportions of cyanidin-3-glucoside (78.9 ± 5.4%) and cyanidin-3-rutinoside (21.1 ± 1.3%). Addition of polymeric anthocyanin fractions resulted in dose-dependent decreases in anthocyanin half-lives (Table 21).

Table 21. Kinetic parameters of cyanidin-3-glucoside and cyanidin-3-rutinoside degradation during storage (35°C) of blackberry anthocyanin models.

Anthocyanin model	Cyanidin-3-glucoside		Cyanidin-3-rutinoside	
	β_1 [a]	$t_{1/2}$ [b]	β_{11}	$t_{½}$
100% blackberry monomers	27.5	25.2 a [c]	19.2	36.2 a
90% blackberry monomers, 10% açai polymers	56.0	12.4 b	21.1	32.9 ab
80% blackberry monomers, 20% açai polymers	61.3	11.3 b	21.3	32.6 ab
70% blackberry monomers, 30% açai polymers	69.8	9.94 bc	25.7	27.0 b
50% blackberry monomers, 50% açai polymers	85.1	8.15 c	29.8	23.3 c

[a] Anthocyanin degradation rate constant ($\beta_1 \times 10^3$ $days^{-1}$). [b] Half-life (days) of initial absorbance for each juice model. [c] Values with different letters within the same column are significantly different (student's t test, $p<0.05$).

Similar to previous observations in açai-based models, the presence of anthocyanin polymers (10 to 50% v/v) induced 2.0 to 3.1-fold higher cyanidin-3-

glucoside degradation, while only increased cyanidin-3-rutinoside degradation by 1.1 to 1.6-fold (Table 21). Anthocyanin losses were correlated to increased resistance to sulfite bleaching ($p<0.05$, $r=0.67$, initially 10.1 ± 1.1%), and decreased antioxidant capacity ($p<0.05$, $r=0.44$, initially 19.1 ± 1.8 µmol TE/mL). Losses were also related to an increase in unresolved absorption at ~500 nm, and to 10 to 33% higher ion signals for previously identified (Table 17) anthocyanin adducts, in agreement with prior observations in açai-based models (Table 19). Possible mechanisms for the formation of anthocyanin-flavanol adducts are addressed below.

Potential mechanisms for the formation of anthocyanin-based adducts were further investigated. Aldehyde-mediated condensation of anthocyanins and flavanols was initially considered, as pasteurization of fruit juices may result in thermal decomposition of sugars, and the subsequent formation of aldehydic derivatives, most commonly 5-(hydroxymethyl)furfural (HMF) (Lee & Nagy, 1990; Burdulu & Karadeniz, 2003; Babsky, Toribio, & Lozano, 2006); and interactions between anthocyanins and furfural derivatives have been reported in model systems (Es-Safi et al., 2000; Es-Safi et al., 2002). Thus, the presence of HMF in a variety of commercial açai pulps and both of the anthocyanin fractions used in these trials was evaluated by tandem HPLC-ESI-MS^n. However, HMF concentrations were below quantitation limits (<10 µmol). Furthermore, MS fragmentation patterns of previously observed anthocyanin adducts did not correspond to those typical aldehyde-mediated anthocyanin-flavanol adducts, which are commonly linked by ethyl bridges (Timberlake & Bridle, 1976; Fulcrand, Docco, Es-Safi, Cheynier, & Moutounet, 1996; Dallas et al., 1996;

Francia-Aricha et al., 1997) and are generally characterized by the detection of ethanol-flavanol adducts (*m/z* 333, $[M-H]^-$) in ESI-MS^n analyses (Es-Safi, Fulcrand, Cheynier, & Moutounet, 1999; Es-Safi et al., 2000; Es-Safi et al., 2002).

Additional mechanisms for the formation of anthocyanin-based adducts were considered based on the obtained MS fragmentation data (Table 17), which suggested the presence of direct anthocyanin-polymer linkages in these polymers. The linkage connecting the anthocyanin to the polymer may be one or more of several types known to occur in condensed tannins, including the acid and heat-catalyzed 2-6 or 2-8 C-C links between flavonoid units and heterocyclic ring opening of the 2-substituent (Jurd, 1969), the 4-6 or 4-8 C-C link, as in most natural polymeric flavanols, or the products of oxidative free-radical condensation, forming both C-C and C-O B-ring linkages (Singleton, 1972). According to Jurd (1969), the 4-6 or 4-8 C-C leucoanthocyanidin type is the most likely for the incorporation of an anthocyanin into a polymer, although it would be possible for all to incorporate anthocyanins, at least as end units.

Formation of anthocyanin-flavanol adducts in dilute acid solutions (pH 3-5), both in presence and absence of oxygen, has been previously reported (Jurd, Waiss, & Bergot, 1965; Jurd, 1967; Somers, 1971), and a direct condensation reaction has been proposed. Direct condensation reactions would also increase anthocyanin resistance to sulfite bleaching, commonly used to estimate polymeric anthocyanin contents, as linkages in the 4-position makes anthocyanins resistant to bleaching by sulfite (Singleton, 1972). Dimers resulting from the direct condensation between anthocyanins and (epi)catechins have also been detected in red wines (Remy et al., 2000; Vivar-Quintana, Santos-Buelga,

& Rivas-Gonzalo, 2002; Remmy-Tanneau et al., 2003; Alcalde-Eon, Escribano-Bailon, Santos-Buelga, & Rivas-Gonzalo, 2006), and their formation has been attributed to anthocyanin reactions with flavanols, particularly procyanidins, which result in new pigment species during wine storage (Salas, Le Guerneve, Fulcrand, Poncet-Legrand, & Cheynier, 2004b).

Two basic mechanisms leading to the formation of (1) anthocyanin-flavanol (A-F) or (2) flavanol-anthocyanin (F-A) adducts have been proposed: (1) nucleophilic addition of the flavanol onto the flavylium cation of the anthocyanin, leading to the colorless flavene, which is then oxidized to a red flavylium, resulting in an anthocyanin-flavanol dimer and a monomeric flav-2-ene, as shown in Fig. 24 (Jurd, 1969; Somers, 1971; Salas, 2004b) and (2) acid-catalyzed cleavage of the interflavanic bond of procyanidins, releasing an intermediate carbocation at C4 that reacts with the hydrated hemiketal form of anthocyanins via nucleophilic addition, resulting in a colorless dimer that dehydrates to a red flavylium form (Haslam, 1980; Salas, 2004b), as shown in Fig. 25. These reactions would lead to two possible structures: (1) an anthocyanin linked by its C-4 position to the C-6 or C-8 positions of a flavanol (A-F adduct, Fig. 24) or (2) an anthocyanin linked by its C-8 or C-6 position to the C-4 of a flavanol (F-A adduct, Fig. 25). Evidence in favor of a flavanol-anthocyanin structure has been recently obtained by MS^n fragmentation patterns (Salas et al., 2004a; Gonzalez-Paramas et al., 2006; Dueñas, Fulcrand, & Cheynier, 2006) and hemisynthesis (Salas et al., 2004a; Salas et al., 2004b). A complete structural characterization of similar pigments isolated from strawberries (Fossen, Rayyan, & Andersen, 2004) and from purple corn (Gonzalez-Manzano et al.,

2008) using NMR techniques has also confirmed their flavanol-anthocyanin (F-A) nature.

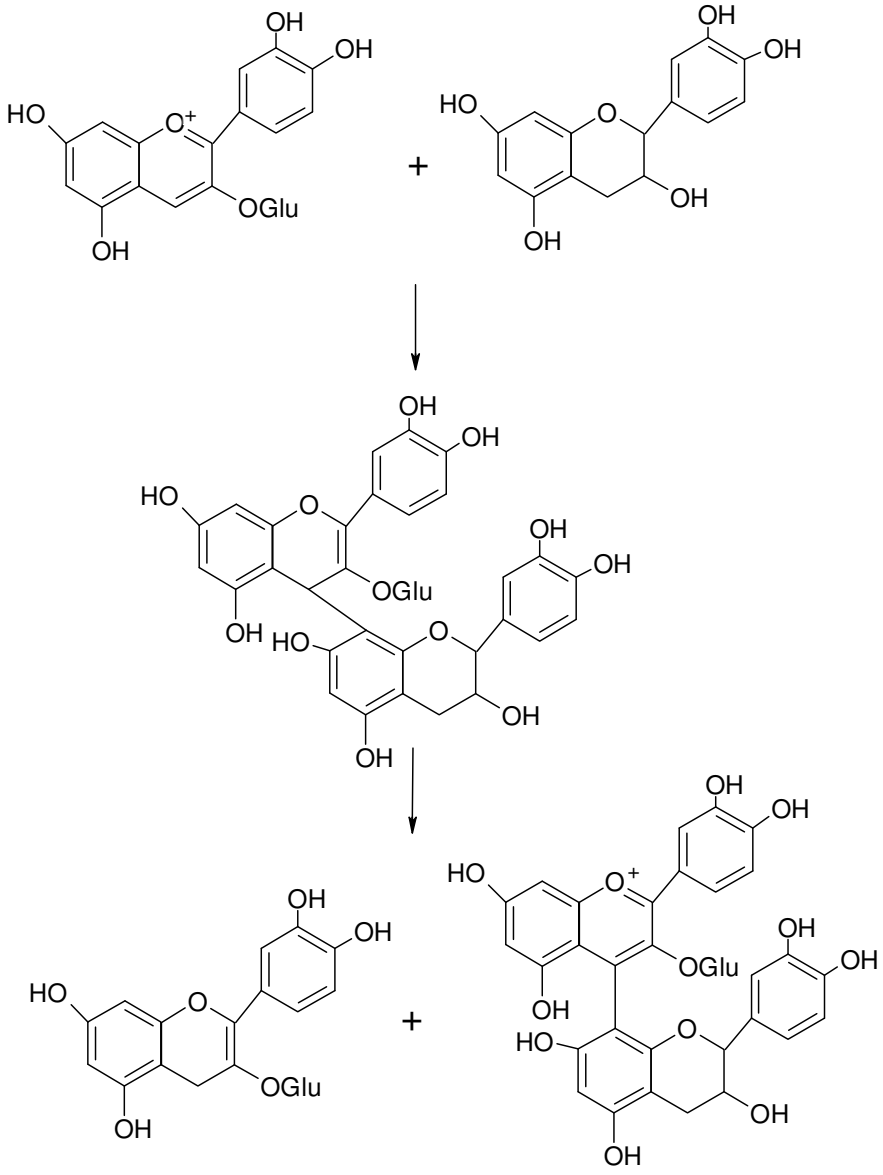


Fig. 24. Proposed mechanism for the formation of anthocyanin-flavanol adducts.

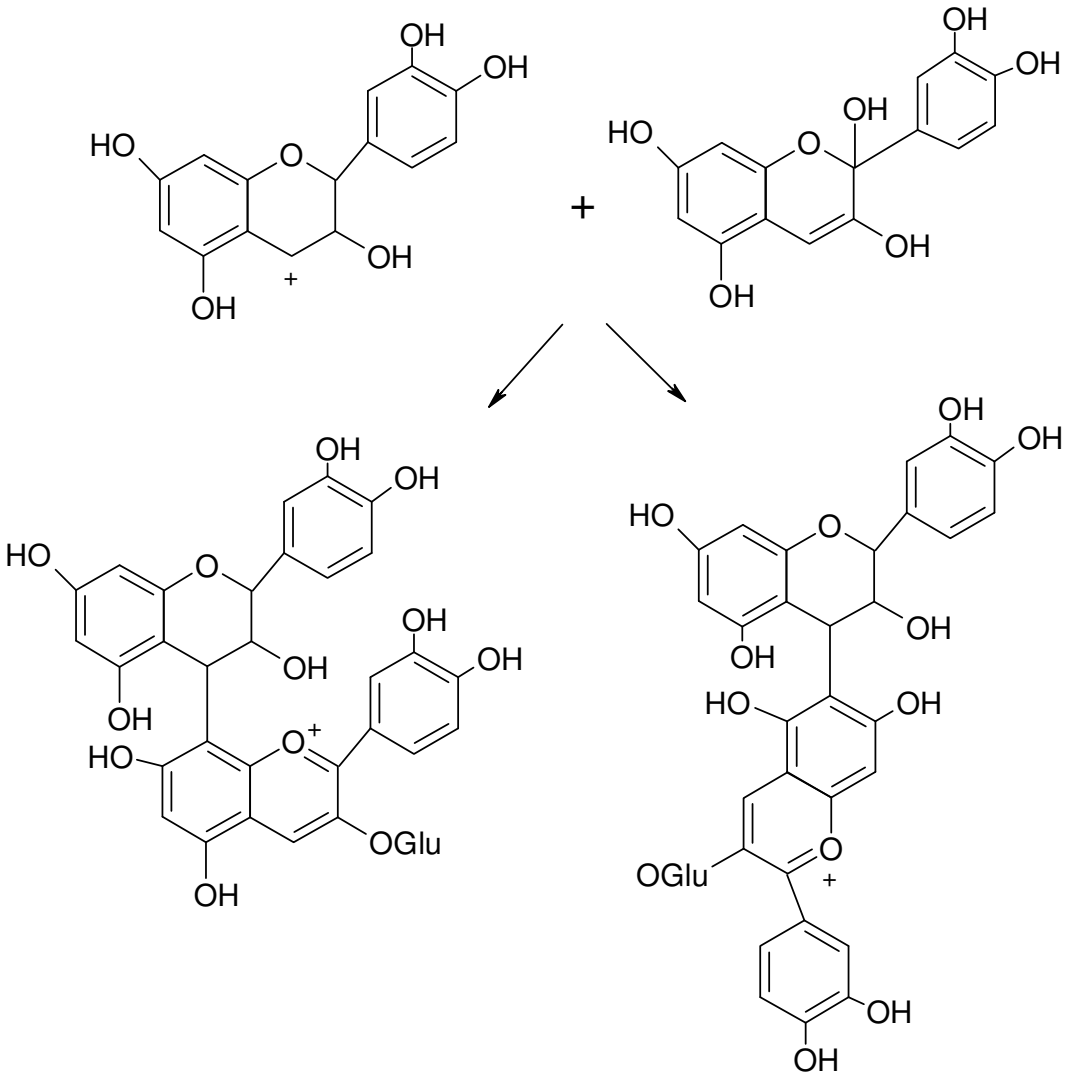


Fig. 25. Proposed mechanism for the formation of flavanol-anthocyanin adducts.

The formation of similar flavanol-anthocyanin adducts during storage of açai fruit juices arising from direct reactions between anthocyanins and (+)-catechins or procyanidins would therefore be feasible. However, monomeric flavanol derivatives, (+)-catechin and (-)-epicatechin, are only present in low concentrations (<10 mg/L) in fresh açai fruit (chapter III), and are stable during storage of açai fruit juices (Pacheco-

Palencia et al., 2007a). Our previous investigations using açai fruit anthocyanin models with added phenolic cofactors (chapter VI), also indicated that the presence of procyanidins does not influence anthocyanin degradation or polymerization reactions during storage, suggesting additional mechanisms may be responsible for the formation of anthocyanin-flavanol adducts in açai fruit juices.

The natural occurrence of flavanol-anthocyanin condensed pigments in açai fruit was investigated, as these condensed pigments could participate in similar mechanisms to those leading to the formation of flavanol-anthocyanin adducts (Fig. 25). Anthocyanin-flavanol condensed pigments have been reported to occur naturally in strawberry (Fossen et al., 2004; Gonzalez-Paramas et al., 2006; Lopes da Silva, Escribano-Bailon, Perez-Alonso, Rivas-Gonzalo, & Santos-Buelga, 2007), black currants (McDougall, Gordon, Brennan, & Stewart, 2005), runner beans, grape skin extracts (Gonzalez-Paramas et al., 2006) and purple corn (Gonzalez-Paramas et al., 2006; Gonzalez-Manzano et al., 2008) and their presence in fresh açai fruit pulps (Fig. 21) may indicate that they are natural pigments in açai fruit, and not exclusive products of anthocyanin polymerization reactions occurring during storage. It was therefore hypothesized that naturally occurring anthocyanin-flavanol pigments in açai fruit promote the formation of flavanol-anthocyanin adducts via similar mechanisms to those observed in the presence of procyanidins. Hence, formation of flavanol-anthocyanin adducts would be initiated by cleavage of interflavanic bonds and subsequent release of intermediate nucleophilic carbocations at C-4 (F^+), prone to react with the hydrated form

of anthocyanins (AOH) at positions C-8 or C-6, leading to 4-8 or 4-6 flavanol-anthocyanin adducts (Fig. 25).

Additional reactions involving the carbocations (F^+) resulting from cleavage of anthocyanin-flavanol adducts could also lead to polymerization with other phenolic compounds, including anthocyanins, and could result in the formation of large, structurally diverse polymers during storage of açai fruit juices. These polymers may also be responsible for increased anthocyanin sulfite resistance, hue changes towards dark red colors, and the appearance of a wide, unresolved peak in the HPLC chromatograms of açai fruit juices during storage. Additional studies using NMR techniques may assist in the complete structural characterization of these polymers in açai fruit juices and facilitate their identification in other fruit matrices.

Conclusion

Anthocyanin polymerization reactions occurring during storage of açai fruit juices were investigated. Several anthocyanin-flavanol adducts based on cyanidin or pelargonidin aglycones were tentatively identified by HPLC-ESI-MS^n analyses and their presence was related to increased anthocyanin sulfite bleaching resistance and to the appearance of a wide, unresolved peak in HPLC chromatograms. A potential reaction mechanism involving nucleophilic addition of anthocyanins in their hydrated form to flavanol carbocations resulting from cleavage of interflavanic bonds was proposed for the formation of flavanol-anthocyanin adducts in açai fruit juices. The natural occurrence of condensed flavanol-anthocyanin pigments in açai fruit was also suggested

to promote the formation of additional anthocyanin adducts and further polymerization with other phenolic components, potentially resulting in a variety of structurally diverse anthocyanin polymers during storage of açai fruit juices. Results from these studies provide new insights on the nature and occurrence of anthocyanin polymerization reactions in açai fruit juices.

CHAPTER VIII

IN-VITRO ABSORPTION AND ANTIPROLIFERATIVE ACTIVITY OF MONOMERIC AND POLYMERIC ANTHOCYANIN FRACTIONS FROM AÇAI FRUIT

Introduction

Anthocyanins are considered one of the largest and most important plant pigments and are responsible for most red, blue, and purple hues in fruits, vegetables, and flowers (Kong et al., 2003). Nutritional interest in anthocyanins is based on their considerable daily intake (180 to 215 mg/day in the United States), which is significantly higher than the estimated intake of other flavonoids (23 mg/day), including quercetin, kaempferol, myricetin, apigenin, and luteolin (Galvano et al., 2004). Yet numerous studies have reported the relatively low absorption (generally <1.0%) of anthocyanins from rich dietary fruit sources, in both animal and human trials (Miyazawa, Nakagawa, Kudo, Muraishi, & Someya, 1999; Bub, Watzl, Heeb, Rechkemmer, & Briviba, 2001; Matsumoto, Inaba, Kishi, Tominaga, Hirayama, & Tsuda, 2001; Felgines, Texier, Besson, Fraisse, Lamaison, & Remesy, 2002). Anthocyanins were also shown to be absorbed mostly without change in their glycosylated form directly into the bloodstream (Cao, Muccitelli, Sanchez-Moreno, & Prior, 2001) with peak plasma concentrations observed within 1-2 hours of ingestion (Matsumoto et al., 2001). However, anthocyanins can also be metabolized in vivo to modified forms, including methylated, glucuronated,

and sulfo-conjugated anthocyanin metabolites, which have been reported in urine and blood plasma (Felgines et al., 2003).

Information on the absorption of anthocyanin glycosides is widely available, yet the literature is devoid of information concerning the absorption or mere presence of anthocyanin polymers despite a preponderance of studies that show that anthocyanin polymerization reactions readily occur during the processing and storage of anthocyanin-containing fruits and vegetables. Studies on the absorption of structurally similar compounds, such as flavanols, have revealed high absorption levels for monomers that decrease as the degree of polymerization increases (Shoji et al., 2006). Scalbert et al. (2000) reported (+)-catechin, procyanidin dimer B3, and procyanidin trimer C2 were transported through the human intestinal epithelial Caco-2 cell monolayer yet polymers with a higher degree of polymerization were not. Additional studies using LC-MS techniques have suggested that procyanidin dimers were absorbed in vitro (Spencer, Schroeter, Shenoy, Srai, Debnam, & Rice-Evans, 2001) and also into the bloodstream in both animals (Baba, Osakabe, Natsume, & Terao, 2002) and humans (Sano, Yamakoshi, Tokutake, Tobe, Kubota, & Kikuchi, 2003; Shoji et al., 2006). However, the limits of polyphenolic absorption in relation to the degree of polymerization are still unclear.

Açai fruit (*Euterpe oleracea* Mart.) was selected as an anthocyanin source for these trials, mainly due to its inherently high polymeric anthocyanin content, commonly between 25 and 45% in fresh açai fruit pulp (Pacheco-Palencia et al., 2007a; Pacheco-Palencia et al., 2007b), and its high anthocyanin polymerization rates during storage, reaching polymeric anthocyanin contents of 70-80% in 8 days at 35°C (Pacheco-

Palencia et al., 2007a). Thus, a few days of storage may result in significant variations in anthocyanin composition in açai fruit juices, potentially leading to changes in anthocyanin bioactive and absorption properties. These studies were conducted to evaluate the influence of anthocyanin monomer and polymers on the in vitro intestinal absorption and antiproliferative activity of anthocyanins from açai fruit and to determine a dose-response for anthocyanin forms potentially absorbed by the human body. Caco-2 cell monolayers were used as in vitro models for intestinal absorption, while antiproliferative activities were evaluated in HT-29 human colon adenocarcinoma cells. Results from these investigations are aimed at promoting postharvest, processing, and storage conditions that retain absorbable and bioactive anthocyanin forms in juices, beverages, and other anthocyanins-containing food products.

Materials and Methods

Anthocyanin models were based from 100% açai fruit pulp anthocyanin isolates. Pasteurized açai pulp was donated by the Bossa Nova Beverage Group (Los Angeles, CA) and shipped frozen to the Department of Nutrition and Food Science at Texas A&M University. Prior to anthocyanin isolation, açai pulp was clarified according to a previously described procedure (Pacheco-Palencia et al., 2007b) to remove lipids and insoluble solids. Clarified açai pulp was then loaded onto activated C18 Sep-Pak 6 cc cartridges (Waters Corporation, Milford, MA) and eluted sequentially with water and ethyl acetate to remove sugars, organic acids, metals, proteins, phenolic acids, and other flavonoids (Pacheco-Palencia et al., 2007a). Anthocyanins were recovered with acidified

methanol (0.01% HCl) and redissolved in a 0.1M citric acid buffer (pH 3.0) following solvent evaporation under vacuum (<40°C). The resulting anthocyanin isolate was adsorbed onto a second Sep-Pak cartridge and eluted with an alkaline borate solution (0.1 N, pH 9.0), until a colorless eluent was obtained. Eluted anthocyanin monomers were immediately diluted with 4N HCl to favor anthocyanin equilibrium toward the more stable red flavylium form, loaded onto a third Sep-Pak cartridge, and recovered with methanol (0.01% HCl). Polymeric anthocyanins adsorbed to the second cartridge were likewise eluted with acidified methanol (0.01% HCl), and both fractions were concentrated under vacuum at <40°C until complete solvent removal and reconstituted in a 0.1 M citric acid buffer (pH 3.0). Anthocyanin monomer and polymer fractions were standardized to a final concentration of ~2500 mg cyanidin-3-glucoside equivalents/L, determined spectrophotometrically (Pacheco-Palencia et al., 2007b). All anthocyanin isolates were kept at -20°C and sterile-filtered prior use in cell culture experiments.

Polyphenolic isolates were analyzed by reversed phase HPLC and mass spectrometric analyses, performed on a Thermo Finnigan LCQ Deca XP Max MSn ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA). Separations were conducted using an Acclaim 120 5µm 120Å (4.6 x 250 mm) column (Dionex Corporation, Sunnyvale, CA). Mobile phases consisted of 0.5% formic acid in water (phase A) and 0.5% formic acid in methanol (phase B) run at 0.6 mL/min. Polyphenolics were separated with a gradient elution program in which phase B changed from 5 to 10% in 3 min, from 10 to 30% in the following 17 min, from 30 to 50% in the following 20 min, from 50 to 70% in the following 15 min, and from 70 to 100% in the

following 10 min. Initial conditions were then restored and held constant for 20 min prior each injection. Ionization was conducted in the negative ion mode under the following conditions: sheath gas (N2), 60 units/min; auxiliary gas (N2), 5 units/min; spray voltage, 3.5 kV; capillary temperature, 250 °C; capillary voltage, 1.5 V; tube lens offset, 0 V. Identification and quantitation of polyphenolics was based on their spectral characteristics, retention time, and mass spectrometric properties, as compared to authentic cyanidin-3-glucoside and cyanidin-3-rutinoside standards (Sigma Chemical Co., St. Louis, MO). Antioxidant capacity was determined by the oxygen radical absorbance capacity assay using a BMG Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission), as described previously (Pacheco-Palencia et al., 2007b). Results were expressed in μmol Trolox equivalents, TE/mL. Total soluble phenolic contents were determined by the Folin-Cioucalteau assay, as a measure of the reducing ability of anthocyanin fractions, as noted in Chapter IV.

Antiproliferative activity of anthocyanin fractions against HT-29 human colon adenocarcinoma cells was evaluated. HT-29 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA), cultured in Dulbecco's modified Eagle's medium (1X) (DMEM) containing 5% fetal bovine serum, 1% non-essential amino acids, 100 units/mL penicillin G, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, and 10 mM sodium pyruvate (Gibco BRL Life Technology, Grand Island, NY). Cells were incubated at 37°C under 5% CO_2, and used between passages 10-20. Cells were seeded at a density $2x10^4$ cells/well, into 24-well tissue culture plates. Following incubation for 24 h, the growth medium was replaced with 500 μL of media containing

different concentrations of standardized anthocyanin extracts (from 0.5 to 100 µg cyanidin-3-glucoside equivalents/mL). Cell numbers were determined following incubation for 48 h, using a Beckman Coulter Particle Counter (Fullerton, CA). Cell numbers were expressed as a percentage of the untreated control and the anthocyanin extract concentration at which cell proliferation was inhibited by 50% (IC_{50}) was calculated by linear regression analyses for each anthocyanin fraction.

Transepithelial transport models using Caco-2 colon carcinoma cells were used to evaluate in-vitro absorption properties of anthocyanin fractions. Caco-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (1X) high glucose (DMEM) containing 10% fetal bovine serum, 1% non-essential amino acids, 100 units/mL penicillin G, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate (all chemicals supplied by Sigma-Aldrich Co., St. Louis, MO). Cells between passages 5-10 were seeded in 12-mm transparent polyester cell culture insert well plates (Transwell, Corning Costar Corp., Cambridge, MA) at 1.0 x 10^5 cells per insert, with 0.5 mL of growth medium in the apical side and 1.5 mL in the basolateral side. Cells were grown and differentiated to confluent monolayers for 21 days, as previously described (Hidalgo, Raub, & Borchardt, 1989). Transepithelial electrical resistance (TEER) values were monitored with an EndOhm Volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments Inc., Sarasota, FL). Monolayers with TEER values >450 Ω cm^2 after correction for the resistance in control wells were used for transport experiments. To insure monolayer integrity, TEER values

were also measured at the conclusion of transport experiments and data was only collected from monolayers with corrected TEER values >350 Ω cm^2. Prior transport experiments, growth media was replaced by Hank's balanced salt solution (HBSS, Fischer Scientific, Pittsburgh, PA) containing 10 mM 2-(*N*-morpholino)ethanesulfonic acid solution (MES, Gibco BRL Life Technology, Grand Island, NY) adjusted to pH 6.0 in the apical side, and HBSS containing *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] buffer solution (1 M) (HEPES, Gibco BRL Life Technology, Grand Island, NY) adjusted to pH 7.4 in the basolateral side, creating a pH gradient similar to the small intestine environment. Sample aliquots (200 µL) were taken from the basolateral compartment at 0.5, 1.0, 1.5, and 2.0 h, immediately acidified with a known volume of 4N HCl, kept frozen (-20°C), and analyzed within one week. Samples were finally filtered through 0.45µm PTFE membranes (Whatman, Florham Park, NJ) and injected directly into the HPLC-ESI-MS system.

Statistical analyses were performed in all data and included one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL) using α=0.05. Mean separations were conducted by post-hoc LSD ($p<0.05$) pairwise comparisons. A significance level of 0.05 was also used for parametric correlations and linear regression analyses.

Results and Discussion

Polyphenolic composition of monomeric and polymeric anthocyanin fractions was evaluated. Anthocyanins present in monomeric and polymeric fractions from açai

Table 22. HPLC-ESI-MS^n of monomeric and polymeric anthocyanin fractions from açai fruit.

Monomeric Anthocyanin Fractions			
Retention Time (min)	**Anthocyanin**	**[M-H]$^-$ (*m/z*)**	**MS/MS (*m/z*)^1**
29.7	Cyanidin-3-glucoside	447.0	285.1, 257.1, 183.0
31.5	Cyanidin-3-rutinoside	593.1	285.1, 257.1, 183.0
34.1	Pelargonidin-3-glucoside	431.0	269.0, 225.0, 183.1
Polymeric Anthocyanin Fractions			
Retention Time (min)	**Anthocyanin**	**[M-H]$^-$ (*m/z*)**	**MS/MS (*m/z*)^1**
19.1	Cyanidin glycoside adduct	611.0	593.0, 449.1, 431.1, 285.2, 257.1
20.7	Pelargonidin glycoside adduct	721.1	685.1, 524.0, 431.1, 269.1, 225.1
21.8	Cyanidin glycoside adduct	883.0	721.1, 685.1, 524.0, 431.1, 269.1, 225.1
22.5	Cyanidin glycoside adduct	865.1	685.0, 431.1, 285.0, 257.0, 183.1
23.4	Cyanidin glycoside adduct	611.0	593.0, 449.1, 285.2, 257.1
26.8	Cyanidin glycoside adduct	611.1	593.0, 449.1, 285.2, 257.1
28.7	Pelargonidin glycoside adduct	793.0	595.0, 449.1, 431.1, 269.0, 225.1
29.5	Cyanidin-3-glucoside	447.0	285.1, 257.1, 183.0
31.5	Cyanidin-3-rutinoside	593.1	285.1, 257.1, 183.0
34.1	Pelargonidin-3-glucoside	431.0	269.0, 225.0, 183.1
35.6	Peonidin-3-glucoside	461.0	299.1, 284.1, 240.0

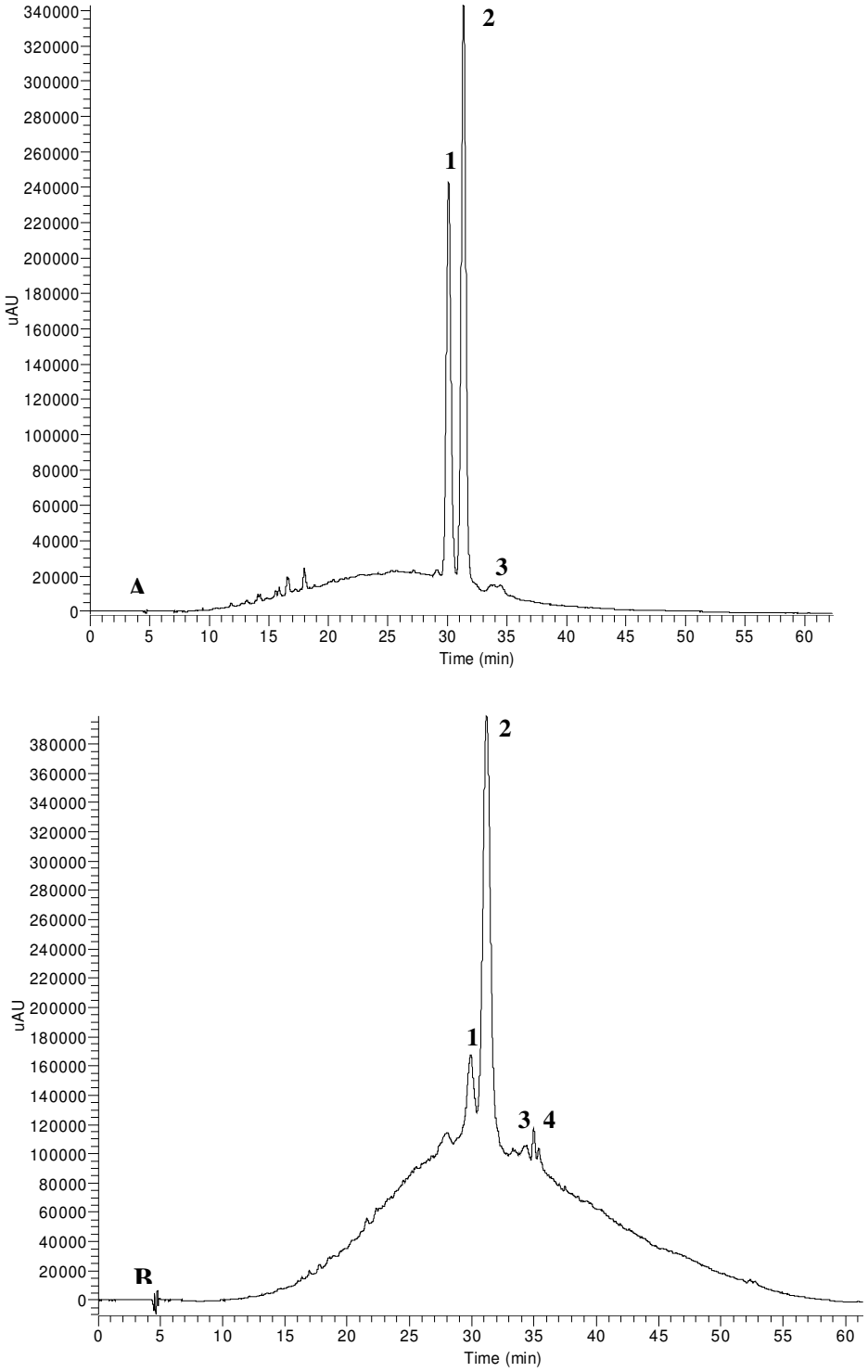


Fig. 26. HPLC chromatogram (520 nm) of anthocyanin monomer extracts (A) and anthocyanin polymer extracts (B). Peak assignments: 1. cyanidin-3-glucoside; 2. cyanidin-3-rutinoside; 3. pelargonidin-3-glucoside; 4. peonidin-3-glucoside.

fruit were normalized to equivalent total anthocyanin contents (2500 ± 100 mg cyanidin-3-glucoside equivalents/L) and characterized by HPLC-ESI-MS^n analyses. Mass fragmentation patterns for anthocyanins in both fractions are shown in Table 22. Cyanidin-3-rutinoside (1397.7 ± 64.3 mg/L) and cyanidin-3-glucoside (992.8 ± 10.9 mg/L) were predominant in monomeric fractions, along with trace amounts of pelargonidin-3-glucoside (Fig. 26). Polymeric anthocyanin fractions were characterized by the presence of pelargonidin and cyanidin adducts, which eluted as a large, unresolved peak in the reversed phase HPLC chromatograms, with strong UV absorption ~520 nm (Fig. 26), responsible for the characteristic dark red color observed in these fractions.

Cyanidin adducts present in polymeric anthocyanin fractions were identified by negative ion signals at m/z 611.0, 883.0, and 865.1, yielding common fragments at m/z 285.2 and 257.1, corresponding to the fragmentation pattern of cyanidin glycosides. Likewise, pelargonidin adducts were characterized by negative ion signals at m/z 721.1 and 793.0 and common fragments at m/z 431.1, 269.1, and 225.1, corresponding to the fragmentation of pelargonidin glycosides (Table 22). Cyanidin-3-rutinoside (332.8 ± 24.4 mg/L), cyanidin-3-glucoside (84.6 ± 5.9 mg/L), and traces of pelargonidin-3-glucoside, and peonidin-3-glucoside were also detected in polymeric anthocyanin fractions. Hence, anthocyanin monomers were responsible for 95.6 ± 3.7% of total anthocyanin contents in monomeric fractions, but only accounted for 16.7 ± 0.6% of total anthocyanin contents in polymeric fractions. Polymeric anthocyanin fractions also contained higher proportions of cyanidin-3-rutinoside (79.7 ± 5.8%) and relatively

smaller amounts of cyanidin-3-glucoside (20.3 ± 1.4%) when compared to monomeric fractions (58.5 ± 4.6% cyanidin-3-rutinoside, 41.5 ± 1.1% cyanidin-3-glucoside).

Variations in anthocyanin composition were hypothesized to be important contributing factors to differences in absorption and bioactive properties between monomeric and polymeric anthocyanin fractions. Thus, mixtures containing varying proportions of monomers and polymers (75/25 and 50/50 monomer/polymer ratios) were also evaluated in order to assess the influence of polymeric anthocyanins on the absorption and bioactive properties of monomeric anthocyanin glycosides.

Total antioxidant capacity of anthocyanin fractions and fraction mixtures were also related to total anthocyanin contents. Both anthocyanin fractions were comparable in terms of antioxidant capacity, with respective values of 256.9 ± 12.6 µmol TE/mL for monomeric fractions and 206.8 ± 6.7 µmol TE/mL for polymeric fractions. Comparable reducing capacities were also observed for both monomeric and polymeric anthocyanin fractions in the Folin-Ciocalteu assay, ranging from 5220 to 6577 mg gallic acid equivalents/mL. Analogous observations have been reported in anthocyanin extracts from *Hibiscus sabdariffa*, where degradation of monomeric anthocyanins to brown polymeric forms resulted in a <10% change in antioxidant capacity (Tsai & Huang, 2004).

The antiproliferative activities of anthocyanin monomer and polymer fractions were evaluated in a cell culture model using HT-29 colon carcinoma cells. Cell counts were related to HT-29 cell proliferation and declines in cell numbers were considered reflective of the cytotoxic effects of anthocyanin extracts. Both monomeric and

polymeric anthocyanin fractions and their mixtures decreased ($p<0.01$) total cell numbers in a concentration-dependent manner (Fig. 27). Monomeric anthocyanin fractions (5-20 µg/mL) were more effective in reducing cell proliferation when compared to similar concentrations of mixtures containing polymeric fractions. Likewise, anthocyanin concentrations at which cell proliferation was inhibited by 50% (IC_{50}) were lower ($p<0.05$) for monomeric fractions (IC_{50}= 12.1 µg/mL) than for polymeric fractions (IC_{50}= 14.4 µg/mL). Mixtures containing both monomeric and polymeric fractions had intermediate IC_{50} values, varying from 12.2 to 13.6µg/mL for mixtures with up to 25 and 50% anthocyanin polymers, respectively.

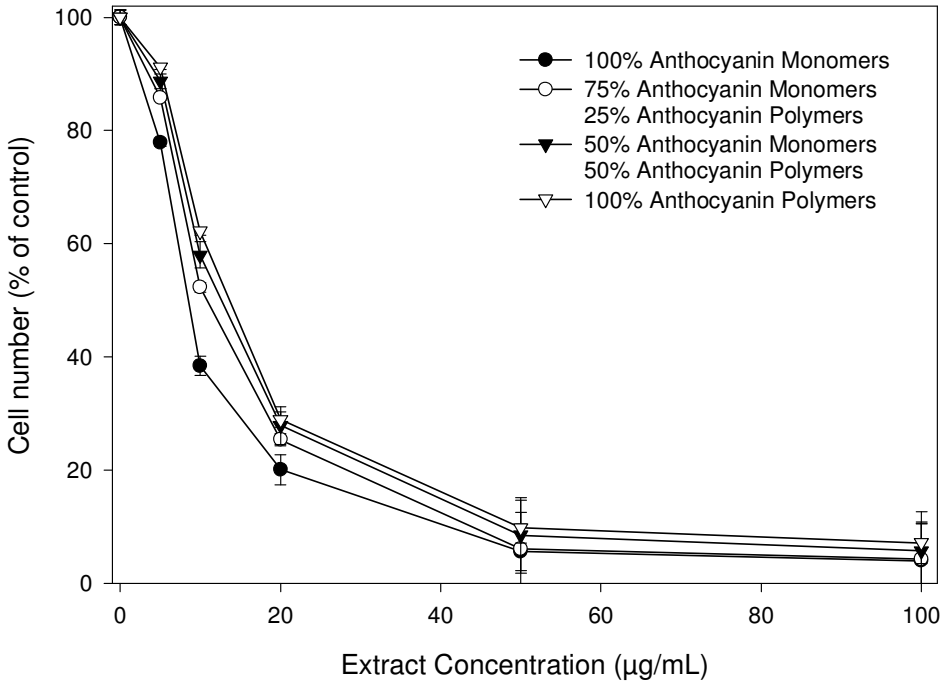


Fig. 27. Percent changes in total HT-29 cell numbers expressed as a ratio to control cells following treatment of cells with anthocyanin monomer and polymer fractions adjusted to different concentrations (µg/mL) for 48 h. Error bars represent the standard error of the mean (n=6).

Differences in the inhibitory effects of monomeric and polymeric anthocyanin extracts may be due to variations in their anthocyanin composition, as the availability of functional groups and ability to access target sites within the cells is likely higher for monomeric anthocyanin glycosides. Anthocyanin chemical structure has been found to influence the chemopreventive and antiproliferative activities of anthocyanin-rich extracts in similar cell models, with non-acylated, monoglycosylated anthocyanins having a higher inhibitory effect on HT-29 cell growth when compared to their acylated counterparts (Zhao, Giusti, Malik, Moyer, & Magnuson, 2004; Jing, Bomser, Schwartz, He, Magnuson, & Giusti, 2008). Inhibitory concentrations (IC_{50}) for purple corn extracts, rich in cyanidin-3-glucoside, were estimated at ~14 µg/mL (Jing et al., 2008), in agreement with those calculated for açai fruit anthocyanin fractions in this study (IC_{50}= 12.1-14.4 µg/mL).

Antiproliferative activity of monomeric anthocyanin glycosides on cancer cells has been attributed to their effects on growth stimulatory signals across the cell membrane, including inhibition of the epidermal growth factor receptor (EGFR) (Meiers, Kemeny, Weyand, Gastpar, Von Angerer, & Marko, 2001) and to their ability to interfere with various stages of the cell cycle by effects on regulator proteins such as $p21^{WAF1}$, an inhibitor of cell proliferation (Malik, Zhao, Schoene, Guisti, Moyer, & Magnuson, 2003; Wu et al., 2007; Wang & Stoner, 2008). Additional mechanisms, including induction of apoptosis in malignant cells are also likely to be involved in the antiproliferative action of anthocyanin monomers (Hou, 2003; Chen, Chu, Chiou, Chiang, Yang, & Hsieh, 2005; Seeram et al., 2006; Srivastava, Akoh, Fischer, &

Krewer, 2007; Fimognari, Lenzi, & Hrelia, 2008; Neto, Amoroso, & Liberty, 2008). Similar mechanisms might be responsible for the inhibitory effects of anthocyanin polymers on colon cancer cell growth, yet this is the first report on the antiproliferative activity of polymeric anthocyanin fractions on cancer cells in-vitro. Results from this study suggest both monomeric and polymeric anthocyanin fractions may inhibit colon cancer cell proliferation and potentially exert other important biological functions in tissues exposed to these fractions.

Caco-2 cell monolayers were used as in-vitro models to assess intestinal absorption of monomeric and polymeric anthocyanin fractions from açai fruit. Unidirectional anthocyanin transport was assessed from the apical to basolateral side of differentiated cell monolayers. Transport efficiencies were expressed as the percentage of anthocyanin concentrations initially loaded into the apical side detected on the basolateral side of cell monolayers following incubation for 0.5, 1.0, 1.5, and 2.0 h. Analytical HPLC chromatograms of anthocyanins present in the basolateral side of cell monolayers following incubation with monomeric and polymeric anthocyanin fractions for 2 h are presented in Fig. 28. Cyanidin-3-glucoside and cyanidin-3-rutinoside present in all açai fruit anthocyanin fractions were transported from apical to basolateral sides of cell monolayers, while additional polymeric anthocyanin fraction components were not transported following incubation for up to 2 h (Fig. 28). Transport efficiencies of monomeric and polymeric anthocyanin fractions and their mixtures are shown in Table 23.

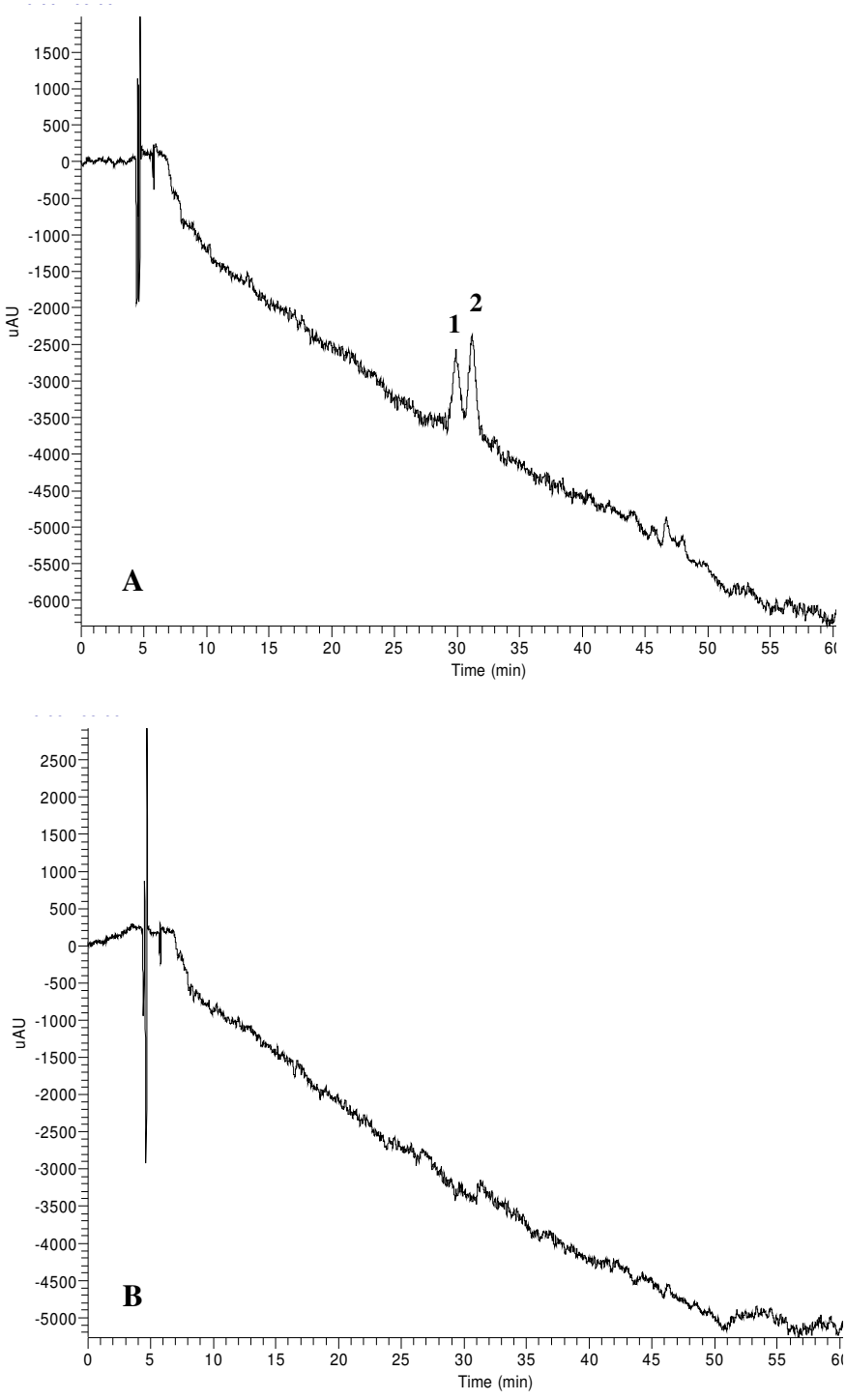


Fig. 28. Typical HPLC chromatogram (520 nm) of anthocyanins present in the basolateral compartment of Caco-2 cell monolayers following incubation with anthocyanin monomer fractions (A) and anthocyanin polymer fractions (B) for 2h. Peak assignments: 1. cyanidin-3-glucoside; 2. cyanidin-3-rutinoside.

Table 23. Percent transport of anthocyanins from apical to basolateral side of Caco-2 cell monolayers following incubation for 2 h with monomeric and polymeric anthocyanin fractions from açai fruit.

Anthocyanin Fraction	% Anthocyanin Transport			
	Cyanidin-3-glucoside		Cyanidin-3-rutinoside	
	50 µg/mL [1]	500 µg/mL	50 µg/mL	500 µg/mL
100% anthocyanin Monomers	4.94 ± 0.35 a[2]	4.89 ± 0.31 a	4.51 ± 0.33 a	4.43 ± 0.31 a
75% anthocyanin monomers, 25% anthocyanin polymers	3.84 ± 0.26 b	3.80 ± 0.25 b	3.79 ± 0.27 b	3.51 ± 0.24 b
50% anthocyanin monomers, 50% anthocyanin polymers	3.04 ± 0.20 c	2.92 ± 0.23 c	3.11 ± 0.22 c	2.86 ± 0.19 c
100% anthocyanin Polymers	---	0.49 ± 0.09 d	---	0.54 ± 0.10 d

[1] Anthocyanin concentrations initially loaded into the apical side of Caco-2 cell monolayers.
[2] Values with different letters within the same column are significantly different (LSD test, $p<0.05$).

Percent transport of monomeric anthocyanin glycosides following incubation for 2 h ranged from 0.5 to 4.9% in all anthocyanin fractions tested, and was higher for monomeric fractions (4.4 to 4.9%). Decreased monomeric anthocyanin transport efficiencies were observed for anthocyanin mixtures containing 25% to 50% polymeric fractions (2.9 to 3.8%), while anthocyanins in polymeric fractions had the lowest transport efficiencies, at 0.5 ± 0.1% (Table 23). Similar results were also observed following incubation for 0.5, 1.0, and 1.5 h (Fig. 29). Decreased monomeric anthocyanin transport (16.0 to 40.3%) in anthocyanin fraction mixtures containing polymeric fractions (25 to 50%) indicated anthocyanin polymers may interfere with monomeric anthocyanin transport mechanisms. Mechanisms for absorption and transport of monomeric anthocyanin glycosides through cell monolayers are not yet clear, and both active and passive transport mechanisms have been proposed (Cao et al., 2001; Brown, Khodr, Hider, & Rice-Evans, 1998; Hollman & Katan, 1998; Hollman, Bijsman, van Gameren, Cnossen, de Vries, & Katan, 1999; Gee et al., 2000; Williamson, Day, Plumb, & Couteau, 2000; Mulleder, Murkovic, & Pfannhauser, 2002; Manach, Williamson, Morand, Scalber, & Remesy, 2005). Results from this study suggest the presence of anthocyanin polymers may interfere with either active or passive transport mechanisms involved in the absorption of anthocyanin glycosides.

To determine a dose response on transport efficiency, 50 or 500 µg/mL of both monomeric and polymeric anthocyanin fractions and two combination ratios were loaded into the apical compartment of the cell monolayers. No differences were found for the relative transport rates of cyanidin-3-glucoside or cyanidin-3-rutinoside for either

fraction, suggesting intestinal transport efficiency of anthocyanin glycosides was not dose-dependent (Table 23). Moreover, both target anthocyanins were equally ($p<0.05$) transported from the apical to basolateral side of the cell monolayers, indicating that the glycosidic moiety (glucose to rutinose) had no influence on absorption of these cyanidin-based anthocyanins (Table 23).

Average transport rates (µg/L·h) of anthocyanin glycosides following incubation with monomeric and polymeric anthocyanin fractions and their mixtures (50 or 500 µg/mL) were calculated based on the relative amount of anthocyanins transported from apical to basolateral side of cell monolayers over time (0.5, 1.0, 1.5, and 2.0 hrs), as shown in Fig. 29. Anthocyanin transport rates varied with anthocyanin composition, and ranged from 3.2 to 19.6 µg/L·h (Table 24). Transport rates were higher for anthocyanin glycosides present in monomeric fractions (18.9-19.6 µg/L·h), and decreased to 11.6-16.2 µg/L·h as the relative proportion of polymeric fractions increased from 25% to50%. Transport rates were lowest (3.2-3.6 µg/L·h) in the polymeric anthocyanin fraction. Similar to previous observations on anthocyanin transport efficiencies, variations in anthocyanin concentration levels loaded into the apical compartments of cell monolayers (50 or 500 µg/L) did not influence transport rates of cyanidin-3-glucoside or cyanidin-3-rutinoside and no differences were found between transport rates for cyanidin-3-glucoside and cyanidin-3-rutinoside in any of the fractions.

A strong correlation ($r=0.98$) was found between anthocyanin transport efficiencies (%) and anthocyanin transport rates (µg/L·h), suggesting that factors responsible for increased anthocyanin transport likely influenced the rate of anthocyanin

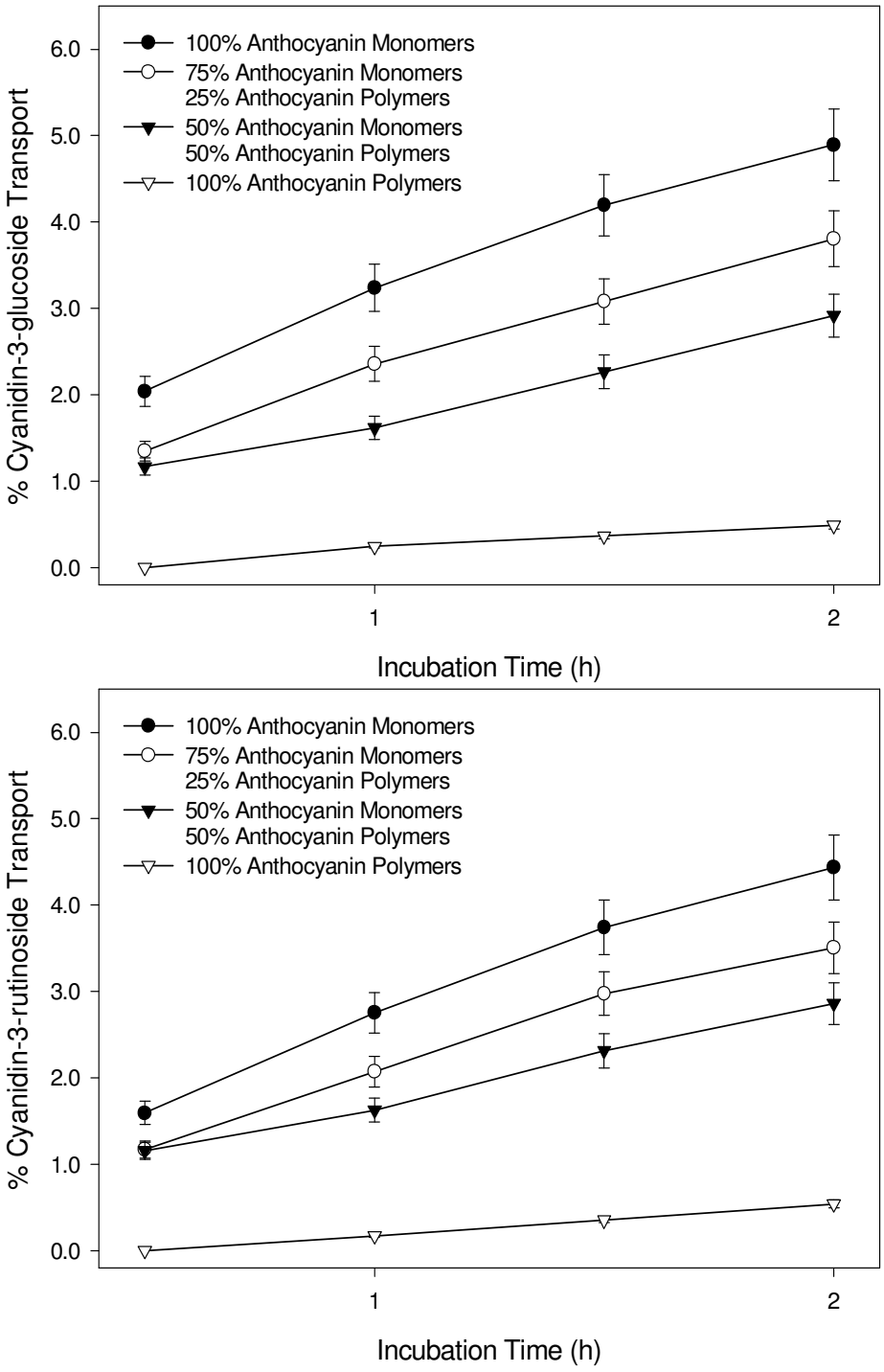


Fig. 29. Percent transport of cyanidin-3-glucoside and cyanidin-3-rutinoside from apical to basolateral side of Caco-2 cell monolayers following incubation with monomeric and polymeric anthocyanin fractions from açai.

Table 24. Average anthocyanin transport rates (µg/L·h) from the apical to the basolateral side of Caco-2 cell monolayers, following incubation with monomeric and polymeric anthocyanin fractions from açai fruit.

Anthocyanin Fraction	Anthocyanin Transport Rate (µg/L·h)			
	Cyanidin-3-glucoside		Cyanidin-3-rutinoside	
	50 µg/mL [1]	500 µg/mL	50 µg/mL	500 µg/mL
100% anthocyanin Monomers	19.6 ± 1.5 a[2]	19.0 ± 1.5 a	18.9 ± 1.4 a	19.0 ± 1.4 a
75% anthocyanin monomers, 25% anthocyanin polymers	15.7 ± 1.1 b	16.2 ± 1.3 b	16.0 ± 1.2 b	15.8 ± 1.2 b
50% anthocyanin monomers, 50% anthocyanin polymers	12.6 ± 1.0 c	11.8 ± 0.9 c	12.5 ± 1.1 c	11.6 ± 1.0 c
100% anthocyanin Polymers	---	3.18 ± 0.3 d	---	3.62 ± 0.3 d

[1] Anthocyanin concentrations initially loaded into the apical side of Caco-2 cell monolayers.
[2] Values with different letters within the same column are significantly different (LSD test, $p<0.05$).

transport in these models. Thus, higher transport efficiencies may be associated with transport mechanisms targeting monomeric anthocyanin glycosides, potentially inhibited or disrupted by the presence of polymeric anthocyanin fractions. Polymeric anthocyanin fractions may decrease transport of monomeric anthocyanin glycosides due to the presence of polar hydroxyl group ends, which have been associated with increased hydrogen-bond formation at the surface of the cell membrane, resulting in lower transport efficiencies (Saija, Scalese, Lanza, Marzullo, Bonina, & Castelli, 1995; Van Dijk, Driessen, & Recourt, 2000; Ollila et al., 2002). Although mechanisms for anthocyanin transport are still in debate and may be influenced by numerous factors including molecular weight or substituent moieties, this is the first report to show that polymeric anthocyanins are prevented from absorption in-vitro and that polymeric anthocyanins exert an inhibitory response to the absorption of monomeric anthocyanin glycosides.

Conclusion

The influence of anthocyanin polymerization reactions on the chemical composition, antioxidant properties, antiproliferative activity, and in-vitro absorption of anthocyanins from açai fruit were evaluated. Monomeric anthocyanin fractions were characterized by the predominant presence of cyanidin-3-glucoside and cyanidin-3-rutinoside, while several anthocyanin adducts were found in polymeric fractions. Both fractions (0.5-100 µg cyanidin-3-glucoside equivalents/mL) inhibited HT-29 colon cancer cell proliferation in a concentration-dependent manner by up to 95.2%. In-vitro

absorption trials using Caco-2 intestinal cell monolayers demonstrated that cyanidin-3-glucoside and cyanidin-3-rutinoside were similarly transported from the apical to the basolateral side of the cell monolayers (0.5-4.9% efficiency), while no anthocyanin adducts were transported following incubation for up to 2 h. The presence of polymeric anthocyanin fractions also decreased transport of monomeric anthocyanin glycosides in a dose-dependent manner by up to 40.3%. Results from this study suggest the presence of anthocyanin polymers may significantly influence anthocyanin absorption properties in açai fruit products.

CHAPTER IX

SUMMARY

Açai fruit are native to the Amazon region of South America and two predominant species are commercially exported as fruit pulps for use in food and beverage applications. Detailed characterization of the polyphenolic compounds present in the fruits of *Euterpe oleracea* and *Euterpe precatoria* species were conducted by HPLC-ESI-MS^n analyses and their thermal stability and overall influence on antioxidant capacity determined. Anthocyanins were the predominant polyphenolics in both *E. oleracea* (2,247 ± 23 mg/kg) and *E. precatoria* (3,458 ± 16 mg/kg) species, and accounted for nearly 90% of the antioxidant capacity in both *E. oleracea* (87.4 ± 4.4 μmol TE/g) and *E. precatoria* (114 ± 6.9 μmol TE/g) fruits. Various flavones, including isoorientin, orientin, taxifolin deoxyhexose, and isovitexin; flavanol derivatives, including (+)-catechin, (-)-epicatechin, procyanidin dimers and trimers; and phenolic acids, including protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, and ferulic acid, were also present in both species. Thermal stability was evaluated following a thermal holding cycle (80ºC for up to 60 min) in the presence and absence of oxygen. Both species experienced minor changes (<5%) in non-anthocyanin polyphenolic contents during all thermal processes whereas 34 ± 2.3% of anthocyanins in *E. oleracea* and 10.3 ± 1.1% of anthocyanins in *E. precatoria* were lost under these conditions, regardless of the presence of oxygen. Proportional decreases (10 to 25%) in antioxidant capacity accompanied the anthocyanin changes. Both açai species were characterized by similar

polyphenolic profiles, comparable antioxidant capacities, yet only moderate phytochemical stability during heating.

Polyphenolic compounds present in crude oil extracts from açai fruit were identified for the first time. Stability of açai oil that contained three concentrations of polyphenolics was evaluated under a short and long-term storage for lipid oxidation and polyphenolic retention impacting antioxidant capacity. Similar to açai fruit, açai oil isolates contained phenolic acids such as vanillic acid (1,616 ± 94 mg/kg), syringic acid (1,073 ± 62 mg/kg), *p*-hydroxy benzoic acid (892 ± 52 mg/kg), protocatechuic acid (630 ± 36 mg/kg), and ferulic acid (101 ± 5.9 mg/kg) at highly enriched concentrations in relation to açai pulp, as well as (+)-catechin (66.7 ± 4.8 mg/kg) and various procyanidin oligomers (3,102 ± 130 mg/kg). Phenolic acids experienced up to 16% loss after 10 weeks storage at 20 or 30°C and up to 33% loss at 40°C. Procyanidin oligomers degraded more extensively (23% at 20°C, 39% at 30°C, and 74% at 40°C), in both high- and low-polyphenolic açai oils. The hydrophilic antioxidant capacity of açai oil isolates with the highest polyphenolic concentration was 21.5 ± 1.7 µmol TE/g and total soluble phenolic content was 1,252 ± 11 mg gallic acid equivalents, GAE/kg and each decreased by up to 30 and 40%, respectively during long-term storage. Short-term heating stability at 150 and 170 °C for up to 20 min exhibited only minor losses (<10%) in polyphenolics and antioxidant capacity. Due to its high polyphenolic content, the phytochemical enriched açai oil from açai fruit offers a promising alternative to traditional tropical oils for food, supplement, and cosmetic applications.

Polyphenolic extracts from various fruits and vegetables have been shown to exert growth inhibitory effects in cell culture studies. Whereas individual polyphenolic compounds have been extensively evaluated, understanding the biological activity of polyphenolic extracts from natural sources are limited and critical to understanding their potential effects on the human body. Thus, the absorption and anti-proliferative effects of phytochemical extracts from açai pulp and a polyphenolic-enriched açai oil obtained from the fruit pulp of the açai berry were investigated. Chemical composition, antioxidant properties, and polyphenolic absorption of phytochemical fractions in a Caco-2 monolayer were determined, along with their cytotoxicity in HT-29 human colon adenocarcinoma cells. Extracts were characterized by their predominance of hydroxybenzoic acids, monomeric flavan-3-ols, and procyanidin dimers and trimers. Polyphenolic mixtures (0-12 µg GAE/mL) from both açai pulp and açai oil extracts inhibited cell proliferation by up to 90.7%, which was accompanied by up to 2.1 fold increase in reactive oxygen species. Absorption experiments using a Caco-2 intestinal cell monolayer demonstrated that phenolic acids such as *p*-hydroxy benzoic, vanillic, syringic, and ferulic acids were readily transported from the apical to the basolateral side along with monomeric flavanols such as (+)-catechin and (-)-epicatechin. Results indicated açai fruit extracts are sources of bioactive and absorbable polyphenolics.

The influence of different classes of previously characterized polyphenolic cofactors naturally occurring in açai on the phytochemical and color stability of anthocyanins in açai fruit was also investigated. Model systems were based on anthocyanin isolates from açai fruit, rich in cyanidin-3-rutinoside (311 ± 27 mg/L) and

cyanidin-3-glucoside (208 ± 18 mg/L), and isolated groups of naturally occurring polyphenolic cofactors in açai fruit (phenolic acids, procyanidins, and flavone-*C*-glycosides, each adjusted to ~50 mg/L). Anthocyanin degradation kinetics were assessed as a function of pH (3.0, 3.5, and 4.0) and storage temperature (5, 20 and 30°C). During storage, anthocyanins experienced pH and temperature-dependent losses, and the half life cyanidin-3-rutinoside (t½= 2.67 to 210 days) was consistently longer than cyanidin-3-glucoside (t½= 1.13 to 144 days). The presence of flavone-*C*-glycosides induced significant hyperchromic shifts and enhanced anthocyanin stability at all pH and temperature combinations, while no effects were attributed to the presence of phenolic acids or procyanidins. Additional models using externally added cofactors from rooibos tea, also rich in flavone-*C*-glycosides, resulted in up to 45.5% higher anthocyanin color and up to 40.7% increased anthocyanin stability compared to uncopigmented anthocyanin isolates and had similar copigmentation effects to a commercial rosemary-based color enhancer. Results suggest flavone-*C*-glycosides offer potential for their use as color enhancers and stabilizing agents in products rich in cyanidin glycosides, particularly açai fruit-containing juice blends and beverages.

Anthocyanin polymerization reactions occurring during storage of açai fruit juices were additionally investigated. Several anthocyanin-flavanol adducts based on cyanidin or pelargonidin aglycones were tentatively identified by HPLC-ESI-MS^n analyses and their presence was related to increased anthocyanin sulfite bleaching resistance and to the appearance of a wide, unresolved peak in HPLC chromatograms. The proposed reaction mechanism involved the nucleophilic addition of anthocyanins in

their hydrated form to flavanol carbocations resulting from cleavage of interflavanic bonds, and leading to the formation of flavanol-anthocyanin adducts in açai fruit juices. It was also hypothesized that condensed flavanol-anthocyanin pigments are not likely exclusive products of polymerization reactions during storage, but may be naturally occurring compounds in açai fruit. Moreover, the natural occurrence of condensed flavanol-anthocyanin pigments in açai fruit pulp was also thought to promote the formation of additional anthocyanin adducts and further polymerization with other phenolic components, potentially resulting in a variety of structurally diverse anthocyanin polymers during storage of açai fruit juices.

The role of anthocyanin polymerization reactions on anthocyanin absorption and anti-cancer properties was also evaluated. Phytochemical composition, antioxidant properties, antiproliferative activity, and in-vitro absorption of monomeric and polymeric anthocyanin fractions from açai fruit were determined. Cyanidin-3-rutinoside (58.5 ± 4.6%) and cyanidin-3-glucoside (41.5 ± 1.1%) were the predominant compounds found in monomeric fractions, while a mixture of anthocyanin adducts were found in polymeric fractions and characterized using HPLC-ESI-MS^n analyses. Monomeric fractions (0.5-100 µg cyanidin-3-glucoside equivalents/mL) inhibited HT-29 colon cancer cell proliferation by up to 95.2% while polymeric anthocyanin fractions induced up to 92.3% inhibition. In-vitro absorption trials using Caco-2 intestinal cell monolayers demonstrated that cyanidin-3-glucoside and cyanidin-3-rutinoside were equally transported from the apical to the basolateral side of the cell monolayers (0.5-4.9% efficiency), while no anthocyanin adducts were transported following incubation for up

to 2h. Polymeric anthocyanin fractions also decreased monomeric anthocyanin transport by up to 40.3 ± 2.8%. Results indicated that the presence of anthocyanin polymers may significantly influence anthocyanin absorption properties in açai fruit products.

REFERENCES

Abedamowo, C., Cho, E., Sampson, L., Katan, M., Spiegelman, D., Willett, W., & Holmes, M. (2004). Dietary flavonols and flavonol-rich foods intake and the risk of breast cancer. *International Journal of Cancer, 114,* 628-633.

Adams, J. B. (1972). Changes in the polyphenols of red fruits during heat processing: the kinetics and mechanism of anthocyanin degradation. Campden Food Preservation Research Association Technology Bulletin No. 22.

Adams, J. B. (1973). Thermal degradation of anthocyanins with particular reference to the 3-glycosides of cyanidin. I. In acidified aqueous solution at 100°C. *Journal of the Science of Food and Agriculture, 24*, 747–762.

Alcalde-Eon, C., Escribano-Bailon, M., Santos-Buelga, C., & Rivas-Gonzalo, J. (2006). Changes in the detailed pigment composition of red wine during maturity and ageing. A comprehensive study. *Analytica Chimica Acta, 563,* 238-254.

Artursson, P. (1990). Epithelial transport of drugs in cell culture. I. A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. *Journal of Pharmaceutical Science, 79,* 476-482.

Asen , S., Stewart, R., Norris, K., & Massie, D. (1970). A stable blue non-metallic copigment complex of delphanin and C-glycosylflavones in Prof. Blaauw Iris. *Phytochemistry, 9,* 619-627.

Baba, S., Osakabe, N., Natsume, M., & Terao, J. (2002). Absorption and urinary excretion of procyanidin B2 [epicatechin-(4β-8)-epicatechin] in rats. *Free Radical Biology and Medicine, 33*, 142-148.

Babsky, N., Toribio, J., & Lozano, J. (2006). Influence of storage on the composition of clarified apple juice concentrate. *Journal of Food Science, 51,* 564-567.

Bakker, J., & Timberlake, C. (1997). Isolation, identification, and characterization of ne color-stable anthocyanins occurring in some red wines. *Journal of Agricultural and Food Chemistry, 45,* 35-43.

Balimane, P., Chong, S., & Morrison, R. (2000). Current methodologies used for evaluation of intestinal permeability and absorption. *Journal of Pharmacology and Toxicology Methods, 44,* 301-312.

Baranac, J., Petranovic, N., & Dimitric-Markovic, J. (1996). Spectrophotometric study of anthocyanin copigmentation reactions. *Journal of Agricultural and Food Chemistry, 44,* 1333-1336.

Baublis, A., Spomer, A., & Berber-Jimenez, M. (1994). Anthocyanin pigments: comparison of extract stability. *Journal of Food Science, 59*, 1219-1233.

Benavides, A., Montoro, P., Bassarello, C., Piacente, S., & Pizza, C. (2006). Catechin derivatives in *Jatropha macrantha* stems: characterization and LC/ESI/MS/MS quail-quantitative analysis. *Journal of Pharmaceutical and Biomedical Analysis, 40,* 639-647.

Berg, H., & Akiyoushi, M. (1975). On the nature of reactions responsible for color behavior in red wine: a hypothesis. *Journal of Enology and Viticulture, 26,* 134-143.

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: a critical review. *American Journal of Enology and Viticulture*, *52*, 67-86.

Bramati, L., Minoggio, M., Gardana, C., Simonetti, P., Mauri, P., & Pietta, P. (2002). Quantitative characterization of flavonoid compounds in Rooibos tea (*Aspalathus linearis*) by LC-UV/DAD. *Journal of Agricultural and Food Chemistry, 50,* 5513-5519.

Brenes, C., Del Pozo-Insfran, D., & Talcott, S. T. (2005). Stability of copigmented anthocyanins and ascorbic acid in a grape juice model system. *Journal of Agricultural and Food Chemistry, 53,* 49-56.

Briviba, K., Pan, L., & Rechkemmer, G. (2002). Red wine polyphenols inhibit the growth of colon carcinoma cells and modulate the activation pattern of mitogen-activated protein kinases. *Journal of Nutrition, 132,* 2814-2818.

Brondizio, E., Safar, C., & Siqueira, A. (2002). The urban market of açaí fruit (*Euterpe oleracea* Mart.) and rural land use change: ethnographic insights into the role of price and land tenure constraining agricultural choices in the Amazon estuary. *Urban Ecosystems*, *6*, 67-97.

Brouillard, R. (1983). The in vivo expression of anthocyanin color in plants. *Phytochemistry, 22,* 1311-1323.

Brown, J., Khodr, H., Hider, R., & Rice-Evans, C. (1998). Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochemistry Journal, 330*, 1173–1178.

Bub, A., Watzl, B., Heeb, D., Rechkemmer, G., & Briviba, K. (2001). Malvidin-3-glucoside bioavailability in humans after ingestion of red wine, dealcoholized red wine and red grape juice. *European Journal of Nutrition, 40,* 113-120.

Burdulu, H., & Karadeniz, F. (2003). Effect of storage on nonenzymatic browning of apple juice concentrates. *Food Chemistry, 80,* 91-97.

Cao, G., Muccitelli, H., Sanchez-Moreno, C., & Prior, R. (2001). Anthocyanins are absorbed in glycated forms in elderly women: a pharmacokinetic study. *American Journal of Clinical Nutrition, 73*, 920-926.

Careri, M., Bianchi, F., & Corradini, C. (2002). Recent advances in the application of mass spectrometry in food-related analysis. *Journal of Chromatography A, 970,* 3-64.

Careri, M., Mangia, A., & Musci, M. (1998). Overview of the applications of liquid chromatography mass spectrometry interfacing systems in food analysis: naturally occurring substances in food. *Journal of Chromatography A, 794,* 263-297.

Caristi, C., Bellocco, E., Gargiulli, C., Toscano, G., & Leuzzi, U. (2006). Flavone-di-*C*-glycosides in citrus juices from Southern Italy. *Food Chemistry, 95,* 431-437.

Chang, Y., Huang, H., Hsu, J., Yang, S., & Wang, C. (2005). Hibiscus anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. *Toxicology and Applied Pharmacology, 205,* 201-212.

Chen, P., Chu, S., Chiou, H., Chiang, C., Yang, S., & Hsieh, Y. (2005). Cyanidin-3-glucoside and peonidin-3-glucoside inhibit tumor cell growth and induce apoptosis in vitro and suppress tumor growth in vivo. *Nutrition and Cancer, 53,* 232-243.

Cheynier, V., Duenas-Paton, M., Salas, E., Maury, C., Souquet, P., Sarni-Manchado, P., & Fulcrand, H. (2006). Structure and properties of wine pigments and tannins. *American Journal of Enology and Viticulture, 57,* 298-305.

Chun, O., Dim, D., Smith, N., Schroeder, D., Han, J., & Lee, C. (2005). Daily consumption of phenolics and total antioxidant capacity from fruit and vegetables in the American diet. *Journal of the Science of Food and Agriculture, 85,* 1715-1724.

Clay, J. & Clement, C. (1993). Selected species and strategies to enhance income generation from Amazonian forests. Food and Agriculture Organization of the United Nations Corporate Document Repository V0784/E. Rome, Italy.

Clifford, M. (2000). Anthocyanins: nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture, 80,* 1063-1072.

Coisson, J., Travaglia, F., Piana, G., Capasso, M., & Arlorio, M. (2005). *Euterpe oleracea* juice as a functional pigment for yogurt. *Food Research International, 38*, 893-897.

Cook, N. & Samman, S. (1996). Flavonoids-chemistry, metabolism, cardioprotective effects and dietary sources. *Journal of Nutritional Biochemistry, 7,* 66-76.

Cos, P., De Bruyne, T., Hermans, N., Apers, S., Vanden-Berghe, D., & Vlietinck, A. (2003). Proanthocyanidins in health care: current and new trends. C*urrent Medicinal Chemistry, 10,* 1345-1359.

Cuyckens, F., & Claeys, M. (2004). Mass spectrometry in the structural analysis of flavonoids. *Journal of Mass Spectrometry, 39,* 1-15.

Dallas, C., Ricardo-da-Silva, J., & Laureano, O. (1996). Interactions of oligomeric procyanidins in model wine solutions containing malvidin-3-glucoside and acetaldehyde. *Journal of the Science of Food and Agriculture, 70,* 493-500.

Dao, L., Takeoka, G., Edwards, R., & Berrios, J. (1998). Improved method for the stabilization of anthocyanidins. *Journal of Agricultural and Food Chemistry, 46*, 3564-3569.

De Ancos, B., Gonzales, E., & Cano, M. (1999). Differentiation of raspberry varieties according to anthocyanin composition. *Food Research and Technology*, *208*, 33-38.

De Bruyne, T., Pieters, L., Deelstra, H., & Vlietinck, A. (1999). Condensed vegetable tannins: Biodiversity in structure and biological activities. *Biochemical Systematics and Ecology, 27,* 445-459.

De Pascual-Teresa, S., Santos-Buelga, C., & Rivas-Gonzalo, J. (2002). LC-MS analysis of anthocyanins from purple corn cob. *Journal of the Science of Food and Agriculture, 82,* 1003-1006.

Del Pozo, D., Brenes, C., & Talcott, S. (2004). Phytochemical composition and pigment stability of Açai (*Euterpe oleracea* Mart.). *Journal of Agricultural and Food Chemistry, 52,* 1539-1545.

Delgado-Vargas, F., Jiménez, A.R., & Paredes-Lopez, O. (2002). Natural pigments: carotenoids, anthocyanins and betalains, characteristics, biosynthesis, processing, and stability. *Critical Reviews in Food Science and Nutrition*, *40*, 173–289.

Deprez, S., Mila, I., Humeau, J.F., Tome, D., & Scalbert, A. (2001). Transport of proanthocyanidin dimer, trimer, and polymer across monolayers of human intestinal epithelial Caco-2 cells. *Antioxidants and Redox Signaling, 3,* 957-967.

Dragsted, L., Strube, M., & Leth, T. (1997). Dietary levels of plant phenols and other non-nutritive components: could they prevent cancer? *European Journal of Cancer Prevention, 6,* 522-528.

Dueñas, M., Fulcrand, H., & Cheynier, V. (2006). Formation of anthocyanin-flavanol adducts in model solutions. *Analytica Chimica Acta, 563,* 15-25.

Eiro, M., & Heinonen, M. (2002). Anthocyanin color behavior and stability during storage: effect of intermolecular copigmentation. *Journal of Agricultural and Food Chemistry, 50,* 7461-7466.

Elattar, T., & Viriji, A. (1999). The effect of red wine and its components on growth and proliferation of human oral squamous carcinoma cells. *Anticancer Research, 19,* 5407-5414.

Es-Safi, N., Cheynier, V., & Moutounet, M. (2000). Study of the reactions between (+)-catechin and furfural derivatives in the presence or absence of anthocyanins and their implication in food color change. *Journal of Agricultural and Food Chemistry, 48*, 5946-5954.

Es-Safi, N., Cheynier, V., & Moutounet, M. (2002). Interactions between cyanidin-3-O-glucoside and furfural derivatives and their impact on food color changes. *Journal of Agricultural and Food Chemistry, 50*, 5586-5595.

Es-Safi, N., Fulcrand, H., Cheynier, V., & Moutounet, M. (1999). Studies on the acetaldehyde-induced condensation of (-)-epicatechin and malvidin-3-O-glucoside in a model solution system. *Journal of Agricultural and Food Chemistry, 47,* 2096-2102.

Felgines, C., Talavera, S., Gothier, M., Texier, O., Scalbert, A., Lamaison, J., & Remesy, C. (2003). Strawberry anthocyanins are recovered in urine as glucuro-and sulfoconjugates in humans. *Journal of Nutrition, 133,* 1296-1301.

Felgines, C., Texier, O., Besson, C., Fraisse, D., Lamaison, J., & Remesy, C. (2002). Blackberry anthocyanins are slightly bioavailable in rats. *Journal of Nutrition, 132,* 1249-1253.

Ferguson, P., Kurowska, E., Freeman, D., Chambers, A., & Koropatnick, D. (2004). A flavonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines. *Journal of Nutrition, 134*, 1529-1535.

Ferreres, F., Silva, B., Andrade, P., Seabra, R., & Ferreira, M. (2003). Approach to the study of *C*-glycosyl flavones by ion trap HPLC-PDA-ESI/MS/MS: application to seeds of quince (*Cydonia oblonga*). *Phytochemical Analysis, 14,* 352-259.

Fimognari, C., Lenzi, M., & Hrelia, P. (2008). Chemoprevention of cancer by isothiocyanates and anthocyanins: mechanisms of action and structure-activity relationship. *Current Medicinal Chemistry, 15,* 440-447.

Flamini, R. (2003). Mass spectrometry in grape and wine chemistry. Part I: Polyphenols. *Mass Spectrometry Reviews, 22,* 218-221.

Fossen, T., Rayyan, S., & Andersen, O. (2004). Dimeric anthocyanins from strawberry (*Fragaria ananassa*) consisting of pelargonidin-3-glucoside covalently linked to four flavan-3-ols. *Phytochemistry, 65,* 1421-1428.

Francia-Aricha, E., Guerra, M., Rivas-Gonzalo, J., & Santos-Buelga, C. (1997). New anthocyanin pigments formed after condensation with flavanols. *Journal of Agricultural and Food Chemistry, 45,* 2262-2266.

Francis, F. (1989). Food colorants: anthocyanins. *Critical Reviews in Food Science and Nutrition, 28*, 273-314.

Friederich, W., Eberhardt, A., & Galensa, R. (2000). Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *European Food Research and Technology, 211,* 56-64.

Fulcrand, H., Benabdeljalil, C., Rigaud, J., Cheynier, V., & Moutounet, M. (1998). A new class of wine pigments yielded by reactions between pyruvic acid and grape anthocyanins. *Phytochemistry, 47*, 1401-1407.

Fulcrand, H., Cameira dos Santos, P., Sarni-Manchado, P., Cheynier, V., & Bonvin, J. (1996). Structure of new anthocyanin-derived wine pigments. *Journal of Chemical Society and Perkin Transations, 1,* 735-739.

Fulcrand, H., Docco, T., Es-Safi, N., Cheynier, V., & Moutounet, M. (1996). Study of acetaldehyde induced polymerization of flavan-3-ols by liquid chromatography-ion spray mass spectrometry. *Journal of Chromatography A, 752,* 85-91.

Gallori, S., Bilia, A., Bergonzi, M., Barbosa, L., & Vincieri, F. (2004). Polyphenolic constituents of fruit pulp of *Euterpe oleracea* Mart. (açai palm). *Chromatographia, 59,* 739-743.

Galotta, A., & Boaventura, M. (2005). Chemical constituents from roots and leaf stalks of açai (*Euterpe precatoria* Mart., Arecaceae). *Quimica Nova, 28*, 610-613.

Galvano, F., La Fauci, L., Lazzarino, G., Fogliano, V., Ritieni, A., Ciappellano, S., Battistini, N., Tavazzi, B., & Galvano, G. (2004). Cyanidins: metabolism and biological properties. *Journal of Nutritional Biochemistry, 15,* 2-11.

Garzon, G., & Wrolstad, R. (2002). Comparison of the stability of pelargonidin-based anthocyanins in strawberry juice and concentrate. *Journal of Food Science, 67*, 1288-1299.

Gattuso, G., Barreca, D., Gargiulli, C., Leuzzi, U., & Caristi, C. (2007). Flavonoid composition of citrus juices. *Molecules, 12,* 1641-1673.

Gee, J., DuPont, M., Day, M., Day, A., Plumb, G., Williamson, G., & Johnson, I. (2000). Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *Journal of Nutrition, 130*, 2765–2771.

Gil-Izquierdo, A., & Mellenthin, A. (2001). Identification and quantitation of flavonols in rowanberry (*Sorbus aucuparia* L.) juice. *European Food Research and Technology, 213*, 1438-2377.

Gonzalez-Manzano, S., Perez-Alonso, J., Salinas-Moreno, Y., Mateus, N., Silva, A., De Freitas, V., & Santos-Buelga, C. (2008). Flavanol-anthocyanin pigments in corn: NMR characterisation and presence in different purple corn varieties. *Journal of Food Composition and Analysis, 21,* 521-526.

Gonzalez-Paramas, A., Lopes da Silva, F., Martin-Lopez, P., Macz-Pop, G., Gonzalez-Manzano, S., Alcalde-Eon, C., Perez-Alonso, J., Escribano-Bailon, M., Rivas-Gonzalo, J., & Santos-Buelga, C. (2006). Flavanol-anthocyanin condensed pigments in plant extracts. *Food Chemistry, 94,* 428-436.

Grayer, R., Kite, G., Abou-Zaid, M., & Archer, L. (2000). The application of atmospheric pressure chemical ionization liquid chromatography-mass spectrometry in the chemotaxonomic study of flavonoids: characterization of flavonoids from *Ocimum gratissimum* var. Gratissimum. *Phytochemical Analysis,11,* 257–267.

Gu, L., Kelm, M., Hammerstone, J., Beecher, G., Cunningham, D., Vannozzi, S., & Prior, R. (2002). Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. *Journal of Agricultural and Food Chemistry, 50,* 4852-4860.

Gu, L., Kelm, M., Hammerstone, J., Zhang Z., Beecher, G., Holden, H., Haytowitz, D., & Prior, R. (2003). Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. *Journal of Mass Spectrometry, 38,* 1272-1280.

Guyot, S., Doco, T., Souquet, J., Moutounet, M., & Drilleau, J. (1997). Characterization of highly polymerized procyanidins in cider apple (*Malus sylvestris* var. kermerrien) skin and pulp. *Phytochemistry, 44,* 351-357.

Guyot, S., Marnet, N., & Drilleau, J. (2001). Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *Journal of Agricultural and Food Chemistry, 49,* 14-20.

Hammerstone, J., Lazarus, S., Mitchell, A., Rucker, R., & Schmitz, H. (1999). Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using

high-performance liquid Chromatography/Mass Spectrometry. *Journal of Agricultural and Food Chemistry, 47,* 490-496.

Han, J., Ye, M., Xu, M., Sun, J., Wang, B., & Guo, D. (2007). Characterization of flavonoids in the tradicional Chinese herbal medicine Huangqin by liquid chromatography coupled with electrospray ionization mass spectrometry. *Journal of Chromatography B, 848*, 355–362.

Harborne, J. (1964). Plant polyphenols. XI. Structure of acylated anthocyanins. *Phytochemistry, 3,* 151-160.

Haslam, E. (1980). In vino veritas: oligomeric procyanidins and the ageing of red wines. *Phytochemistry, 19,* 2577-2582.

Herrmann, K. (1995). The shikimate pathway: early steps in the biosynthesis of aromatic compounds. *Plant Cell, 7,* 907-919.

Hertog, M., Bueno-de-Mesquita, H., & Fehily, A. (1996). Fruit and vegetable consumption and cancer mortality in the Caerphilly strudy. *Cancer Epidemiology Biomarkers and Prevention, 5,* 673-677.

Hidalgo, I., Raub, T., & Borchardt, R. (1989). Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology*, *96*, 736-749.

Hollman, P., & Katan, M. (1998). Bioavailability and health effects of dietary flavonols in man. *Archives of Toxicology Supplement, 20,* 237–248.

Hollman, P., Bijsman, M., van Gameren, Y., Cnossen, E., de Vries, J., & Katan, M. (1999). The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Research, 31,* 569–573.

Hong, E., & Wrolstad, R. (1990). Use of HPLC separation/photodiode array detection for the characterization of anthocyanins. *Journal of Agricultural and Food Chemistry, 38,* 708-715.

Horwitz, W., Ed. (2000). *Official Methods of Analysis of AOAC International*, 17th ed.; AOAC International, Gaithersburg, MD.

Hou, D. (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Current Molecular Medicine, 3,*149-159.

Jackman, R., Yada, R., Tung, M., & Speers R. (1987). Anthocyanins as food colorants- a review. *Journal of Food Biochemistry, 11*, 201-247.

Jing, P., Bomser, J., Schwartz, S., He, J., Magnuson, B., & Giusti, M. (2008). Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. *Journal of Agricultural and Food Chemistry, 56,* 9391-9398.

Jurd, L. (1967). Anthocyanidins and related compounds. XI. Catechin-flavylium salt condensation reactions. *Tetrahedron, 23,* 1057-1064.

Jurd, L. (1969). Review of polyphenol condensation reactions and their possible occurrence in the aging of wines. *American Journal of Enology and Viticulture, 20,* 191-195.

Jurd, L. (1972). Some advances in the chemistry of anthocyanin-type plant pigments. *Advances in Food Research Supplement, 3,* 123-142.

Jurd, L., & Asen, S. (1966). The formation of metal and co-pigment complexes of cyanidin 3-glucoside. *Phytochemistry*, *5*, 1263-1271.

Jurd, L., Waiss, A., & Bergot, B. (1965). Condensation reactions of flavylium salts. *Bulletin of National Institute of Sciences India, 31,* 69-77.

Kader, F., Irmouli, M., Nicolas, J., & Metche, M. (2001). Proposed mechanism for the degradation of pelargonidin 3-glucoside by caffeic acid o-quinone. *Food Chemistry, 75,* 139-144.

Kammerer, D., Carle, R., & Schieber, A. (2003). Detection of peonidin and pelargonidin glycosides in black carrots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry, 17,* 2407-2412.

Katsube, N., Iwashita, K., Tsushida, T., Yamaki, K., & Kobori, M. (2003). Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. *Journal of Agricultural and Food Chemistry, 51*, 68-75.

Kim, D., & Lee, C. (2004). Comprehensive study on vitamin C equivalent antioxidant capacity (VCEAC) of various polyphenolics in scavenging a free radical and its structural relationship. *Critical Reviews in Food Science and Nutrition, 44,* 253–273.

Kobayashi, Y., Suzuki, M., Satsu, H., Arai, S., Hara, Y., Suzuki, K., Miyamoto, Y., & Shimizu, M. (2000). Green tea polyphenols inhibit the sodium-dependent glucose transport of intestinal epithelial cells by a competitive mechanism. *Journal of Agricultural and Food Chemistry, 48,* 5618-5623.

Kong, J., Chia, L., Goh, N., Chia, T., & Brouillard, R. (2003). Analysis and biological activities of anthocyanins. *Phytochemistry, 64,* 923-933.

Konishi, Y., & Shimizu, M. (2003). Transepithelial transport of ferulic acid by monocarboxylic acid transporter in Caco-2 cell monolayers. *Bioscience, Biotechnology and Biochemistry, 67,* 856-862.

Konishi, Y., Kobayashi, S., & Shimizu, M. (2003). Tea polyphenols inhibit the transport of dietary phenolic acids mediated by the monocarboxylic acid transporter (MCT) in intestinal Caco-2 cell monolayers. *Journal of Agricultural and Food Chemistry, 51,* 7296-7302.

Krafczyk, N., & Glomb, M. (2008). Characterization of phenolic compounds in Rooibos tea. *Journal of Agricultural and Food Chemistry, 56,* 3368-3376.

Krifi, B., Chouteau, F., Boudrant, J., & Metche, M. (2000). Degradation of anthocyanins from blood orange juices. *International Journal of Food Science and Technology, 35,* 275-283.

Le Marchand, L. (2002). Cancer preventive effects of flavonoids- a review. *Biomedicine & Pharmacotherapy, 56,* 296-301.

Le Roux, E., Doco, T., Sarni-Manchado, P., Lozano, Y., & Cheynier, V. (1998). A-type proanthocyanidins from pericarp of *Litchi chinensis*. *Phytochemistry, 48,* 1251-1258.

Lee, H., & Nagy, S. (1990). Relative reactivities of sugars in the formation of 5-hydroxymethylfurfural in sugar-catalyst model systems. *Journal of Food Processing and Preservation, 14,* 171-252.

Li, Q., Van den Heuvel, H., Delorenzo, O., Corthout, J., Pieters, A., Vlietinck, A., & Claeys, M. (1991). Mass spectral characterization of C-glycosidic flavonoids isolated from a medicinal plant (*Pasiflora incarnata*). *Journal of Chromatography, 562,* 435-446.

Lichtenthaler, R., Belandrino, R., Maia, J., Papagiannopoulos, M., Fabricius, H., & Marx, F. (2005). Total antioxidant scavenging capacities of *Euterpe oleracea* Mart. (Açai) fruits. *International Journal of Food Science and Nutrition, 56,* 53-64.

Liu, R. (2003). H. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *American Journal of Clinical Nutrition, 78*, 517S-520S.

Lopes da Silva, F., De Pascual-Teresa, S., Rivas-Gonzalo, J., & Santos-Buelga, C. (2002). Identification of anthocyanin pigments in strawberry (cv Camarosa) by LC using DAD and ESI-MS detection. *European Food Research and Technology, 214*, 248–253.

Lopes da Silva, F., Escribano-Bailon, M., Perez-Alonso, J., Rivas-Gonzalo, J., & Santos-Buelga, C. (2007). Anthocyanin pigments in strawberry. *LWT Food Science and Technology, 40,* 374-382.

Lubrano, C., Robin, J., & Khaiat, A. (1994). Composition en acides gras, sterols, et tocopherols d'huiles de pulpe de fruits de six especes de palmiers de Guyane. *Oleagineux, 49,* 59-65.

Maccarone, E., Maccarone, A., & Rapisarda, P. (1985). Stabilization of anthocyanins of blood orange fruit juice. *Journal of Food Science, 50*, 901-904.

Malien-Aubert, C., Dangles, O., & Amiot, M. (2001). Color stability of commercial anthocyanin-based extracts in relation to the phenolic composition: protective effects by intra- and intermolecular copigmentation. *Journal of Agricultural and Food Chemistry, 49,* 170-176.

Malik, M., Zhao, C., Schoene, N., Guisti, M., Moyer, M., & Magnuson, B. (2003). Anthocyanin-rich extract from *Aronia meloncarpa* E. induces a cell cycle block in colon cancer but not normal colonic cells. *Nutrition and Cancer, 46,* 186-196.

Manach, C., Williamson, G., Morand, C., Scalbert, A., & Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition, 81,* S230–S242.

Markakis, P. (1974). Anthocyanins and their stability in food. *Critical Reviews in Food Technology, 4,* 437-456.

Markakis, P. (1982). Stability of anthocyanins in foods. In: *Anthocyanins as Food Colors*. Markakis, P., Ed., Academic Press Inc., London, UK, 163-180.

Marnewick, J., Joubert, E., Joseph, S., Swanevelder, S., Swart, P., & Gelderblom, W. (2005). Inhibition of tumor promotion in mouse skin by extracts of rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), unique South African herbal teas. *Cancer Letters, 224,* 193-202.

Mateus, N., Silva, A., Santos-Buelga, C., Rivas-Gonzalo, J., & De Freitas, V. (2002). Identification of anthocyanin-flavanol pigments in red wines by NMR and mass spectrometry. *Journal of Agricultural and Food Chemistry, 50,* 2110-2116.

Matsumoto, H., Inaba, H., Kishi, M., Tominaga, S., Hirayama, M., & Tsuda, T. (2001). Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *Journal of Agricultural and Food Chemistry, 49*, 1546-1551.

Mazza, G., & Brouillard, R. (1990). The mechanism of co-pigmentation of anthocyanins in aqueous solutions. *Phytochemistry, 29,* 1097-1102.

McDougall, G., Gordon, S., Brennan, R., & Stewart, D. (2005). Anthocyanin-flavanol condensation products from black currant (*Ribes nigrum* L.). *Journal of Agricultural and Food Chemistry, 53,* 7878-7885.

Meiers, S., Kemeny, M., Weyand, U., Gastpar, R., Von Angerer, E., & Marko, D. (2001). The anthocyanidins cyanidin and delphinidin are potent inhibitors of the epidermal growth-factor receptor. *Journal of Agricultural and Food Chemistry, 49,* 958-962.

Mertens-Talcott, S., & Percival, S. (2005). Ellagic acid and quercetin interact synergistically with resveratrol in the induction of apoptosis and cause transient cell cycle arrest in human leukemia cells. *Cancer Letters, 218,* 141-151.

Mertens-Talcott, S., Bomser, J., Romero, C., Talcott, S., & Percival, S. (2005). Ellagic acid potentiates the effect of quercetin on p21wafl/cip1, p53, and MPA-kinases without affecting intracellular generation of reactive oxygen species in vitro. *Journal of Nutrition, 135,* 609-614.

Mertens-Talcott, S., Lee, J., Percival, S., & Talcott, S. (2006). Induction of cell death in Caco-2 human colon carcinoma cells by ellagic acid rich fractions from muscadine grapes (*Vitis rotundifolia*). *Journal of Agricultural and Food Chemistry, 54,* 5336-5343.

Miketova, P., Schram, K., Whitney, J., Li, M., Huang, R., Kerns, E., Valcic, S., Timmermann, B., Rourick, R., & Klohr, S. (2000). Tandem mass spectrometry studies of green tea catechins: identification of three minor components in the polyphenolic extract of green tea. *Journal of Mass Spectrometry, 35,* 860-869.

Mintel International U.S. Functional Beverage Report. (2008). Mintel International Group Ltd., Chicago.

Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K., & Someya, K. (1999). Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. *Journal of Agricultural and Food Chemistry, 47*, 1083-1091.

Monagas, M., Gomez-Cordoves, C., & Begoña, B. (2006). Evolution of the phenolic content of red wines from *Vitis vinifera* L. during ageing in bottle. *Food Chemistry, 95*, 405-412.

Mulleder, U., Murkovic, M., & Pfannhauser, W. (2002). Urinary excretion of cyanidin glycosides. *Journal of Biochemical and Biophysical Methods, 53,* 61–66.

Muñiz-Miret, N., Vamos, R., Hiraoka, M., Montagnini, F., & Mendelsohn, R. (1996). The economic value of managing the Açai palm (*Euterpe oleracea Mart*.) in floodplains of the Amazon estuary, Pará, Brazil. *Forest Ecology and Management, 87*, 163-173.

Neto, C., Amoroso, J., & Liberty, A. (2008). Anticancer activities of cranberry phytochemicals: an update. *Molecular Nutrition & Food Research, 52,* S18-S27.

Nichenametla, S., Taruscio, T., Barney, D., & Exon, J. (2006). A review of the effects and mechanisms of polyphenolics in cancer. *Critical Reviews in Food Science and Nutrition, 46*, 161-183.

Niessen, W. (1999). *Liquid Chromatography Mass Spectrometry*. 2nd. ed. Chromatographic Science Series, Vol. 79, Marcel Dekker, New York.

Oliveira, J., Fernandes, V., Miranda, C., Santos-Buelga, C., Silva, A., De Freitas, V., & Mateus, N. (2006). Color properties of four cyanidin-pyruvic acid adducts. *Journal of Agricultural and Food Chemistry, 54,* 6894-6903.

Ollila, F., Halling, K., Vuorela, P., Vuorela, H., Slotte, J. (2002). Characterization of flavonoid-biomembrane interactions. *Archives of Biochemistry and Biophysics, 399,* 103-108.

Pacheco-Palencia, L., Hawken, P., & Talcott, S. (2007a). Juice matrix composition and ascorbic acid fortification effects on the phytochemical, antioxidant, and pigment stability of açai (*Euterpe oleracea* Mart.). *Food Chemistry, 105*, 28-35.

Pacheco-Palencia, L., Hawken, P., & Talcott, S. (2007b). Phytochemical, antioxidant and pigment stability of açai (*Euterpe oleracea* Mart.) as affected by clarification, ascorbic acid fortification and storage. *Food Research International*, *40*, 620-628.

Pati, S., Losito, I., Gambacorta, G., La Notte, E., Palmisano, F., & Zambonin, P. (2006). Simultaneous separation and identification of oligomeric procyanidins and anthocyanin-derived pigments in raw red wine by HPLC-UV-ESI-MSn. *Journal of Mass Spectrometry, 41,* 861-871.

Pereira, C., Yariwake, J., & McCullagh, M. (2005). Distinction of the *C*-glycosylflavone isomer pairs orientin/isoorientin and vitexin/isovitexin using HPLC-MS exact mass measurement and in-source CID. *Phytochemical Analysis, 16*, 295-301.

Petsalo, A., Jalonen, J., & Tolonen, A. (2006). Identification of flavonoids of *Rhodiola rosea* by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A, 1112,* 224-231.

Pietta, P. (2000). Flavonoids as antioxidants. *Journal of Natural Products, 63,* 1035-1042.

Pinto, M., Robineleon, S., Appay, M., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simonassmann, P., Haffen, K., Fogh, J., & Zweibaum, A. (1983). Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biology of the Cell, 47,* 323-330.

Plotkin, M., & Balick, M. (1984). Medicine uses of South American palms. *Journal of Ethnopharmacology, 10,* 157-179.

Poei-Langston, M., & Wrolstad, R. (1981). Color degradation of ascorbic-anthocyanin-flavanol model system. *Journal of Food Science, 46,* 1218-1222.

Poon, G. (1998). Analysis of catechins in tea extracts by liquid-chromatography-electro spray ionization mass spectrometry. *Journal of Chromatography A, 794,* 63–74.

Rein, M., Ollilainen, V., Vahermo, M., Yli-Kauhaluoma, J., & Heinonen, M. (2005). Identification of novel pyranoanthocyanins in berry juices. *European Food Research and Technology, 220,* 239-244.

Remy, S., Fulcrand, H., Labarbe, B., Cheynier, V., & Moutounet, M. (2000). First confirmation in red wine of products resulting from direct anthocyanin-tannin reactions. *Journal of the Science of Food and Agriculture, 80*, 745-751.

Remy-Tanneau, S., Le Guerneve, C., Meudec, E., & Cheynier, V. (2003). Characterization of a colorless anthocyanin-flavan-3-ol dimer containing both carbon-carbon and ether interflavanoid linkages by NMR and mass spectrometry. *Journal of Agricultural and Food Chemistry, 51,* 3592-3597.

Riboli, E., & Norat, T. (2003). Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. *American Journal of Clinical Nutrition, 78,* 559S-569S.

Rice-Evans, C., Miller, N., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20,* 933-956.

Rijke, E., Out, P., Niessen, W., Ariese, F., Gooijer, C., & Brinkman, U. (2006). Analytical separation and detection methods for flavonoids. *Journal of Chromatrography A***,** *1112,* 31-63.

Rios, L., Bennett, R., Lazarus, S., Remesy, C., Scalbert, A., & Williamson, G. (2002). Cocoa procyanidins are stable during gastric transit in humans. *American Journal of Clinical Nutrition, 76*, 1106–1110.

Rivas-Gonzalo, J., Bravo-Haro, S., & Santos-Buelga, C. (1995). Detection of compounds formed through the reaction of malvidin 3-monoglucoside and catechin in the presence of acetaldehyde. *Journal of Agricultural and Food Chemistry, 43,* 1444-1449.

Robards, K., Prenzler, P., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry, 66,* 401-436.

Robbins, R. (2003). Phenolic acids in foods: An overview of analytical methodology. *Journal of Agricultural and Food Chemistry, 51,* 2666-2687.

Rodrigues, R., Lichtenthäler, R., Zimmermann, B., Papagiannopoulos, M., Fabricius, H., Marx, F., Maia, J., & Almeida, O. (2006). Total oxidant scavenging capacity of *Euterpe oleracea* Mart. (açai) seeds and identification of their polyphenolic compounds. *Journal of Agricultural and Food Chemistry, 54,* 4162-4167.

Rodriguez-Saona L., Guisti, M., & Wrolstad, R. (1999). Color and pigment stability of red radish and red fleshed potato anthocyanins in juice model systems. *Journal of Food Science, 64*, 451-456.

Rogez, H. (2000). Açai: preparo, composiçao e melhoramento da conservaçao. PhD thesis, Chemical Engineering Department, Federal University of Pará. Pará, Brazil.

Rubiskiene, M., Jasutiene, I., Venskutonis, P., & Viskelis, P. (2005). HPLC determination of the composition and stability of blackcurrant anthocyanins. *Journal of Chromatographic Science, 43*, 478-482.

Saija, M., Scalese, M., Lanza, D., Marzullo, Bonina, F., & Castelli, F. (1995). Flavonoids as antioxidant agents: Importance of their interaction with biomembranes. *Free Radical Biology and Medicine, 19,* 481-486.

Salas, E., Atanasova, V., Poncet-Legrand, C., Meudec, E., Mazauric, J., & Cheynier, V. (2004a). Demonstration of the occurrence of flavanol-anthocyanin adducts in wine and in model solutions. *Analytica Chimica Acta, 513,* 325-332.

Salas, E., Dueñas, M. Schwarz, M., Winterhalter, P., Cheynier, V., Fulcrand, H. (2005). Characterization of pigments from different high speed countercurrent chromatography wine fractions. *Journal of Agricultural and Food Chemistry, 53*, 4536-4546.

Salas, E., Fulcrand, H., Meudec, E., & Cheynier, V. (2003). Reactions of anthocyanins and tannins in model solutions. *Journal of Agricultural and Food Chemistry, 51,* 7951-7961.

Salas, E., Le Guerneve, C., Fulcrand, H., Poncet-Legrand, C., & Cheynier, V. (2004b). Structure determination and colour properties of a new directly linked flavanol-anthocyanin dimer. *Tetrahedron Letters, 45,* 8725-8729.

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M., Stammati, A., & Zucco, F. (2005). The Caco-2 cell line as a amodel of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biology and Toxicology, 21,* 1-26.

Sano, A., Yamakoshi, J., Tokutake, S., Tobe, K., Kubota, Y., & Kikuchi, M. (2003). Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. *Bioscience, Biotechnology, Biochemistry, 67*, 1140-1143.

Santos-Buelga, C., & Williamson, G. (2003). *Methods in Polyphenol Analysis*. Royal Society of Chemistry, Cambridge, U.K.

Sarni-Manchado, P., Cheynier, V., & Moutounet, M. (1997). Reaction of enzymically generated quinones with malvidin-3-glucoside. *Phytochemistry, 45,* 1365-1369.

Scalbert, A., Deprez, S., Mila, I., Albrecht, A., Huneau, J., & Robot, S. (2000). Proanthocyanidins and human health: Systemic effects and local effects in the gut. *BioFactors, 13*, 115-120.

Schauss, A., Wu, X., Prior, R., Ou, B., Patel, D., Huang, D., & Kababick, J. (2006). Phytochemical and nutrient composition of the freeze-dried amazonian palm berry, *Euterpe oleracea* Mart. (açai). *Journal of Agricultural and Food Chemistry, 54,* 8598-8603.

Seeram, N., Adams, L., Hardy, M., & Heber, D. (2004). Total cranberry extract versus its phytochemical constituents: antiproliferative and synergistic effects against human tumor cell lines. *Journal of Agricultural and Food Chemistry, 52,* 2512-2517.

Seeram, N., Adams, L., Zhang, Y., Lee, R., Sand, D., Scheuller, H., & Heber, D. (2006). Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *Journal of Agricultural and Food Chemistry, 54,* 9329-9339.

Shahidi, F., & Wanasundara, P. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition, 32,* 67-103.

Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., Kanda, T., Ohtake, Y., & Goda, Y. (2006). Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the Porter method and high-performance liquid chromatography/tandem mass spectrometry. *Journal of Agricultural and Food Chemistry, 54,* 884-892.

Shwarz, M., Wabnitz, T., & Winterhalter, P. (2003). Pathway leading to the formation of anthocyanin-vinylphenol adducts and related pigments in red wines. *Journal of Agricultural and Food Chemistry, 51,* 3682-3687.

Singleton, V. L. (1972). Common plant phenols other than anthocyanins, contributions to coloration and discoloration. *Advances in Food Research Supplement, 3*, 143-191.

Singleton, V., & Rossi, J. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16,* 144-153.

Skerget, M., Kotnik, P., Hadolin, M., Hras, A., Simonic, M., & Knez, Z. (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chemistry 89,* 191-98.

Skrede, G., Wrolstad, R., Lea, P., & Enersen, G. (1992). Color stability of strawberry and black currant syrups. *Journal of Food Science, 57*, 172-177.

Somers, T. (1971). The polymeric nature of wine pigments. *Phytochemistry, 10,* 2175-2186.

Spencer, J., Schroeter, H., Shenoy, B., Srai, S., Debnam, E., & Rice-Evans, C. (2001). Epicatechin is the primary bioavailable form of the procyanidin dimers B2 and B5 after transfer across the small intestine. *Biochemical and Biophysical Research Communications, 285,* 588-593.

Srivastava, A., Akoh, C., Fischer, J., & Krewer, G. (2007). Effect of anthocyanin fractions from selected cultivars of Georgia-grown blueberries on apoptosis and phase II enzymes. *Journal of Agricultural and Food Chemistry, 55,* 3180-3185.

Stintzing, F., Stintzing, A., Carle, R., Frei, B., & Wrolstad, R. (2002). Color and antioxidant properties of cyanidin-based anthocyanin pigments. *Journal of Agricultural and Food Chemistry, 50*, 6172-6181.

Talcott, S., & Lee, J. (2002). Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. *Journal of Agricultural and Food Chemistry*, *50*, 3186-3192.

Talcott, S., Brenes, C., Pires, D., Del Pozo-Insfran, D. (2003). Phytochemical stability and color retention of copigmented and processed muscadine grape juice. *Journal of Agricultural and Food Chemistry, 51,* 957-963.

Talcott, S. (2007). Phytochemical-Rich Oils and Methods Related Thereto. Provisional U.S. Patent Application, June 2007.

Tammela, P., Laitinen, L., Galkin, A., Wennberg, T., Heczko, R., Vuorela, H., Slotte, J., & Vuorela, P. (2004). Permeability characteristics and membrane affinity of flavonoids and alkyl gallates in Caco-2 cells and in phospholipid vesicles. *Archives of Biochemistry and Biophysics, 425,* 193-199.

Tian, Q., Giusti, M., Stoner, G., & Schwartz, S. (2005). Screening for anthocyanins using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry with precursor-ion analysis, product-ion analysis, common neutral-loss analysis and selected reaction monitoring. *Journal of Chromatography A, 1091,* 72-82.

Timberlake, C., & Bridle, P. (1976). Interactions between anthocyanins, phenolic compounds and acetaldehyde and their significance in red wines. *American Journal of Enology and Viticulture, 27*, 97-105.

Tsai, P., & Huang, H. (2004). Effect of polymerization on the antioxidant capacity of anthocyanins in Roselle. *Food Research International, 37,* 313-318.

Tsai, P., Delva, L., Yu, T., Huang, Y., & Dufosse, L. (2005). Effect of sucrose on the anthocyanin and antioxidant capacity of mulberry extract during high temperature heating. *Food Research International, 38,* 1059–1065.

Turker, N., Aksay, S., & Ekiz, I. (2004). Effect of storage temperature on the stability of anthocyanins of a fermented black carrot *(Daucus carota.)* beverage: Shalgam. *Journal of Agricultural and Food Chemistry, 52*, 3807-3813.

Vaidyanathan, J., & Walle, T. (2001). Transport and metabolism of the tea flavonoid (-)-epicatechin by the human intestinal cell line Caco-2. *Pharmaceutical Research, 18.* 1420-1425.

Van Dijk, C., Driessen, A., & Recourt, K. (2000). The uncoupling efficiency and affinity of flavonoids for vesicles. *Biochemical Pharmacology, 60,* 1593–1600.

Visioli, F., Borsani, L., & Galli, C. (2000). Diet and prevention of coronary heart disease: the potential role of phytochemicals. *Cardiovascular Research, 47*, 419-425.

Vivar-Quintana, A., Santos-Buelga, C., & Rivas-Gonzalo, J. (2002). Anthocyanin-derived pigments and colour of red wines. *Analytica Chimica Acta, 458,* 147-155.

Voirin, B., Sportouch, M., Raymond, O., Jay, M., Bayet, C., Dangles, O., & El Hajji, H. (2006). Separation of flavone C-glycosides and qualitative analysis of *Passiflora incarnata L.* by capillary zone electrophoresis. *Phytochemical Analysis,* 11, 90-98.

Walgren, R., Walle, U., & Walle, T. (1998). Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. *Biochemical Pharmacology, 55,* 1721-1727.

Wang, L., & Stoner, G. (2008). Anthocyanins and their role in cancer prevention. *Cancer Letters, 269,* 281-290.

Waridel, P., Wolfender, J., Ndjoko, K., Hobby, K., Major, H., & Hostettmann, K. (2001). Evaluation of quadrupole time-of-flight tandem mass spectrometry and ion-trap multiple-stage mass spectrometry for the differentiation of *C*-glycosidic flavonoid isomers. *Journal of Chromatography A, 926,* 29-41.

Whiting, D. (2001). Natural phenolic compounds 1900-2000: a bird's eye view of a century's chemistry. *Natural Products Report, 18,* 583-606.

Williamson, G., Day, A., Plumb, G., & Couteau, D. (2000). Human metabolic pathways of dietary flavonoids and cinnamates. *Biochemical Society Transaction, 28,* 16–22.

Wilska-Jeszka, J., & Korzuchowska, A. (1996). Anthocyanins and chlorogenic acid copigmentation: influence on the color of strawberry and chokeberry juices. *Lebensm Unters Forsch*, *203*, 38-42.

Wrolstad, R. E., Durst, R. W., & Lee, J. (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science and Technology*, 16, 423-428.

Wu, Q., Koponen, J., Mykkanen, H., & Torronen, A. (2007). Berry phenolic extracts modulate the expression of p21WAF1 and Bax but not Bcl-2 in HT-29 colon cancer cells. *Journal of Agricultural and Food Chemistry, 55,* 1156-1163.

Wu, Q., Wang, M., & Simon, J. (2003). Determination of proanthocyanidins in grape products by liquid chromatography/mass spectrometric detection under low collision energy. *Analytical Chemistry, 75,* 2440-2444.

Yi, W., Akoh, C., Fischer, J., & Krewe, G. (2006). Absorption of anthocyanins from blueberry extracts by Caco-2 human intestinal cell monolayers. *Journal of Agricultural and Food Chemistry, 54,* 5651-5658.

Zhao, C., Giusti, M., Malik, M., Moyer, M., & Magnuson, B. (2004). Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. *Journal of Agricultural and Food Chemistry, 52,* 6122-6128.

VITA

Lisbeth Alicia Pacheco Palencia received her Bachelor of Science in Agroindustrial Engineering from Zamorano University in Honduras, Central America in December 2004. She pursued graduate studies in Food Science and Human Nutrition at the University of Florida and received her Master of Science degree in May 2006. She finally received her Doctor of Philosophy degree in Food Science and Technology, from Texas A&M University in May 2009.

Lisbeth can be reached at 1500 Research Parkway Centeq Bldg. A, suite 234, College Station, TX, 77845. Her email is lisbethpp@gmail.com.